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EDITORIAL

The term “biomarker”, a combination of “biological marker”, refers to a broad subcategory of medical signs – that is, objective indications of medical state observed from exterior of the patient – which can be measured precisely. Medical signs stand in contrast to medical symptoms, which are limited to those indications of health or illness observed by patients themselves.

A biomarker as “a characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention”. A joint venture on chemical safety, the international programme on chemical safety, led by the World Health Organization (WHO) and in coordination with the United Nations and the International Labour Organization, has defined a biomarker as “any substance, structure, or process that can be measured in the body or its products and influence or predict the incidence of outcome or disease”. An even broader definition takes into account not just incidence and outcome of disease, but also the effects of treatments, interventions, and even unintended environmental exposure, such as to chemicals or nutrients. In their report on the validity of biomarkers in environment risk assessment, the WHO has stated that a true definition of biomarkers includes “almost any measurement reflecting an interaction between a biological system and a potential hazard, which may be chemical, physical, or biological. The measured response may be functional and physiological, biochemical at the cellular level, or a molecular interaction”. Examples of biomarkers include everything from pulse and blood pressure through basic chemistries to more complex laboratory tests of blood and other tissues. Medical signs have a long history of use in clinical practice-as old as medical practice itself and biomarkers are merely the most objective, quantifiable medical signs modern laboratory science allows us to measure reproducibly. Some of the biochemical markers are alpha-fetoprotein, cancer antigen 125, prostate specific antigen and a few examples for molecular markers are BRCA 1 and 2 for breast cancer and point mutation in exon 21(L585R) for lung cancer. The use of biomarkers, and in particular laboratory-measured biomarkers, in clinical research is somewhat newer, and the best approaches to this practice are still being developed and refined. The key issue at hand is determining the relationship between any given measurable biomarker and relevant clinical endpoints.

Dr. Manjula Shantaram

Editor-in-chief
ABSTRACT

Oral cancer is the sixth most common malignancy worldwide. Cancer development and progression requires inactivation of tumor suppressor genes and activation of proto-oncogenes. Expression of these genes is in part dependent on RNA and microRNA-based mechanisms. MicroRNAs are essential regulators of diverse cellular processes including proliferation, differentiation, apoptosis, survival, motility, invasion and morphogenesis. Several microRNAs have been found to be aberrantly expressed in various cancers including oral cancer. The purpose of this article was to review the literature related to microRNA deregulation in the head and neck/oral cavity cancers. A comprehensive review of the available literature from 2000 to 2017 relevant to microRNA deregulation in oral cancer was undertaken using PubMed, Medline, Google Scholar and Scopus. Keywords for the search were: microRNA and oral cancer, microRNA and squamous cell carcinoma, microRNA deregulation. Only full-length articles in English language were included. Strengths and limitations of each study are presented in this review. Several studies were identified that investigated microRNA alternations in the head and neck/oral cavity cancers. Significant progress has been made in identification of microRNA deregulation in these cancers. It has been evident that several microRNAs were found to be deregulated specifically in oral cavity cancers. Among these, several microRNAs have been functionally validated and their potential target genes have been identified. These findings on microRNA deregulation in cancer further enhance our understanding of the disease progression, response to treatment and may assist with future development of targeted therapy.

Keywords: Oral potentially malignant disorders; microRNA; miRNA; expression in tissue; deregulation.

INTRODUCTION

Cancer of the oral cavity accounts for approximately 3% of all malignancies and found in 270,000 patients annually worldwide (1, 2). It is the 12th most common Cancer in women and the 6th in men (3). Almost 4 - 8.1% of females and 8 - 8.5% of males may develop oral cancer in their lives (4, 5). Most of the oral squamous cell Carcinomas develop from potentially malignant disorders (PMDs). Lack of early detection is believed to be responsible for the diagnostic delay of these entities. Early detection of Oral cancer is a multistep process which starts with epithelial hyperplasia, dysplasia and then finally turns into carcinoma (6). In these above transformations a variety of genetic and molecular changes occur. If these changes can be identified at an earlier stage, then prevention of the disease and initiation of treatment can be done (7). MicroRNAs (miRNAs) are a class of endogenous small non-coding RNAs about 19 to 25 nucleotide long that have been found highly conserved among species. MiRNAs are able to negatively regulate gene expression through base pairing of 3’ UTRs of their target genes. Therefore, miRNAs have been shown to play an important role in regulating various cellular activities (8). Over the past decade, substantial evidences have been...
obtained to show that miRNAs are aberrantly expressed in human malignancies and could act as “OncomiRs” or “Tumor suppressor miRs”. Recently microRNA expression profiling has been extensively used in order to screen the expression of the large number of microRNAs through extensive sample collections (9). Several microRNA expression profiling studies in cell lines, tissue samples, serum have revealed the number of microRNAs as the biomarkers in OPMD (Oral Potentially Malignant Disorders). These studies provide hundreds of differentially expressed miRNAs, and finally only a small number of them may have clinical use and act as diagnostic and prognostic biomarkers. Different profiling studies show inconsistency in the identified differentially expressed miRNAs (10). A logical method to identify the most consistently reported differentially expressed miRNAs is to search for the intersections of miRNAs identified in multiple independent studies. In recent years, increasing number of studies have demonstrated the involvement of miRNAs in early detection of oral potentially malignant disorders (11). This systematic review aims to evaluate the expression profiles of miRNA in oral potentially malignant disorders.

METHODOLOGY

Search strategy
An English literature search was done using the MeSH terms in the Pubmed data base, Science direct, Cochrane library, Embase, Google scholar and was hand searched in the reference list of the identified articles. The search was done till November 2016. MeSH terms for key words like oral leukoplakia, oral sub mucous fibrosis and oral lichen planus, oral cancer along with the microRNA, MiRNA, Tissue miRNA and malignant transformation were also used.

Selection criteria

Inclusion criteria
1. Only clinical trials were included.
2. Only studies with tissue microRNA of potentially malignant disorders and oral cancer were included.

Exclusion criteria
1. Studies done in microRNA of saliva and serum levels of both oral potentially malignant disorders and oral cancers were excluded.
2. Animal studies were excluded.
3. Studies in different languages were excluded.
4. Studies done in carcinoma other than the oral carcinoma were excluded.

A total of 574 articles were identified by key word search, one article from Cochrane library was identified, one article from Embase was identified and seven articles were from hand searching. Out of these only 11 articles met the inclusion criteria. The quality assessment of these studies was done by RevMan review manager 5 using QUADAS II tool (Quality Assessment of Diagnostic Accuracy Studies)
Table 1: Data extraction form of tissue miRNA in oral potentially malignant disorders

<table>
<thead>
<tr>
<th>S. No.</th>
<th>MiRNA</th>
<th>Year</th>
<th>Author</th>
<th>Journal</th>
<th>Up/down Regulated</th>
<th>Sample Description</th>
<th>Location</th>
<th>Methodology</th>
<th>Statistics</th>
<th>Results</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>21-181b</td>
<td>2009</td>
<td>Cervigne et al., (12)</td>
<td>Human molecular</td>
<td>Up Regulated</td>
<td>22-progressive</td>
<td>University of Toronto, Canada</td>
<td>Biopsy done. FFPE and fresh frozen specimens were used RNA extracted from QRT-PCR Taq Man low density assay</td>
<td>One way ANOVA test p&lt;0.01 One sided Wilcoxon signed rank test. Linear mixed effect regression analysis.</td>
<td>Micro RNA 21, 181b,345 were upregulated</td>
</tr>
<tr>
<td></td>
<td>181c</td>
<td>2013</td>
<td>Yang et al., (13)</td>
<td>BMC cancer</td>
<td></td>
<td>8-oscc 6-Moderate</td>
<td>Shanghai, China</td>
<td>TaqMan low density array</td>
<td>P value 181c-0.028 10b-0.008 145-5p—0.034 99b-5p—0.0118 708-0.0028</td>
<td>MiRNA 708,10b-upregulated 99,145,181c—downregulated</td>
</tr>
<tr>
<td></td>
<td>10b</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>dysplasia leukoplakia</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td></td>
<td>145-5p</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>1-mild dysplasia</td>
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<tr>
<td></td>
<td>99b-5p</td>
<td></td>
<td></td>
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<td></td>
<td>leukoplakia</td>
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<tr>
<td></td>
<td>708</td>
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<tr>
<td>Page</td>
<td>MiRNA</td>
<td>Year</td>
<td>Org</td>
<td>Tissue</td>
<td>miRNA Target</td>
<td>Source</td>
<td>Control</td>
<td>Assay</td>
<td>Technique</td>
<td>Result</td>
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<tr>
<td>3</td>
<td>miR-375</td>
<td>2015</td>
<td>Shi et al., (14)</td>
<td>Upregulated</td>
<td>control-17 OLP-22 2) OSCC-15</td>
<td>Beijing, China</td>
<td>Total RNA, including miRNA, was isolated using the TRIzol reagent RNA for next generation sequencing was evaluated with an Agilent 2100 Bioanalyzer Cell culturing Immunohistochemistry Quantitative real-time PCR (qPCR Oligonucleotide transfection Cell proliferation assay Western blot analysis</td>
<td>miR-375-P &lt; 0.05 A non-parametric Mann-Whitney U test ANOVA Newman-Keuls Multiple Paired or unpaired Student’s t tests. Linear regression SPSS 20.0</td>
<td>Global miRNA profiling in paired OLP and OSCC tissues reveals the possible involvement of suppressive miRNA, miR-375, in premalignant progression</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>miRNA</td>
<td>2015</td>
<td>LingZuo et al., (15)</td>
<td>TF-miRNA coregulation network in OLP</td>
<td>7- healthy control 7-OLP</td>
<td>China</td>
<td>Gene sequences are obtained from (GEO) database</td>
<td>Student’s t test. The genes with P values less than 0.05 were regarded to be differentially expressed genes Fisher’s exact test (P value cutoff 0.01).</td>
<td>TF-miRNA coregulation network in OLP plays an important role in oral cancer development.</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>MiRNA</td>
<td>2012</td>
<td>Xiao et al., (16)</td>
<td>upregulated</td>
<td>20 -Oral leukoplakia 7- Malignantly transformed oral leukoplakia</td>
<td>Shanghai Jiao Tong University School of Medicine</td>
<td>Total RNA was isolated from all samples using TRIZOL reagent miRCURYTM Locked Nucleic Acid Array version 11.0</td>
<td>FISH Analyses mtOLK (mean=1.2) than in OLK (mean=0.2, p&lt;0.05;</td>
<td>Expression of MiRNA 31 was significantly up-regulated plays an important role in progression of oral leukoplakia</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>MiRNA</td>
<td>2014</td>
<td>Sarkar et al., (17)</td>
<td>Plos one</td>
<td>fhsa-miR-1293, hsa-miR-31, hsa-miR-31* and hsa-miR-7 up-regulated Hsa-miR-206, hsa-miR-204 and hsa-miR-133a down-regulated.</td>
<td>Kolkata</td>
<td>Cancer (n=18) Leukoplakia (n=18) Lichen planus (n=12)</td>
<td>TaqMan Assay RT-PCR Punch biopsy RNA later at - 80c</td>
<td>Kolmogoro v smirnov test One tailed unpaired t test</td>
<td>Expression of MiRNA 31 was significantly up-regulated in both cancer and oral leukoplakia tissue, thus may be one of the markers of leukoplakia which may progress to gingiva-buccal cancer.</td>
</tr>
</tbody>
</table>
### DISCUSSION

The included studies have been done in 128 oral leukoplakia cases, 55 oral squamous cell carcinoma cases, 150 oral *Lichen planus* cases, 6 oral sub mucous fibrosis, 12 malignantly transformed oral leukoplakia and 47 healthy controls. Graph 1 shows sample distribution. All the included studies were done in the following populations, 8 in China, one each in Canada, India, Brazil and Germany. Graph 2 shows studies done in various populations. All the tissue samples were biopsied with the consent of the individuals and miRNA extraction was done using TaqMan Q MicroRNA assay. Out of the total 1407 differentially expressed miRNA, 36 miRNA were statistically significant (14, 18). The present systematic review identified one most consistently reported upregulated miRNA, miRNA-21, which was reported to be differentially expressed in 3 studies followed by miR-181b in two studies (16, 21). The most

<table>
<thead>
<tr>
<th>Study</th>
<th>miRNA</th>
<th>Year</th>
<th>Authors</th>
<th>Journal</th>
<th>Tissue Type</th>
<th>Control</th>
<th>Extracted Method</th>
<th>Analysis Method</th>
<th>p-value</th>
<th>Significant miRNA</th>
<th>p-value</th>
<th>Genomic Location</th>
<th>dmiRNA-Association</th>
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<tr>
<td>7</td>
<td>miR-27b</td>
<td>2012</td>
<td>Zhang et al., (18)</td>
<td>Oral diseases</td>
<td>MiR-27b downregulated</td>
<td>OLP-71 Healthy control-49</td>
<td>Shanghai, China</td>
<td>Microarray analysis, RTQPCR IGH ANLYSIS</td>
<td>Students t test was done to compare normal tissues and OLP tissues</td>
<td></td>
<td>MiRNA-27b was significantly downregulated</td>
<td></td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>miRNA-21,499,205</td>
<td>2015</td>
<td>Zhu et al., (19)</td>
<td>BioMed research international</td>
<td>MiRNA-21-upregulated, MiRNA -499 downregulated, MiRNA -205-upregulated</td>
<td>Healthy control-5</td>
<td>Shanghai, China</td>
<td>Gene sequences are obtained from (GEO) database</td>
<td>Students t test was done p-value cutoff of 0.001</td>
<td>MiRNA-21 is upregulated, MiRNA 499 is associated with reduced risk of OSCC, MiRNA 205 can be used to differentiate OSCC from adenocarcinoma</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>miRNA-203</td>
<td>2015</td>
<td>Zheng et al., (20)</td>
<td>Oncology reports</td>
<td>MiRNA-203-upregulated</td>
<td>Healthy control-6</td>
<td>China</td>
<td>RT-PCR R Neasy plus mini kit for extraction of miRNA</td>
<td>Students t test was done One-way ANOVA was done</td>
<td>MiRNA 203 plays a critical role in arecoline induced OSMF</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>miRNA-146a, 155</td>
<td>2012</td>
<td>Rao et al., (21)</td>
<td>Dermatolog y research,</td>
<td>MiRNA-146a, MiRNA-155-upregulated</td>
<td>OLP-31 Healthy control-6</td>
<td>Brazil</td>
<td>TRizol reagent TaqMan Q MicroRNA ASSAY RTPCR</td>
<td>Spearman correlation coefficient-0.699</td>
<td>Increased expression of MiRNA-146a, MiRNA 155 was significant.</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>11</td>
<td>miRNA-21, 31, 155, 130B, 223</td>
<td>2013</td>
<td>Gassling et al., (22)</td>
<td>Plos one</td>
<td>MiRNA-21, 31, 155, 130B, 223 were upregulated.</td>
<td>OLP-7 Healthy control-7</td>
<td>Germany</td>
<td>Affymetrix Human Gene 1.0 ST Array while miRNA profiling was performed using the microRNA Galaxy Array</td>
<td>Mann-Whitney U-test.</td>
<td>miRNA associated with transcripts which are regulated when comparing OLP patients with healthy control individuals. This suggests that miRNAs may potentially regulate disease-relevant transcripts.</td>
<td></td>
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</tbody>
</table>
consistently reported differentially expressed miRNA of upregulation in our systematic review was miR-21 which had an oncogenic activity. The upregulation of miR-21 significantly promoted cell proliferation and revealed a higher proportion of cells at S phase and knockdown of miR-21 expression resulted in cell-cycle arrest at G2/M phase and inhibited cell proliferation (17, 22). The quality assessment of the included studies was done using Revman review manager and risk of bias and applicability concern percentage are shown in graph 2. There are 4 studies with low risk of bias.

CONCLUSION
The miRNA-21, miRNA-31 are the most consistently reported upregulated microRNAs from the four-low risk of bias studies. MicroRNA 181b and miRNA 375 the most consistently downregulated miRNAs from the low risk of bias studies. The most consistently reported differentially expressed microRNAs may be used as efficient biomarkers and therapeutic targets. Since only one study is done in Indian population,
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the applicability of this result in Indian population is low. Still more investigations are needed for the clinical studies focusing on these miRNAs in order to understand the potential roles of these microRNAs in oral potentially malignant disorders.

REFERENCES

Potential of medicinal plant compounds to targeting Tau protein in the therapy of Alzheimer’s disease – A review

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ABSTRACT

Alzheimer’s disease (AD) is a devastative neurodegenerative disorder with complex etiology. AD is characterized by blood-brain barrier disruption, oxidative stress, mitochondrial impairment, neuro-inflammation, hypo-metabolism; it decreases in acetylcholine levels and a reduction of cerebral blood flow. It is also not solely the end-product of aberrantly processed, misfolded, and aggregated oligomeric amyloid-beta peptides but hyper phosphorylated Tau (tubulin binding protein) which formed senile plaque and intracellular neurofibrillary tangles respectively. However, despite the long-term and worldwide effort for a more effective therapy, the only available treatment is a symptomatic use of acetylcholinesterase inhibitors and memantine. Then, many researchers focused their attention to modulate amyloid-beta peptides. These therapeutic approaches as well as those based on cholinergic or amyloid theory have not brought the desired benefits yet. Thus, the main features related with the Tau pathology found in AD are Tau phosphorylation and aggregation. Based on the biochemically diverse range of pathological Tau protein, a number of approaches have been proposed to develop new potential therapeutics like inhibition of Tau phosphorylation, proteolysis and aggregation; promotion of intra- and extracellular Tau clearance and stabilization of microtubules (MTs). Medicinal plants have been used in different systems of medicine and exhibited their powerful roles in the management and cure of memory disorders. This review paper discusses the potential of medicinal plant molecules to targeting Tau protein in Alzheimer’s disease therapy.

Keywords: Alzheimer’s disease; medicinal plants; Tau protein; hyper phosphorylation; aggregation therapy; natural compound.

INTRODUCTION

Alzheimer’s disease (AD) is a chronic neurodegenerative disorder that leads to progressive disturbance of cognition function including memory, decision making, orientation to physical surrounding and language (1). 47 million people live with dementia worldwide. This number is projected to increase to more than 131 million by 2050, as population age. It is the most common form of neurodegenerative diseases and represents a major public health problem and then, the most common cause of progressive dementia in the aging population globally (2). AD is a progressive neurodegenerative disease characterized by senile plaques, neurofibrillary tangles (NFTs); and loss of neurons and synapses in the brain. NFTs are intra-neuronal aggregations mainly composed of abnormally phosphorylated Tau protein. However, Tau phosphorylation is regulated by a balance between Tau kinase and phosphatase activities. Disruption of this equilibrium was suggested to be at the origin of abnormal Tau
phosphorylation and thereby contributes to Tau aggregation (1).

For instance, natural polyphenols, flavonoids and others compounds have reported anti-aggregating capacity to prevent amyloid formation, and a standardized plant extract reduced both amyloid-β and phosphorylated Tau levels in a many transgenic models (3). Tau expression, stabilizing Tau conformations, or clearing hyper phosphorylated Tau aggregates represent challenges for the therapeutic that targeting Tau toxicity in AD. Another approach is to re-stabilize microtubules for preserving neuronal health and axonal transport (4). Pharmacologically, these polyphenols and their derivatives exhibit potential for preventive and therapeutic purposes against protein aggregation during neurodegeneration. Although compounds act on various biochemical pathways, their role in stabilizing the protein degradation machinery at different stages may be an attractive therapeutical strategy to halt the accumulation of misfolded proteins (5).

However, some studies have shown that medicinal plants with potential sources of molecules (natural polyphenols, flavonoids and alkaloids) have neuroprotective effects (5) and can be helpful for the development of the new drugs for Alzheimer’s disease.

Now, the current treatments of Alzheimer’s disease are symptomatic and do not affect the underlying course of the disease, and clinical trials with research derived molecules have proven inconclusive. Therefore, it is imperative to find new and alternative treatments like phytotherapy which is a science-based approach to the use of natural products with medicinal purposes. It is now widely accepted that Tau is an important therapeutic target in neurodegenerative disease (6). These therapeutic include; (a) reduction of Tau hyper phosphorylation using kinase inhibitors and phosphatase activators, (b) activation of proteasome degradation pathways of Tau, (c) Tau clearance by immunotherapy, (d) inhibition of Tau aggregation using small molecules and stabilizing microtubules (MTs; 7).

In this paper, we review current ideas regarding the use of medicinal plant compounds as a potential source for development of cure of Alzheimer’s disease targeting specifically Tau protein.

Alzheimer disease overview

Alzheimer’s disease (AD) is the most common form of dementia. Ageing is the primary risk for AD. Most common forms of AD are sporadic which start at age 65 around and progressing slowly over. Less than 1% of all cases are early onset familial AD, which is inherited in an autosomal dominant manner and develops similar symptoms as sporadic AD prior to age 65. AD symptoms are associated with progressive loss of neurons and synapses in multiple brain regions, especially in the frontal cortex and hippocampus (8) (Fig. 1A). Literature reports changes in Tau protein and amyloid β oligomers as the most important factors responsible for neuronal dysfunction in the pathogenesis of AD. NFTs observed initially in the entorhinal cortex and hippocampus subsequently extend to the amygdala and cortical areas (temporal, frontal, and parietal) (Fig. 1B; 9).

Fig. 1: Spacio-temporal evolution of the lesions in AD. A: Amyloid deposits progression; B: NFTs progression. I, II; III, IV; V, VI: different regions of brain (10).
AD is a progressive neurodegenerative disease that is characterized by the increased abundance of amyloid beta (Aβ) plaques, and neurofibrillary tangles composed of hyperphosphorylated Tau. Normally, Tau is phosphorylated by kinases like cyclin-dependent kinase 5 (CDK5) and glycogen synthase kinase 3 beta (GSK-3β). When Tau becomes hyperphosphorylated at phosphorylation sites, it can no longer bind to the MTs. This phosphorylation has been reported to be abnormal in AD. Tau has phosphorylation sites (Threonine or Serine) located in the proline-rich region (P-region) (residues 172–251) and the C-terminal tail region (C-region) (residues 368-441). It is reported that these sites of phosphorylation of Tau are Ser396, Ser262, Ser 202/Thr205 (11).

**Alzheimer and TAU protein**

Tau is a member of the microtubule-associated protein (MAPs) family. It is a protein that is highly enriched in neurons and was originally defined by its ability to bind and stabilize MTs. Tau plays a role in mediating axonal transport, neurite outgrowth, synaptic structure and function, and neuronal signaling pathways. Physiologically, Tau involves in neurodegenerative diseases, and most prominently in the pathogenesis of AD. The soluble hyper phosphorylated Tau is, however, clearly distinct from aggregated fibrillary Tau in NFTs, despite both being implicated in Tau toxicity (12).

Tau is a highly soluble and natively unfolded protein that binds and promotes the assembly of MTs. Tau protein contains a tandem repeat of 31 or 32 amino acids in the C-terminal half. The repeat region was shown to have a microtubule binding function, and Tau promotes assembly of tubulin and stabilizes MTs (12; Fig. 2).

![Fig. 2. Domains and structural elements in Tau. Top: Representation of Tau deduced from NMR. Bottom: Approximate location of interaction sites with other proteins (13).](image)

Tau is encoded by a single gene located on chromosome 17 (17q21), possessing 16 exons in its primary transcript. Six different isoforms are expressed by post transcriptional modifications generated by splicing from the primary transcript (6). Mature protein length is about 352 up to 441 amino acid residues, and a molecular-weights of 45–65 k Da depending on the Tau isoforms. The C-terminal region has a domain containing the microtubule binding repeats, which is critical for microtubule assembly, whereas the affinity of Tau for MTs is finely regulated by an orchestrated set of phosphorylation (14; Fig. 3).
In tauopathies, Tau accumulates in NFTs that are visualized within dystrophic neurites and cell bodies. The amount of Tau pathology correlates with progressive neuronal dysfunction, synaptic loss, and functional decline in humans. These tangles are bundles of paired helical filaments composed of hyper phosphorylated Tau. Tau promotes tubulin assembly into MTs and stabilizes them. However, the ability of Tau to stabilize MTs is inversely related to the level of Tau hyper phosphorylation. Moreover, Tau may have other post-translational modifications, including glycosylation, ubiquitination, truncations, and nitration (4).

Disruption of this equilibrium of balance between Tau kinase and phosphatase activities was suggested to be at the origin of abnormal tau phosphorylation and thereby might contribute to Tau aggregation. Both kinases and phosphatases have been implicated in the appearance of abnormally phosphorylated Tau (15).

1. **Kinases of TAU**

It is well-known that some kinases which are involved in the induction of synaptic plasticity modulate Tau phosphorylation (16). Kinases belong to the enzyme group termed “transferases” because they transfer phosphate group from high-energy donor molecules (ATP or GTP) to specific substrates. Tau is a substrate of various protein kinases. Tau kinases include proline-directed Ser/Thr kinases (SP/TP kinases) such as CDK5, GSK3β, and MAPK, as well as non-SP/TP kinases including microtubule affinity-regulating kinase (MARK)/Par-1, AMPK, protein kinases A, C (PKA, PKC), CK (17). So, Tau protein kinases are grouped into proline-directed protein kinases (PDPK), protein kinases non-PDPK and tyrosine protein kinases. Among these kinases, GSK-3β is the major Tau kinase involved in most of the hyper phosphorylated serine/threonine sites in Tau. However, GSK-3β is a target of the phosphatidylinositol-3-kinase (PI3K)/serine/threonine PKB (Akt) signaling pathway (18).

2. **Phosphatases of TAU**
Tau function and subcellular localization are tightly regulated by the orchestrated interplay between phosphorylation and de-phosphorylation events (19). Phosphatases are generally classified into three groups according to their amino acids sequences, the structure of their catalytic site and their sensitivity to inhibitors: phosphoprotein phosphatase (PPP), the metal-dependent protein phosphatase and the protein tyrosine phosphatase (PTP). Tau phosphatases belong to PPP group: PP1, PP2A, PP2B and PP5; and PTP group: phosphatase and tensin homolog (PTEN) (1). Of particular relevance, the phosphatase battlefront is largely led by a distinct pool of protein phosphatase enzymes that are responsible for the bulk of neuronal Tau dephosphorylation particularly protein phosphatase 2A (PP2A) which is the most study. PP2A dysfunction has been linked to Tau hyperphosphorylation and synaptic deficits. Deregulation of PP2A enzymes also affects the activity of many protein kinases implicated in AD (20).

3. Microtubules

Tau contains a number of lysine residues, of which positive charges are critical for binding to negatively charged microtubules. Tau protein with an abnormal high degree of phosphorylation is hindered from binding to microtubules increasing free Tau protein concentration and is missorted to the somatodendritic compartment (21). When appropriate physiological Tau phosphorylation is maintained, Tau maintains also affinity to MTs and structure of MTs, axon integrity and cellular function are preserved. When Tau is hyper phosphorylated, it is thought to lose affinity from MTs, form insoluble aggregates, leading to impaired axonal transport, neuronal damage and cell death (22; Fig. 4).

4. Compound as inhibitors of TAU protein

Efforts to develop effective disease-modifying treatments for AD have mostly targeted the amyloid β protein; however, there has recently been increased interest in other targets including phosphorylated Tau and other forms of Tau. Aggregated Tau appears to spread in a characteristic pattern throughout the brain (23). The complexity of Tau biology provides many potential therapeutic targets to prevent Tau production, aggregation, or spread at the level of transcription, phosphorylation, depolymerization, and transport (4, 23-25).

Now, some literature shows that 14-3-3 proteins interact with Tau and regulate Tau phosphorylation by bridging Tau with various protein kinases (11). 14-3-3 proteins are a family of proteins highly conserved and are mainly expressed in the brain especially in central nervous system. These proteins impact many aspects of brain function like neural signaling, neuronal development and neuroprotection.

a. Immunotherapy

Recently, Tau has become one of the most actively pursued therapeutic targets for AD (26). One strategy used for targeting Tau protein is the immunotherapy. This approach is based in on immunization of subjects against the misfolded Tau protein with the result that hyperphosphorylation and aggregation of Tau are prevented with direct link and strong association between Tau pathology and loss of cognition (27).
b. Therapeutic targeting of Tau hyper-phosphorylation

Another strategy may be the prevention of Tau hyper-phosphorylation by the inhibition of Tau kinases. Tau hyper-phosphorylation is widely known to be induced by increased phosphokinase activity and/or decreased phosphatase activity (28). Therefore, treatment with chemical inhibitors may reduce the rates of NFTs in AD. However, most attention has been paid to the role of GSK-3β because high levels of GSK-3β activity lead to alterations in amyloid beta precursor protein (AβPP) processing and increased neuronal death. Some compound isolated from plants showed strongly inhibition of GSK-3β the enzyme mainly responsible for this process (29).

c. Therapeutic targeting Tau aggregation

Post-translational modifications and loss of microtubule binding lead to elevated levels of cytosolic Tau, thereby increasing the potential for Tau–Tau interactions and polymerization. In humans, Tau aggregation and the presence of NFTs correlate more closely with symptom severity and neuron loss. Large fibrils might contribute to cell dysfunction via molecular crowding and effects on cell metabolism validity of targeting extracellular Tau in the later stages of the disease. Compounds that could facilitate the proteolytic degradation of tau aggregates and prevent propagation of NFTs are very important. Therefore, there are some natural compounds that are able to inhibit Tau aggregation and possibly, make an impact in neurodegenerative diseases especially AD (26) (Fig. 5).

3.4. Therapeutic targeting microtubule stabilization

In physiological conditions, Tau is normally bound to MTs in axons, modulating tubulin assembly and MT stability. However, hyper phosphorylation of Tau reduces the tubulin binding affinity of the protein and detaches normal Tau from MTs, leading to MTs destabilization and impaired axonal transport. Thus, MT-stabilizing agents that can compensate for the loss of Tau function and restore axonal transport have therapeutic potential in AD and other tauopathies (30; Fig. 6).
4. Natural Compound of Medicinal Plants as Inhibitors of Tau protein hyper phosphorylation and Aggregation

In recent decades, great interest has been raised due to the potential of polyphenols to prevent many diseases like neurodegenerative diseases (31). Phosphorylation of Tau takes place on serine/threonine residues principally located in the basic proline-rich domains of the protein. However, these domains are potent targets able to fix polyphenols and that polyphenols could inhibit Tau aggregation. Some polyphenols and others compound from plant were also shown to inhibit phosphorylation and aggregation of Tau. Moreover, polyphenols induce disaggregation of aggregated Tau and modify ultrastructure of paired helical filaments isolated from AD brains, decreasing enlargement of filaments (32) (Table 1).

Table 1: Natural products for the treatment of Alzheimer’s disease targeting Tau protein

<table>
<thead>
<tr>
<th>Compound</th>
<th>Structure</th>
<th>Plant</th>
<th>Activities</th>
<th>Ref</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tolfenamic acid</td>
<td><img src="structure1.png" alt="Structure" /></td>
<td>Corner officinalis</td>
<td>Reduces total Tau as well as in site specific hyperphosphorylation of Tau and lowers tau mRNA and protein, as well as the levels of its phosphorylated form and CDK5.</td>
<td>(33)</td>
</tr>
<tr>
<td>Morroniside</td>
<td><img src="structure2.png" alt="Structure" /></td>
<td>Cornus officinalis</td>
<td>Inhibits Tau hyperphosphorylation in SK-N-SH cells induced by okadaic acid, a PP2A inhibitor.</td>
<td>(34)</td>
</tr>
<tr>
<td>Salidroside</td>
<td><img src="structure3.png" alt="Structure" /></td>
<td>Rhodiola rosea L</td>
<td>Up regulates the level of p-GSK-3β and downregulated p-Tau in Tau transgenic Drosophila and inhibiting neuronal loss</td>
<td>(35)</td>
</tr>
<tr>
<td>Curcumin</td>
<td><img src="structure4.png" alt="Structure" /></td>
<td>Curcuma longa</td>
<td>Up-regulates an anti-Tau co-chaperone BCL2-Associated Athanogene2 (BAG2) and thus, suggest probable benefit of curcumin against AD-associated tauopathy.</td>
<td>(7)</td>
</tr>
<tr>
<td>Chemical</td>
<td>Plant Source</td>
<td>Action</td>
<td>Reference</td>
<td></td>
</tr>
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<td>--------------------------</td>
<td>-------------------------------------------</td>
<td>--------------------------------------------------------------------------------------------------</td>
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<tr>
<td>Rosmarinic acid</td>
<td><em>Rosmarinus officinalis</em> L.</td>
<td>Acts as Tau inhibitors by avoiding fibril formation <em>in vitro</em> and subsequent β sheet formation and is able to stop aggregation of Tau</td>
<td>(3)</td>
<td></td>
</tr>
<tr>
<td>Resveratrol</td>
<td>Red wine, grapes, berries, peanuts</td>
<td>Protects against hyperphosphorylation and/or mediates dephosphorylation of the Tau protein; Inhibits Tau phosphorylation mediated by activation of PP2A and AMPK-induced activation of PI3K/Akt signaling pathway by inhibition of GS3K.</td>
<td>(37, 38)</td>
<td></td>
</tr>
<tr>
<td>Luteolin</td>
<td></td>
<td>Reduces the zinc-induced hyperphosphorylation of the Tau protein, the mechanism of which may be explained by its antioxidant activity and ability to regulate the Tau phosphatase/kinase system;</td>
<td>(37)</td>
<td></td>
</tr>
<tr>
<td>Asiatic acid</td>
<td><em>Centella asiatica</em></td>
<td>Reduces phosphoTau by activating Akt/GSK3β pathway.</td>
<td>(39)</td>
<td></td>
</tr>
<tr>
<td>Fisetin</td>
<td><em>Rhus succedaneae</em> L.</td>
<td>Promotes the <em>in vitro</em> degradation of phosphorylated Tau and reduced the <em>in vivo</em> Tau hyperphosphorylation</td>
<td>(40)</td>
<td></td>
</tr>
<tr>
<td>Morin</td>
<td><em>Maclura pomifera</em></td>
<td>Reduces Tau hyperphosphorylation</td>
<td>(41)</td>
<td></td>
</tr>
<tr>
<td>Caffeic acid</td>
<td></td>
<td>Reduces Tau phosphorylation</td>
<td>(25)</td>
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</table>
CONCLUSION

Medicinal plants have been implicated in health benefits relevant to a number of disease conditions. Much of the evidence has focused on the polyphenol, flavonoid components. These main compounds have been shown to have effects against AD. Therefore, it is important to value medicinal plant, exploring new active compounds against AD.

ACKNOWLEDGEMENT

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A study on pulmonary function tests among rice mill workers in Mysuru, Karnataka

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ABSTRACT

Introduction and Aim: Rice is the largest consumed food grain in south India. Rice cultivation, harvesting and milling is done since ages in India. Respiratory diseases are greatly induced by the occupational dusts that are influenced by the duration of exposure. A large amount of dust is produced from rice milling process and hence the workers breathe air mixed with dust into their lungs every day. This study aims to assess the effect of rice mill dust on lung functions among rice mill workers.

Materials and Methods: Fifty non-smoking male adult workers from rice mills were selected for the study and fifty age and sex matched from the general population who were not exposed to such occupational hazard were included as controls. Pulmonary function parameters (FVC, FEV₁, FEV₁/FVC, PEFR and MVV) were recorded using computerized RMS Helios Spirometer during their working hours in both the groups and were statistically analyzed by using student’s t test. Further the cases were classified according to the duration of exposure and inter group comparisons were made using one-way Anova.

Results: Rice mill workers showed a significantly greater percentage of decline in FVC, FEV₁, PEFR, MVV and also a significant decrease in FEV₁/FVC ratio suggestive of obstructive pulmonary disorder. Also, there is a negative correlation between duration of exposure and deterioration of the lung function.

Conclusion: The respiratory parameters were significantly declined among rice mill workers compared to controls.

Keywords: Pulmonary function; rice mill; occupational dusts; harvesting; milling.

INTRODUCTION

Rice milling is the process where in the rice grain is transformed into a form suitable for human consumption. Dust particles smaller than 10 m are called respirable dust. This could be passed through the human lung and are responsible for the serious respiratory disorders of workers in the industry (1).

A rice grain is made up of an internal endosperm, a bran layer and husk layer externally. Rice husk is the largest by product of rice milling industry which amounts to 20% of the weight of the paddy. De-husking or de-hulling is the process of removal of husk layer from the paddy by friction. Then the separated husk from the paddy is removed by suction and transported to a storage dump outside the milling plant (2). The occupational diseases owing to dust exposure depends on constitution and concentration of dust, duration of exposure to dust and also variation in the personal immunity and thus the workers in different agricultural
industries are exposed to varying amount of dust and is a major occupational health hazard on respiratory system (3). Airborne occupational exposures to irritants, vesicants, and fibrogen have the potential to cause pulmonary function impairment when exposures are not properly controlled over extended periods of time (4). Pulmonary tests are important investigating tool. Although they do not provide a diagnosis per se, different patterns of abnormalities are seen in various respiratory diseases which helps to establish a diagnosis. Spirometry is a simple and quick procedures used as a measure of lung function and it is a measure of volume against time (5).

The present study is undertaken to evaluate the effect of rice mill dust on the respiratory functions of the mill workers and to compare with the duration of working (in years).

MATERIALS AND METHODS

The present study was a cross sectional comparative study, undertaken at various rice mills in Mysore. Study population included male permanent workers in 2 groups - study group and control group. Study group included 50 employees in the age group of 20 -50 years working in rice mills and control group included 50 age and sex matched employees from the offices who are not exposed to rice mills as control. Subjects for both groups were selected based on the inclusion and exclusion criteria. Inclusion criteria - 1) Age of workers between 20 - 50 years. 2) Only male workers. Exclusion criteria: 1. Those have cardiovascular illness in present or past. 2. Those having kyphoscoliosis deformity 3. Those predispose to allergic asthma 4. Smokers and tobacco chewers.

Recording occupational and personal histories of exposed workers - A standard questionnaire proforma was maintained to assess the clinical history, smoking, duration of exposure to dusts, nature of occupation, any respiratory symptoms and smoking habits (6).

Both groups were matched for age, height, weight, body surface area and body mass index. Pulmonary functions were tested according to the guidelines recommended by American thoracic society (7) using the instrument ‘RMS – HELIOS – Medspiror’- a self-calibrating computerized spirometer that fulfills the criteria for standardized lung function tests (8). The following respiratory parameters were studied 1. Forced vital capacity (FVC) 2. Forced expiratory volume in 1 second (FEV1). 3. FEV1/ FVC ratio 4. Peak expiratory flow rate (PEFR) 5. Maximum voluntary ventilation (MVV). The subjects were familiarized with the set up and detailed instructions and demonstrations were given before initiation of the procedure. Under aseptic precautions with the nose closed by nose clip, the subjects were made to breathe forcefully following deep inspiration into the mouthpiece. Expiration was maintained for a minimum period of 3-4 seconds. 3 to 4 trials of maximal inspiratory and expiratory efforts were made and only the highest reading was taken for data processing. Statistical analysis of the data was performed by using SPSS 24. The data of the variables were expressed as Mean ± Standard Deviation. Comparisons were performed using student’s‘t’– test for two group comparisons and one-way ANOVA (Analysis of variance) for multiple groups. The p value of < 0.05 was considered as statistically significant.

RESULTS

Table 1 shows the comparison of demographic characteristics of rice mill workers and controls such as age, height, weight and BMI (calculated by dividing subject’s weight in kilogram and height in square meters). The mean age of the rice mill workers was 32.96 ± 8.12 and controls were 33.52 ± 6.63. The mean height and weight of the rice mill workers were158.7 ± 0.99 and 60.14 ±
8.23 respectively. Similarly mean height and weight of controls were 159.94 ± 0.07 and 62.66 ± 9.66 respectively. The mean BMI of the rice mill workers and control subjects were 24.10 ± 5.26 and 24.53 ± 3.67 respectively. On analyzing these characteristics by unpaired ’t’ test there was no statistically significant difference between the groups.

Table 2 summarizes the comparison of the lung function parameters of rice mill workers and control subjects. The mean observed values of Spirometric parameters were, FVC in rice mill workers was 2.40 ± 0.75 and controls was 3.32 ± 0.59. FEV1 in both rice mill workers and controls were 2.21 ± 0.65 and 3.14 ± 0.53 respectively. The observed value of FEV1 / FVC in rice mill workers and controls were 91.39 ± 6.98 and 95.09 ± 3.53 respectively. PEFR in rice mill workers was 5.65 ± 1.84 and controls was 8.11 ± 1.41. MVV in rice mill workers was 78.71 ± 27.19 and controls was 103.50 ± 32.09. There was statistically significant decline in all the lung function parameters - FVC, FEV1, FVC/FEV1, PEFR, and MVV in rice mill workers when compared to controls.

Table 3 shows the changes in lung volume of the rice mill workers by duration of employment. 11 rice mill workers exposed to rice mill dust for more than 20 years shows the mean value of FVC of 1.65± 0.14, FEV1 of 1.45± 0.13, FVC/FEV1 of 85.51± 8.54, PEFR of 1.65± 0.03 and MVV of 59.17± 16.41. 20 rice mill workers exposed for more than more than 10 years and less than 20 years shows the mean value of FVC of 2.06± 0.29, FEV1 of 1.96± 0.28, FVC/FEV1 of 90.61 ± 6.74, PEFR of 5.83± 1.91 and MVV of 70.41± 28.10. 19 rice mill workers exposed to rice mill dust for less than 10 years shows the mean value of FVC of 3.21± 0.52, FEV1 of 2.92 ± 0.35, FVC/FEV1 of 92.76 ± 6.13, PEFR of 6.15± 1.61 and MVV of 84.39 ± 30.10.

On application of ANOVA it was found that there is a statistically significant difference (p<0.05) in lung volumes (FVC, FEV1, FVC/FEV1, PEFR, MVV) among the rice mill workers as the duration of work exposure to rice mill dust increased.

Figure 1 shows significantly decreased mean spirometric parameters (FVC, FEV1, FVC/FEV1, PEFR, MVV) of rice mill workers on comparison with control group. Fig. 2 shows the mean spirometric parameters (FVC, FEV1, FVC/FEV1, PEFR, MVV) of rice mill workers significantly decreased with the increase in duration of exposure.
### Table 3: Comparison of study group spirometry parameters in relation to duration of exposure to rice mill dust

<table>
<thead>
<tr>
<th>Duration of exposure</th>
<th>n</th>
<th>Mean ± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>FVC</td>
</tr>
<tr>
<td>1-10</td>
<td>19</td>
<td>3.21±0.52</td>
</tr>
<tr>
<td>11-20</td>
<td>20</td>
<td>2.06±0.29</td>
</tr>
<tr>
<td>&gt;20</td>
<td>11</td>
<td>1.65±0.14</td>
</tr>
<tr>
<td>ANOVA</td>
<td></td>
<td>0.00*</td>
</tr>
</tbody>
</table>

p > 0.05 = Not significant, p < 0.05 = Statistically significant; SD – Standard deviation, FVC – Forced vital capacity , FEV1 – Forced expiratory volume in 1 s, FEV1/ FVC – Forced expiratory volume in 1 s / Forced vital capacity, PEFR – Peak expiratory flow rate, MVV – Maximum voluntary ventilation.

### DISCUSSION

Occupational respiratory diseases are caused by extended exposure to ‘Respirable dusts’ that causes acute or chronic respiratory ailments (9). Poor ventilation is the basic problem in rice mills which leads to accumulation of dust. Cumulative exposure to dust results in chronic pulmonary
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diseases. Pulmonary function tests are essential to assess the pulmonary function status and respiratory efficiency in these workers (10). In this study, there was statistically significant difference found with respect to mean values of FVC, FEV1 and FEV1/ FVC ratio, indicated the prevalence of obstructive type of pulmonary impairment (11). Similar findings were reported by Dhillion et al., (12) and Prakash et al., (13). The reduction in mean values of PEFR in study group compared to control group suggests that maximum effect on peripheral airway obstruction (14). PEFR is an index of expiratory airway resistance and is more effort dependent (15). A highly significant decrease in PEFR was also reported from Zodpey et al., (16). This study also showed significant reduction in MVV which indicates increase in airway resistance, reduced compliance or decreased Respiratory muscle force (17). These findings were consistent with those reported Yadav et al.,(18), Bose et al.,(19). This study revealed a clear picture of obstructive respiratory functions and showed a significant relationship in reduction of all the pulmonary function test parameters with increase in the duration of occupational exposure which is consistent with the studies done by Vijayanathitagi et al.,(20)and Rao et al.,(21) which may be due to the chronic exposure of industrial dust, poor ventilation and lack of proper exhaust facility(22). This exposure to industrial dust causes occupational airway obstruction which occurs due to chronic irritation of the airways (23).

CONCLUSION

In conclusion, this study demonstrated obstructive respiratory functions associated with rice mill dust exposure. This study findings support the association between the duration of exposure to rice mill dust and spirometry indices. Strict implementation of the Respiratory protection program is recommended in rice mills and awareness program for the rice mill workers about the same has to be done in a regular basis.

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Anti-inflammatory activity of nutmeg oleoresin mediated silver nanoparticles- An In-vitro study

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ABSTRACT

Introduction and Aim: The most emerging area of research in nanotechnology deals with the synthesis of nanoparticles which are of great importance due to its use in various biological fields. Myristica fragrans is commonly known as “nutmeg”. It is popular as a spice and also possesses various therapeutic properties. It has a characteristic pleasant fragrance and a slightly warm taste. It has various therapeutic uses and is widely used. The aim is to assess the anti-inflammatory activity of nutmeg oleoresin mediated silver nanoparticles.

Materials and Methods: The nutmeg oleoresin mediated silver nanoparticles were synthesized and was confirmed by UV Vis spectroscopy. The anti-inflammatory property of the prepared nutmeg oleoresin mediated silver nanoparticles was assessed using albumin denaturation inhibitory assay technique.

Results: The nutmeg oleoresin mediated silver nanoparticles showed good anti-inflammatory activity with increasing concentration of the nanoparticles.

Conclusion: Although a variety of steroidal and non-steroidal anti-inflammatory drugs have been developed, researchers are focusing on natural substances to develop new anti-inflammatory agents. Nutmeg mediated silver nanoparticles showed a good range of inhibition and can be used against inflammation. The myristicin present in the nutmeg maybe responsible for its anti-inflammatory action. Increased albumin denaturation is reported in conditions like in rheumatoid arthritis, diabetes and cancer. Hence this may pay way to manage such conditions.

Keywords: Myristica fragrans; anti-inflammatory; nanoparticles; oleoresin; albumin denaturation assay technique.

INTRODUCTION

The most emerging area of research in nanotechnology deals with the synthesis of nanoparticles which are of great importance due to its use in various biological fields. The synthesis of nanoparticles is based on the chemical composition, mono-disparsity and dimensions (1, 2). Till date, metallic nanoparticles are mostly prepared from noble metals like gold, silver and platinum. The nanoparticles are used in the field of catalysis, optoelectronics, diagnostic biological problem and these uncovered many significant findings.(1-4) Among the noble metals silver is the metal of choice in the field of biological system, living organism and medicine (5-7). The formation of nanoparticles include sol process, micelles, sol-gel process, chemical precipitation, hydrothermal method pyrolysis chemical vapor deposition, bio-based protocols etc (8). The bio-based protocols are currently under exploitation as it is cost-effective eco-friendly.
Myristica fragrans is commonly known as “nutmeg”. It is popular as a spice and also possesses various therapeutic properties. It has a characteristic pleasant fragrance and a slightly warm taste. It has various therapeutic uses and is widely uses worldwide (9, 10). It is rich source of vitamin A, C and E, electrolytes (sodium and potassium) and minerals (manganese, zinc, copper, iron, calcium and phosphorus). It is used in flavoring many kinds of baked food, confections, puddings, meat, sausages and beverages (11).

M. fragrans has been used as a folklore medicine to treat diarrhea, mouth-sores and insomnia. The essential oil of nutmeg is used externally for sprains, rheumatism and paralysis, which possesses its analgesic and anti-inflammatory properties (12). Compounds isolated from the seeds of this plant have been reported to process strong platelet anti-aggregatory activity (13,14). The myristicin found in M. fragrans has cytotoxic and apoptotic effects in neuroblastoma SK-N-SH cells with an accumulation of cytochrome and activation of Caspase 3 in the cytosol, which shows it’s anti-carcinogenic properties (15). In the present study, the anti-inflammatory activity of nutmeg oleoresin mediated silver nanoparticles was assessed using albumin denaturation inhibition assay.

MATERIALS AND METHODS

The nutmeg oleoresin was gifted by Synthite Industries Ltd, Kerala.

Synthesis of Nutmeg oleoresin mediated silver nanoparticles

90 mL of 1 millimolar of silver nitrate in water was mixed with 10 mL of the nutmeg oleoresin. The solution was kept in orbital stirrer for nanoparticles synthesis. The nanoparticles were centrifuged using lark refrigerated centrifuge at 8000 rpm for 10mts and the pellet is collected and was washed twice with distilled water. The final purified pellet were collected, dried at 60°C and stored in air tight Eppendorff tube (Fig 1, 2).

Inhibition of albumin denaturation assay

BSA (bovine serum albumin) was used as a reagent for the assay. Bovine serum albumin (BSA) makes up approximately 60% of all proteins in animal serum. It is commonly used in cell culture, particularly when protein supplementation is necessary, and the other components of serum are unwanted. BSA undergoes denaturation upon heating and starts expressing antigens associated with type iii hypersensitive reaction which are related to diseases such as rheumatoid arthritis, glomerulonephritis, serum sickness and systemic lupus erythematosus (16). 2 ml of 1% bovine albumin fraction was mixed with 400 ml of plant crude extract indifferent concentrations (20-100 μg/ml) and the pH of reaction mixture was adjusted to 6.8 using in HCL. The reaction mixture was incubated at room temperature for 20 minutes and then heated at 55°C for 20 min in a water bath. The mixture was cooled to room temperature and the absorbance value was recorded at 660 nm. An equal amount of plant extract was replaced with DMSO for control. Diclofenac sodium in different concentrations was used as a standard. The experiment was performed in triplicate (fig. 3).

% inhibition was calculated using the following formulae

\[
\% \text{ inhibition} = \frac{\text{control O.D} - \text{sample O.D}}{\text{Control O.D}} \times 100
\]
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present in aqueous solution of silver complex in the nutmeg extract demonstrated the change in color was due to the formation of silver nanoparticles. When the anti-inflammatory property of the nanoparticles was assessed, it was found that the percentage of inhibition kept increasing with the increase in concentration of the nutmeg silver nanoparticles. This proves the effect of nutmeg against inflammation.

The anti-inflammatory property of nutmeg was checked using the HRBC membrane stabilizing method. Nutmeg seed extract was reported to have anti-inflammatory property. The anti-inflammatory activity of *M. fragrans* (Nutmeg) was carried out using HRBC membrane stabilizing method (17). Plants such as *B. racemosa* have also shown to have anti-inflammatory property by inhibiting albumin denaturation assay (18). Increased albumin denaturation is reported in conditions like in rheumatoid arthritis, diabetes and cancer (19). Similar studies are reported the anti-inflammatory activity of plant extract mediated silver nanoparticles (20).
CONCLUSION

Although a variety of steroidal and non-steroidal anti-inflammatory drugs have been developed, researchers are focusing on natural substances to develop new anti-inflammatory agents. Nutmeg oleoresin mediated silver nanoparticles showed a good range of inhibition and can be used against inflammation. The oil present in nutmeg, myristicin is responsible for its anti-inflammatory action. Hence, this nutmeg oleoresin mediated silver nanoparticles may be used for conditions such as rheumatoid arthritis, cancer and diabetes.

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Significance of serum magnesium levels in the auditory status of type II diabetes mellitus patients
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ABSTRACT

Introduction and Aim: Diabetes mellitus is one of the commonest metabolic causes associated with hypomagnesaemia. Magnesium is said to be one of the chief neuroprotective and vasodilatory mineral of the body. The main aim of this study was to establish the potential correlation between the serum magnesium levels and the auditory acuity in type II Diabetes mellitus patients.

Materials and Methods: It is a cross-sectional study done at Sree balaji medical college & hospital, Chromepet, Chennai. In this study we evaluated a total of 200 type II DM cases consisting of 100 cases with poor glycaemic control, and 100 cases under glycaemic control, all are recently diagnosed cases of type II Diabetes mellitus, in the age groups of 40-55 years who had come to the hospital for their regular annual master check-ups. Their serum magnesium levels were measured using the xylidyl blue dye binding method and pure tone audiometry was also done.

Results: Results showed that of the 100 poorly controlled type II DM subjects (i.e. HbA1c >/= 7), 65 patients with hypomagnesaemia had sensory neural type of hearing loss & of the 100 type II DM subjects with controlled HbA1c levels (i.e. HbA1c<7), 8 out of the 10 subjects with hypomagnesaemia again had, sensory neural type of hearing loss.

Conclusion: The results of this cross-sectional study proved that hypomagnesaemia results in the impairment of hearing, amidst the type II diabetic population, especially the poorly controlled diabetics are more vulnerable to develop hypomagnesaemia and its associated deafness. Thus periodic assessment of auditory status and serum magnesium levels is also required in all type II DM cases, as an early measure to prevent diabetes and hypo magnesia induced deafness.

Keywords: Type II Diabetes mellitus; serum magnesium levels; auditory status.

INTRODUCTION

In today’s sedentary, gadget dominated society with increased uptake of unhealthy hyper palatable junk foods along with a positive genetic predisposition, has all resulted in the increased prevalence of diabetes at an unprecedented rate globally. In India around 62 million diabetic individuals have been diagnosed & many more are expected to be associated with the subclinical/undiagnosed/neglected forms of diabetes in accordance with the iceberg phenomenon of Diabetes mellitus (1). Chronic DM patients with poor glycaemic control have been proved to be associated with multiple complications affecting almost all the systems of the body over time. Among the various known complications, hearing impairment/ hearing loss and deafness which refers to the partial/total inability to hear is becoming more and more common in type II diabetes patients. Though hearing loss could also
result from multiple other causes such as advancing age associated with repeated exposure to excessive loud noise, local ear infections, ototoxic drugs, physical trauma etc. Most of the diabetic patients with poor metabolic control, identified by their increased glycosylated haemoglobin (HbA1c) levels, had history of bilateral, sensory neural type of hearing loss which is off sudden onset, progressive and irreversible in nature as proven by various previous studies (2).

Diabetes mellitus is one of the commonest metabolic causes associated with hypomagnesaemia (3). Magnesium is said to be one of the chief neuroprotective and vasodilatory mineral of the body. $\text{Mg}^{2+}$ aids hearing by crossing the hematocochlear barrier thereby counterchecks the cochlear ischemia which plays a significant role in pathological hearing loss (4). Thus any derangements in the magnesium levels, in general makes the person more susceptible to noise induced hearing loss (NIHL).

Most of the chronic diabetic patients with relatively poorly controlled glycaemic profiles, have been found to have magnesium deficits, mainly due to the increased loss of magnesium in urine & due to the decreased intake of magnesium that ultimately results in $\text{Mg}^{2+}$ depletion in the diabetic patients (5).

**MATERIALS AND METHODS**

The main aim of this study was to establish the potential correlation between the serum magnesium levels and the auditory acuity in type II Diabetes mellitus patients.

It is a cross-sectional study done at Sree Balaji Medical College and Hospital, chrome pet, Chennai. In this study, we evaluated a total of 200 type II DM cases consisting of 100 cases, with poor glycaemic control, and 100 under glycaemic control cases, all were recently diagnosed cases of type II diabetes mellitus, in the age groups of 40-55 years who had come to the hospital for their regular annual master check-up. The participants past history and significant family history were all taken. Exclusion criteria included: other potential causes of hearing loss such as past trauma to the ear/head injury, persons working in highly noisy-constant machinery involved environment, any history of significant ear infection in the past, any drug intake of ototoxic drugs like streptomycin etc.,

We explained the need and the significance of the study to all the participants, and all their written consents were obtained prior to the study. We measured their Glycaemic index-HbA1c levels by ion exchange chromatography method using the subjects venous blood samples, obtained under sterile aseptic conditions. We also measured their serum $\text{Mg}^{2+}$ levels, in the biochemistry department of the central laboratory of Sree balaji medical college &hospital. And pure tone audiometry was also done for all the participants in co-ordination with the ENT department of Sree balaji Hospital in Chrome pet, in order to access their auditory status.

Their serum magnesium levels was measured using the xylidyl blue dye binding method, that has a standard normal serum magnesium, reference range of 1.5 to 2.5 mEq/L.

**Statistical analysis**

All the data entry was done in MS excel sheet. Statistical analysis was done using the software package for the social sciences version Spss 18.

**RESULTS**

**Table 1:** Overall comparison of serum magnesium levels in all the 200 type II diabetic subjects

<table>
<thead>
<tr>
<th>Poor glycemic control (HbA1c ≥ 7)</th>
<th>Under glycemic control (i.e HbA1c &lt; 7)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serum magnesium levels</td>
<td>No. of subjects</td>
</tr>
<tr>
<td></td>
<td>Serum magnesium levels</td>
</tr>
<tr>
<td></td>
<td>No. of subjects</td>
</tr>
</tbody>
</table>
Table 2: Comparison of serum magnesium and auditory status in the 100 diabetic subjects with poor glycaemic control (i.e. HbA1c >/= 7)

<table>
<thead>
<tr>
<th>Serum Magnesium Levels</th>
<th>Auditory Status</th>
<th>Total</th>
<th>Chi Square Value</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hypomagnesaemia (&lt;1.5 mEq/L)</td>
<td>2</td>
<td>65</td>
<td>67</td>
<td>34.56</td>
</tr>
<tr>
<td>Normal Range (1.5-2.5 mEq/L)</td>
<td>25</td>
<td>8</td>
<td>33</td>
<td></td>
</tr>
</tbody>
</table>

Table 3: Comparison of serum magnesium and auditory status in the 100 diabetic subjects who are under glycaemic control (i.e. HbA1c < 7)
**DISCUSSION**

All the subjects included in this study were in the age group of 40-55 years so as to avoid any sensory neural type of hearing loss that occurs above 55 years mainly due to presbycusis (6). The results showed that of the 100 type II DM subjects with poor glycaemic control (i.e. HbA1c < 7), a total of 67 subjects had hypomagnesaemia where their serum Mg$^{2+}$ levels was below 1.5 mEq/L and the remaining 33 subjects had normal serum Mg$^{2+}$ levels. Of the 67(hypomagnesaemia) subjects, 65 subjects had sensory neural type of hearing loss. And of the remaining 33 subjects with normal magnesium levels, only 8 subjects had hearing loss.

Similarly of the 100, type II DM subjects under glycaemic control (HbA1c levels < 7), only 10 subjects had hypomagnesaemia (where their serum Mg$^{2+}$ levels<1.5 mEq/L) and the remaining 90 subjects had normal serum Mg$^{2+}$ levels. Of the 10 subjects with hypomagnesaemia, almost 8 subjects had sensory neural type of hearing loss. While of the remaining 90 subjects with normal magnesium levels, only 3 subjects had hearing loss. In both the groups there was a significant p value of 0.0001 (i.e. p value of .05) which is considered to be statistically significant.

Thus the results suggested that hypomagnesaemia was more likely associated with hearing loss in type II DM subjects than in the subjects with normal magnesium range. It emphatically proved the association between hypomagnesaemia and hearing loss in type II DM subjects.

Magnesium is one of the chief minerals, that is believed to be an integral part of the various biochemical reactions, involving the multiple systems of the human body such as-for the normal functioning of the nerves and muscles, to boost the body’s defence mechanism-the immune system, adds strength to the bones, enhances the pumping activity of the heart etc. It is also required to maintain certain basic functions such as to regulate the normal blood glucose levels and for protein and energy production in the body (7). Thus it plays an integral part in the body’s homeostasis. The well-known common features of Mg$^{2+}$ deficiency include muscle weakness, fatigue, twitching &cramps, it could also be associated with osteoporosis, high BP, asthma, irregular heartbeats, mental disorders etc.,

One of most neglected complications of hypomagnesaemia seen in type II DM subjects is the sensory neural type of irreversible hearing loss which is off sudden onset and progressive nature. As magnesium deficiency causes increase in the calcium channel permeability, of the hair cells. Thereby increasing the influx of calcium

---

**Fig. 4:** Comparison of serum magnesium and auditory status in the 100 diabetic subjects who are under glycaemic control (i.e. HbA1c < 7)

<table>
<thead>
<tr>
<th>Serum Magnesium Levels</th>
<th>Auditory Status</th>
<th>Total</th>
<th>Chi Square Value</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hypomagnesaemia(&lt;1.5 mEq/L)</td>
<td>Normal Hearing</td>
<td>2</td>
<td>8</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td>Hearing Loss</td>
<td>8</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Normal Range(1.5-2.5 mEq/L)</td>
<td>Normal Hearing</td>
<td>87</td>
<td>3</td>
<td>90</td>
</tr>
<tr>
<td></td>
<td>Hearing Loss</td>
<td>3</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
resulting in the increased release of glutamate and over stimulation of N methyl D aspartame receptors(NMDA) which are basically glutamate gated iron channels located chiefly in the auditory nerve and cochlea (8). Thus all these cascading metabolic events caused by hypomagnesaemia results in the impairment of hearing.

CONCLUSION

There are various universally accepted diabetic complications such as the micro vascular and the macro vascular complications. The results of this cross-sectional study proved that hypomagnesaemia results in the impairment of hearing, amidst the type II diabetic population. Especially the poorly controlled diabetics (i.e. HbA1c >/= 7), are more vulnerable to develop this hypomagnesaemia and associated deafness. Thus, this study emphasizes the significance and the potential health benefits of maintaining a normal serum magnesium levels in the type II DM patients, who are otherwise prone to develop sensory neural type, of irreversible, sudden onset hearing loss which could hamper their day to day activities.

We would also like to emphasize the need, to have audiometry and serum magnesium levels tested, along with their regular glycaemic parameters, at least once in every three months’ time interval in case of type II DM patients. As awareness and early subsequent intervention plays a significant role in preventing any illness, as prevention is any day always better than cure.

REFERENCES

Effect of antiepileptic drugs on various lipid fractions and certain liver enzymes in epileptic patients of Punjab origin

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ABSTRACT

Introduction and Aim: Epilepsy requires lifelong therapy with antiepileptic drugs (AEDs) & having medical and psychological consequence. Present study was conducted to observe the effect of different AEDs on lipid profile and certain liver enzymes on epileptic patients.

Material and Methods: 50 epileptic patients receiving AEDs for minimum 1 year were recruited as study group and 50 healthy subjects considered as control group. These subjects were recruited from general community of Punjab. Fasting blood samples were drawn from patients and healthy subjects for the evaluation of total cholesterol, triglycerides, LDL-cholesterol, VLDL-cholesterol, HDL-cholesterol, AST, ALT & ALP.

Results: Significant increase in total cholesterol, triglycerides, LDL-cholesterol, VLDL- cholesterol was observed in epileptic patients while no significant change was recorded in HDL- cholesterol. Maximum increase in total cholesterol, TGs, LDL-cholesterol, VLDL- cholesterol levels was observed in phenytoin treated patients with reference tovalproic acid, carbamazepine, levetiracetam treated patients. Levels of AST, ALT and ALP were significantly increased in epileptic patients. Maximum increase in AST & ALT was observed in levetiracetam treated epileptic patients and maximum rise in ALP levels was found in carbamazepine treated patients with reference to other AEDs treated patients.

Conclusion: Aforementioned observations suggested that epileptic patients treated with phenytoin, valproic acid, carbamazepine & levetiracetam for long time could cause dyslipidemia, might be responsible for various CHD’s and hepatotoxicity in epileptic patients. Routine screening of lipid profile and hepatic enzymes during chronic use of AEDs is recommended.

Keywords: Epilepsy; anti-epileptic drugs (AEDs); aspartate aminotransferase (AST); alanine aminotransferase (ALT); alkaline phosphatase (ALP).

INTRODUCTION

Epilepsy is the most common, chronic neurological disorder and about 65 million people affected worldwide. In India, epilepsy affects 5 to10 people’s out of every 1000 people. Epilepsy usually begins in childhood, potentially impeding education, employment, social relationships and development of a sense of self-worth (1). It is estimated that there are more than 10 million peoples with epilepsy in India (2). In the elderly, epilepsy is the third most common neurologic disorder, and they are at higher risk of new onset epilepsy. Psychiatric and other
comorbidities are common among patients with epilepsy (3). The prevalence among Indian males (5.1 per 1000) was much higher than females (2.2 per 1000) (4). Epilepsy requires long-term or lifelong therapy with antiepileptic drugs (AEDs), particularly for those patients with refractory epilepsy (5). It is a disorder of recurrent and spontaneous seizures resulting clinically into permanent alterations of normal function and morphology of neuronal cells and even cell death (6). Epilepsy imposes a large economic burden on health care systems of countries. Approximately, 70-80% of patients who develop epilepsy may expect to have their seizures controlled with optimal antiepileptic therapy (7).

Therapy with antiepileptic drugs remains the mainstay of treatment of patients with epilepsy. The major groupings of antiepileptic drugs can be defined as: 1) Those which facilitate γ-aminobutyric acid (GABA) transmission by various mechanisms; 2) Those which block voltage-gated ion channels and thus reduce excitatory transmission; 3) Those whose mechanism of action is still open to debate (8). Most antiepileptic drugs exert their antiepileptic effects via the Na⁺ or the Ca²⁺ channel or via GABAergic transmission. In addition to the major action site, the new antiepileptic drugs tend to have several minor action sites. In the chronic phase, the incidence of adverse effects with the new antiepileptic drugs is low, but TPM and PER, drugs that potentiate glutamatergic transmission, may elicit behavioral changes and cognition disorders (9). Patients with epilepsy are often required to take antiepileptic drugs for a long period of time. Carbamazepine and phenytoin were amongst the most prescribed antiepileptic drugs as monotherapy and as combination therapy as well as valproic acid while levetiracetam and lamotrigine were found frequently prescribed amongst newer antiepileptic drugs (4). Antiepileptic drugs act either by increasing inhibition through sustaining the release of GABA or glycine or decreasing excitation by inhibiting glutamate release. However, some antiepileptic drugs reduce membrane excitability by interrelating with neurotransmitter receptors or ion channels but the methods of action for most of them are not fully understood. Many studies (10) have shown prolonged use of antiepileptic drugs is known to be associated with adverse effects such as metabolic and organ toxicity, endocrine disturbance, negative cognitive effects, and psychiatric problems. So, present study was designed to evaluate the role of AEDs like phenytoin, valproic acid, carbamazepine and Levetiracetam on various lipid fractions along with certain liver enzymes in epileptic patients of Punjab origin.

**MATERIALS AND METHODS**

The present case control prospective study comprising total of 100 subjects was carried out in the Department of Biochemistry, Government Medical College, Amritsar, in collaboration with Department of Medicine, Guru Nanak Dev Hospital, Amritsar. The subjects for the present study were selected from rural as well as urban community from general population of male and females. A detailed history, physical and systemic examination including measurement of height, weight, heart rate, blood pressure and body mass index (BMI) was taken and every case was thoroughly interviewed.

**Ethical Issues**

The study protocol is approved by the Institutional Ethics Committee. The study details and potential risks and benefits were explained to individuals taking part in the study and at least to one attendant. A written informed consent was obtained from subjects before entering into the study.

**Selection of epileptic patients**

**Inclusion criteria:**
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50 epileptic patients receiving one of the following antiepileptic drugs (phenytoin, valproic acid, carbamazepine and levetiracetam) for a minimum period of 1 year were included in the study. The epileptic patients must have five or more epileptic attacks. The epileptic patients in the age range of 20 to 50 years of both sex (male & female) from rural/urban community of Punjab origin were included.

Exclusion criteria:
Epileptic patients who had concomitant liver diseases, using other drugs causing elevation of liver enzymes (e.g. antibiotics, anti-rheumatic drugs, statins and non-steroidal anti-inflammatory drugs) or those who were alcohol drinkers were excluded from the present study.

Selection of normal healthy control subjects
50 normal healthy subjects in the age range of 20-50 years of both sexes were recruited from urban/rural general population of Punjab origin.

Measurement of anthropometric Parameters
The examination body weight was done by taking weight in kilogram (kg) and height was measured in centimeters. The Body Mass Index (BMI) was calculated from the formula:

\[ \text{BMI} = \frac{\text{Weight}}{\text{Height}^2} \text{ (kg/m}^2) \]

Table: 1 Effect on anthropometric profile in epileptic patients on different epileptic drugs (Phenytoin, Valproic acid, Carbamazepine, Levetiracetam) and normal healthy subjects of Punjab origin

<table>
<thead>
<tr>
<th>Anthropometric profile</th>
<th>Healthy control subjects (Mean ± S.D.)</th>
<th>Epileptic patients (Mean ± S.D.)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Male (n=28)</td>
<td>Female (n=22)</td>
</tr>
<tr>
<td>Age (years)</td>
<td>35.34±8.87</td>
<td>38.22±7.52</td>
</tr>
<tr>
<td>Height (cm)</td>
<td>156.19 ± 5.93</td>
<td>151 ± 4.87</td>
</tr>
<tr>
<td>Body Weight (Kg.)</td>
<td>55.12 ± 3.11</td>
<td>46 ± 5.21</td>
</tr>
<tr>
<td>BMI (Kg/m^2)</td>
<td>25.76 ± 5.32</td>
<td>26.21 ± 5.01</td>
</tr>
</tbody>
</table>

Collection and processing of blood samples
Five ml of venous blood was taken from all subjects after 12 hours overnight fast in a dry disposable syringe under all aseptic conditions by venipuncture in the antecubital vein in a sterile, dry acid washed vial for biochemical assays.

Preparation of serum
The blood was allowed to stand for half an hour. After clot formation, the supernatant was centrifuged. All the samples were processed for thyroid hormones, lipid profile, and liver enzymes.

Biochemical assays
Estimation of fasting blood glucose: Fasting blood glucose levels in plasma were estimated by using the commercially available kit manufactured by Transasia Pvt. Ltd based on GOD-POD Method, End Point as described by Trinder, 1969 (11).

Estimation of lipid profile: Total cholesterol, triglycerides, HDL-cholesterol levels in serum were determined by using the commercially available kit manufactured by Transasia Pvt. Ltd based on Allianet al.,(12) GPO method as described by McGowan (13) and Grillo and Izzo,1985 (14) respectively. VLDL-Cholesterol were estimated by dividing triglycerides with 5 (15) and LDL-cholesterol levels were determined by using Friedwald’s and Fredrickson’s formula (15).

Serum Liver Enzymes:
Determination of Aspartate Aminotransferase (AST) The levels AST in serum of epileptic patients and normal healthy control subjects were determined by using commercially available standardized kits manufactured by using the commercially available kit manufactured by Transasia Pvt. Ltd based on the principle of Moss and Henderson in 1999, kinetic IFCC method (16).
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Determination of Alanine Aminotransferase (ALT):
ALT levels in serum of epileptic patients and normal healthy control subjects were determined by using commercially available standardized kits manufactured by using the commercially available kit manufactured by Transasia Pvt. Ltd based on the principle of Bessey, 1946(18).

Determination of Alkaline Phosphatase (ALP):
ALP levels in the serum of epileptic patients and normal healthy control subjects were determined by using the commercially available kit manufactured by Transasia Pvt. Ltd based on the kinetic IFCC method (17).

Statistical Analysis:
The data was expressed as Mean ± SD. Differences between the epileptic patients and normal healthy control subjects were evaluated using the Student’s independent samples “t” test. Differences were considered statistically significant at p <0.05.

Table 2: Effect on fasting blood glucose and blood pressure in epileptic patients on different epileptic drugs (Phenytoin, Valproic acid, Carbamazepine, Levetiracetam) and normal healthy subjects of Punjab origin

<table>
<thead>
<tr>
<th>Anthropometric Profile</th>
<th>Healthy Control subjects (Mean ± S.D.)</th>
<th>Epileptic Patients (Mean ± S.D.)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Male (n=28)</td>
<td>Female (n=22)</td>
</tr>
<tr>
<td>Fasting Blood Glucose (mg/dL)</td>
<td>79.67 ± 4.76</td>
<td>81.34 ± 3.78</td>
</tr>
<tr>
<td>Systolic blood Pressure (mmHg)</td>
<td>126.17 ± 8.32</td>
<td>125.22 ± 7.89</td>
</tr>
<tr>
<td>Diastolic blood Pressure (mmHg)</td>
<td>83.05 ± 4.33</td>
<td>81.09 ± 4.21</td>
</tr>
</tbody>
</table>

Table 3: Changes in lipid profile levels in epileptic patients on different epileptic drugs (Phenytoin, Valproic acid, Carbamazepine, Levetiracetam) and normal healthy subjects of Punjab origin

<table>
<thead>
<tr>
<th>Lipid Profile</th>
<th>Healthy control subjects (Mean ± S.D.)</th>
<th>Epileptic patients (Mean ± S.D.)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Male (n=28)</td>
<td>Female (n=22)</td>
</tr>
<tr>
<td>Total Chol. (mg/dL) Reference Range:140-250 mg/dl</td>
<td>169.0±16.92</td>
<td>172.10±15.21</td>
</tr>
<tr>
<td>Triglyceride (mg/dL) Reference &lt;150 mg/dl</td>
<td>80.70±7.23</td>
<td>82.20±6.99</td>
</tr>
<tr>
<td>VLDL-Chol. (mg/dL) Reference 2-30 mg/dl</td>
<td>16.16±1.10</td>
<td>16.44±2.15</td>
</tr>
<tr>
<td>HDL-Chol. (mg/dL) Reference Range Male: 30-65 mg/dL; Female: 35-80 mg/dL</td>
<td>41.10±4.21</td>
<td>45.11±5.04</td>
</tr>
</tbody>
</table>
RESULTS

Effect on antiepileptic drugs on lipids profile
A significant increase in total cholesterol by 58.83% (from 170.55 ± 16.06mg/dL to 270.90 ± 16.55mg/dL), Triglycerides by 121.60% (from 81.45 ± 7.11mg/dL to 180.50 ± 10.60mg/dL), LDL-cholesterol by 76.11% (from 111.46 ± 9.20mg/dL to 196.30 ± 9.83mg/dL) and VLDL-cholesterol by 121.47% (from 16.30 ± 1.62mg/dL to 36.10 ± 4.09mg/dL) levels were recorded in epileptic patients on various antiepileptic drugs with respect to normal healthy control subjects. A similar trend of significant increase in total cholesterol, triglycerides, LDL-cholesterol and VLDL-cholesterol levels was also observed in male and females on antiepileptic drug treated epileptic patients of Punjab origin in comparison to normal healthy male and females while a nominal decrease by 10.54% (from 45.11 ± 5.04mg/dL to 38.56 ± 4.23mg/dL) was recorded in epileptic patients on antiepileptic drugs treatment patients with respect to normal healthy subjects (Table 2). A Maximum increase in total cholesterol (302.60 ± 12.92mg/dL), Triglycerides (189.71 ± 7.84mg/dL), LDL-cholesterol (224.26 ± 10.92mg/dL), VLDL-cholesterol (37.94± 3.21mg/dL) was recorded in serum of phenytoin treated epileptic patients in comparison to valproic, carbamazepine and levetiracetam drugs treated epileptic patients. A maximum fall in HDL-cholesterol (43.41 ± 5.11mg/dL) was also observed in phenytoin treated epileptic patients in comparison to valproic, carbamazepine and levetiracetam drugs treated epileptic patients (Table4).

Effect on antiepileptic drugs on certain liver enzymes
A significant increase in AST levels from 32.68±5.30 U/L to 41.80± 6.62 U/L (by 27.90%, p≤0.05); ALT from 22.08± 4.76 U/L to 41.97± 6.71 U/L (by 90.08%, p≤0.001); ALP from 75.65±8.64 U/L to 362.55±12.47 U/L (by 379.24 %, p≤0.001) were recorded in epileptic patients on various antiepileptic drugs with respect to normal healthy control subjects. A similar trend of significant increase in AST, ALT & ALP levels was also observed in male and females on antiepileptic drug treated epileptic patients of Punjab origin in comparison to normal healthy male and females (Table 4). A maximum rise in AST (41.45 ± 12.81U/L) was observed in phenytoin treated epileptic patients, ALT (43.22 ± 8.57(U/L) was recorded in levetiracetam drugs treated epileptic patients and a maximum rise in ALP (375.97 ± 41.91U/L) was also observed in carbamazepine treated epileptic patients in comparison to other antiepileptic drugs treated epileptic patients (Table 4).

Table 4: Effect of different epileptic drugs (Phenytoin, Valproic acid, Carbamazepine, Levetiracetam) on lipid profile levels in epileptic patients of Punjab origin

<table>
<thead>
<tr>
<th>Lipid Profile</th>
<th>Phenytoin (n=12)</th>
<th>Valproic Acid (n=14)</th>
<th>Carbamazepine (n=11)</th>
<th>Levetiracetam (n=13)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total Chol. (mg/dL)</td>
<td>302.6 ± 12.92</td>
<td>251.70 ± 21.19</td>
<td>283.10 ± 16.21</td>
<td>274.21 ± 14.99</td>
</tr>
<tr>
<td>Triglycerides (mg/dL)</td>
<td>189.71 ±7.84</td>
<td>167.21 ± 7.92</td>
<td>176.60 ± 9.37</td>
<td>172.21 ± 9.03</td>
</tr>
<tr>
<td>VLDL-Chol. (mg/dL)</td>
<td>37.94± 3.21</td>
<td>33.50 ± 3.13</td>
<td>35.32 ± 4.89</td>
<td>34.44 ± 3.76</td>
</tr>
<tr>
<td>HDL-Chol. (mg/dL)</td>
<td>40.41 ± 5.11</td>
<td>40.70 ± 4.78</td>
<td>37.69 ± 6.52</td>
<td>32.16 ± 4.14</td>
</tr>
</tbody>
</table>
DISCUSSION

The present case control study was conducted on 50 epileptic patients out of them 12 epileptic patients taking phenytoin; 14 patients using valproic acid; 11 epileptic patient taking carbamazepine and 13 epileptic patients on levetiracetam therapy. In present study a significant increase was found in total cholesterol, triglycerides, LDL-cholesterol & VLDL cholesterol in epileptic patients in comparison to normal healthy subjects and a nominal decrease in HDL-cholesterol was observed in epileptic patients in comparison to normal healthy subjects. A maximum increase in lipid fraction was recorded in phenytoin treated patients with respect to valproic acid, carbamazepine & levetiracetam treated patients (Table 4). The literature reports (19-20) exploring the effects of different antiepileptic drugs on lipid metabolism are inconsistent and reported either increased, decreased or no change in the levels of serum total cholesterol or triglycerides or LDL –cholesterol or HDL-cholesterol level on drug monotherapy.

The previous studies (21) revealed an increase in total cholesterol, triglycerides, VLDL- cholesterol, LDL-cholesterol epileptic patients on long-term treatment with phenytoin. So, particular attention has been paid on the effect of pancreatic β-cells, where it inhibits the release of insulin and suppresses the response of plasma insulin to various stimuli, thereby increasing the serum lipid levels. The effect of phenytoin may also be due to the induction of CYP enzyme. They are the inducers of CYP51 enzyme. CYP51 is a housekeeping gene of the Cytochrome- P450 super family, which is involved in cholesterol biosynthesis in humans. The CYP450 enzyme system is involved in the synthesis and metabolism of cholesterol. In particular; CYP51A1 plays a key role in cholesterol synthesis (22). A significant increase in various lipid fraction levels like total cholesterol, triglycerides, LDL-cholesterol and VLDL- cholesterol levels suggested that treatment of epileptic patients with phenytoin for long time could cause dyslipidemia in epileptic patients which in turn initiate various cardiovascular diseases like atherosclerosis.

A significant increase was recorded in liver enzymes like AST, ALT and ALP in epileptic patients treated with antiepileptic drugs such as phenytoin, valproic acid, carbamazepine, levetiracetam with respect to normal healthy subjects (Table 5). Liver enzymes such as AST, ALT, and ALP can serve as markers of hepatocellular injury or of an obstruction in the bile flow cholestasis. The significant elevations of liver enzymes are usually transitory or dose-related and might be associated with hepatocellular injury hence could lead to death or an acute liver failure which could imperatively require liver transplantation. The hepatotoxicity induced by antiepileptic drug occurs either because of production of reactive toxic metabolite/s or because of induction of immune-allergic reactions (23).

A maximum rise in the levels of AST was recorded in phenytoin treated epileptic patients in comparison to valproic acid, carbamazepine, levetiracetam treated antiepileptic treated drugs and maximum increase in the levels of ALT and ALP was seen in levetiracetam and carbamazepine respectively with respect to other antiepileptic drug treated patients (Table 6). The rise in liver enzymes in different antiepileptic drugs treated patients suggested that hepatotoxicity might be associated with other clinical manifestations of drug allergy (fever, rash and eosinophilia). This reaction is typical of carbamazepine and phenytoin. Another idiosyncratic hepatotoxic reaction comes from hepatotoxic metabolites because of aberrant metabolism (24).
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Bjornsson et al., (25) reported there was no correlation between the duration of therapy of carbamazepine and elevated liver enzymes.

Table 5: Effect on certain liver enzymes in epileptic patients on different epileptic drugs (Phenytoin, Valproic acid, Carbamazepine, Levetiracetam) and normal healthy subjects of Punjab origin

<table>
<thead>
<tr>
<th>Liver Enzymes</th>
<th>Healthy control subjects (Mean ± S.D.)</th>
<th>Epileptic patients (Mean ± S.D.)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Male (n=28)</td>
<td>Female (n=22)</td>
</tr>
<tr>
<td>AST (U/L)</td>
<td>31.11 ± 5.47</td>
<td>34.26 ± 5.13</td>
</tr>
<tr>
<td>[Reference Range: Male: &lt;35U/L Female: &lt;31 U/L]</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ALT (U/L)</td>
<td>21.98 ± 4.25</td>
<td>22.19 ± 5.27</td>
</tr>
<tr>
<td>[Reference Range: Male: &lt;45 U/L]</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ALP (U/L)</td>
<td>74.68 ± 8.11</td>
<td>76.63 ± 9.18</td>
</tr>
<tr>
<td>[Reference Range: Male: 53-128 U/L Female: 42-98U/L]</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Values in parentheses represent percentage changes w. r. t. normal healthy subjects. *P≤ 0.05, **P≤ 0.01, ***P≤ 0.001

Table 6: Effect of different epileptic drugs (Phenytoin, Valproic acid, Carbamazepine, Levetiracetam) on certain liver enzymes in epileptic patients of Punjab origin

<table>
<thead>
<tr>
<th>Liver Enzymes</th>
<th>Phenytoin (n=12)</th>
<th>Valproic Acid (n=14)</th>
<th>Carbamazepine (n=11)</th>
<th>Levetiracetam (n=13)</th>
</tr>
</thead>
<tbody>
<tr>
<td>AST (U/L)</td>
<td>41.45 ± 12.81</td>
<td>36.37 ± 15.32</td>
<td>39.42 ± 11.83</td>
<td>40.29 ± 14.06</td>
</tr>
<tr>
<td>ALT (U/L)</td>
<td>40.22 ± 11.01</td>
<td>36.43 ± 16.46</td>
<td>38.71 ± 14.35</td>
<td>43.22 ± 8.57</td>
</tr>
<tr>
<td>ALP (U/L)</td>
<td>363.03 ± 58.50</td>
<td>339.64 ± 38.21</td>
<td>375.97 ± 41.91</td>
<td>356.45 44.40</td>
</tr>
</tbody>
</table>

CONCLUSION

Aforementioned observations suggested that epileptic patients treated with phenytoin, valproic acid, carbamazepine & levetiracetam for long time could cause dyslipidemia, might be responsible for the pathophysiology of various cardiovascular diseases like atherosclerosis and increases in liver enzymes could cause hepatotoxicity in epileptic patients. So, Routine screening of complete lipid profile and hepatic enzymes level during the chronic use of antiepileptic drugs is recommended. The controlled studies with larger samples size should be carried out to reveal the further frequency and the risk factors of serious hepatotoxicity.

ACKNOWLEDGEMENTS

Authors are thankful to all the participants for giving us opportunity for being the part of this study.

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Evaluation of cytotoxic potential of L-asparaginase from Scopulariopsis brevicaulis on cell lines in vitro

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ABSTRACT

Introduction and Aim: This study reports the cytotoxic potential of L-Asparaginase isolated from the fungus Scopulariopsis brevicaulis.

Materials and Methods: Extracellular L-Asparaginase was isolated from Scopulariopsis brevicaulis and purified by ammonium sulfate precipitation, followed by dialysis, ion exchange and gel filtration chromatography. Varying concentrations (31.25, 62.5, 125, 250, 500 µg/ml) of purified L-Asparaginase was tested on MCF7, HeLa, HepG2 and 3T3L1 cell lines by MTT assay. Curcumin was maintained as a positive control.

Results: Minimum inhibition of 23.57% was observed at an enzyme concentration of 31.25 µg/ml and maximum inhibition (66.41%) was observed at 500 µg/ml against MCF7 cell line. Minimum inhibition of 2.87% was observed at an enzyme concentration 31.25 µg/ml and maximum inhibition (58.49%) was observed at 500 µg/ml against HeLa cell line. Minimum inhibition of 4.58% was shown at an enzyme concentration of 31.25 µg/ml and maximum inhibition (46.14%) was observed at 500 µg/ml against HepG2 cell line. Minimum inhibition of 1.4% was shown by enzyme concentration 31.25 µg/ml and maximum inhibition (50.9%) was observed at 500 µg/ml against 3T3L1 cell line.

Conclusion: We report for the first time the cytotoxic potential of L-Asparaginase from Scopulariopsis brevicaulis.

Keywords: L-Asparaginase; cytotoxicity; MCF-7; HeLa; HepG2; 3T3L1 cell lines; MTT; Scopulariopsis brevicaulis.

INTRODUCTION

L-Asparaginase (L-asparagine amidohydrolase EC 3.5.1.1) is a hydrolase that plays a major role in the metabolism of all living organisms specifically catalyzing the hydrolysis of L-asparagine to L-aspartic acid and ammonia (1). The reaction is irreversible when maintained at physiological conditions. The enzyme is found widespread in the nature and has been isolated from various sources such as plant tissues, bacteria, fungi, yeasts, actinomycetes, algae and the serum of rodents (2, 3).

L-Asparaginases has been isolated and purified from several different bacteria such as E. coli (3), Serratia marcescens (4), Vibrio succinogens (5), Pseudomonas acidovorans (6), Pseudomonas geniculata (7), Corynebacterium glutamicum (8) and Staphylococcus sps. (9). Fungal sources of the enzyme include Alternaria sps. (10), Aspergillus nidulans (11), A. niger (12), A. tamarii (13),
Fusarium roseum (14). Among plant species, L-asparaginase enzyme has been reported in Pisum sativum (15) and Withania somnifera (16). Among mammals L-Asparaginase was detected in guinea pigs serum (17) and agouti (2).

The enzyme is proven to have cytotoxic activity and is being used in the treatment of acute lymphoblastic leukemia (18). This is due to the fact that the leukemic cells do not have the property to produce L-asparagine, which is a non-essential amino acid, whereas the normal cells can produce their own (19). Therefore, the leukemic cells are deprived from L-asparagine which is their source of nutrition and they are prevented from malignant growth.

L-Asparaginase from bacterial origin can cause hypersensitivity in the long term leading to allergic reactions in the tissues of patients, resulting in anaphylactic shock (20). Therefore, the search for a new serologically different L-asparaginase with similar therapeutic role and less adverse effects is highly recommended. The study on purification of L-Asparaginase from Scopulariopsis brevicaulis has not been reported so far. This prompted to study further on cytotoxic property of the enzyme with reference to therapeutic purposes. Hence, attempt has been made to purify the L-Asparaginase from strain Scopulariopsis brevicaulis and study its antineoplastic effect.

MATERIALS AND METHODS

The screening, isolation, identification and preparation of crude enzyme extract of Scopulariopsis brevicaulis was carried out as mentioned in the earlier publication (21). Modified Czapek –Dox media was employed to culture the organisms by submerged fermentation. The organism produced maximum enzyme on the 10th day. Optimum temperature for the growth of the organism was found to be 37°C and pH was found to be 7.2.

Purification of L-Asparaginase

The purification of the crude extract was carried out at 4°C according to the method (6). Finely powdered ammonium sulfate was added to 80% saturation. The mixture was left overnight for 12hrs at 4°C, followed by centrifugation at 8000 rpm for 20min at 4°C. The precipitate was dissolved in 0.05M Tris HCl buffer pH 7.2 and dialyzed overnight against the same buffer at 4°C. The dialyzed sample was further purified by passing through a column of DEAE cellulose previously equilibrated with 0.05M Tris HCl buffer, pH 7.2. A total of 55 fractions were collected at the flow rate of 3 mL per 15min. Fractions showing high activity were pooled together and dialysed against the same buffer. The dialysed sample was lyophilized and concentrated. 1ml of the lyophilized sample was loaded on to the Sephacyrl S-200 gel filtration column and was eluted by using the Tris HCl buffer pH 7.2. A total of 45 fractions were collected at the rate of 3mL/15min. Fractions showing high activity were pooled together, dialysed, lyophilized and used for further studies. Total and specific enzyme activity was determined using the method briefly described (22).

In vitro cytotoxicity study

Human Cervical Cancer cell line (HeLa), Human Breast Cancer Cell line (MCF7), Human Liver Cancer Cell line (HePG2) and Mouse Embryo Fibroblast Cell lines (3T3L1) were obtained from NCCS, Pune. The cells were maintained in DMEM medium supplemented with 10% FBS and Penicillin (100U/ml) in a humidified atmosphere of 50µg/ml CO₂ at 37°C. The cytotoxicity of the sample on the cell lines was determined by MTT assay (23). 200µl of cell suspension was seeded in a 96 well plates at required cell density (20,000 cells per well), without the test agent. The cells were allowed to grow for about 24 hours. The various concentrations of the sample (31.25, 62.5, 125, 250,500 µg/mL) were added. The plates were incubated at 37°C for 48hrs in a 5% CO₂ atmosphere.
After the incubation period, the spent media was removed and MTT reagent was added to a final concentration of 0.5 mg/mL of total volume. The plates were returned to the incubator and incubated for 3 hours. The MTT was removed and 100 µl of solubilisation solution (DMSO) was added. The absorbance was read on an ELISA reader (ELX800, Biotek) at 570nm and 630 nm used as reference wavelength. The IC₅₀ value was determined graphically. All experiments were performed in triplicates.

**Assessment of Cell Morphology**

The cytotoxicity induced by purified asparaginase was confirmed microscopy. The cell lines were treated with different concentrations of the purified enzyme (31.25, 62.5, 125, 250, 500 µg/mL), after 24 h of treatment cells were subjected to investigation for morphological changes (24, 25).

**RESULTS**

**Purification of Scopulariopsis brevicaulis L-Asparaginase**

The purification profile of L-Asparaginase from *Scopulariopsis brevicaulis* is represented in (Table 1). It was purified to 107.42-fold, with a specific activity of 116.02 (IU/mL/mg) and a yield of 11.03.

**Table 1: Purity profile of L-Asparaginase**

<table>
<thead>
<tr>
<th>Steps</th>
<th>Total activity (IU/mL)</th>
<th>Total protein Conc. (mg)</th>
<th>Specific activity (IU/mL/mg)</th>
<th>Fold purification</th>
<th>Yield</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude enzyme</td>
<td>74,900</td>
<td>68,720</td>
<td>1.08</td>
<td>1</td>
<td>100</td>
</tr>
<tr>
<td>Ammonium sulphate (80%) Precipitation</td>
<td>62,414</td>
<td>18,945</td>
<td>3.2944</td>
<td>3.05</td>
<td>83.32</td>
</tr>
<tr>
<td>Dialysis</td>
<td>38762</td>
<td>4623</td>
<td>8.38</td>
<td>8.18</td>
<td>51.75</td>
</tr>
<tr>
<td>DEAE cellulose chromatography</td>
<td>9482.94</td>
<td>196.91</td>
<td>48.1580</td>
<td>44.5</td>
<td>12.66</td>
</tr>
<tr>
<td>Sephacryl S-200 gel filtration chromatography</td>
<td>8268.76</td>
<td>71.26</td>
<td>116.02</td>
<td>107.42</td>
<td>11.03</td>
</tr>
</tbody>
</table>

The purified enzyme was tested for cytotoxicity on MCF-7, HeLa, HepG2 and 3T3L1 cell lines (Table2)

**Table 2: IC₅₀ value against various cell lines**

<table>
<thead>
<tr>
<th>Sl. No</th>
<th>Cell lines</th>
<th>IC₅₀ value (µg/ml) L-Asparaginase</th>
<th>Curcumin concentration (5µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>MCF-7</td>
<td>310.12</td>
<td>51.87</td>
</tr>
<tr>
<td>2</td>
<td>HeLa</td>
<td>377.66</td>
<td>45.58</td>
</tr>
<tr>
<td>3</td>
<td>HePG2</td>
<td>&gt;500</td>
<td>35.06</td>
</tr>
<tr>
<td>4</td>
<td>3T3L1</td>
<td>454.47</td>
<td>60.7</td>
</tr>
</tbody>
</table>

**Assessment of cell morphology**

Results showed that morphological changes were increased with increasing dose of L-Asparaginase. Symptoms of apoptosis such as cellular rounding
up, cell shrinkage, membrane blebbing and loss of cell adhesion were observed (Fig 1: b, d f, h). The amount of blebbing and shrinkage of the cells were found to increase dramatically at higher concentrations of enzyme treatment. However, morphological changes were not observed with untreated cells (Fig 1: a, c, e, g). These results further suggest that purified asparaginase induced potential apoptotic effect in dose dependent manner.

**DISCUSSION**

L-Asparaginases are effective against acute lymphocytic leukemia, acute myeloid leukemia and chronic myeloid leukemia (1). Tumor cells synthesize L-asparagine slowly and are dependent on an exogenous supply. L-Asparaginase destroys extracellular source of L-asparagine and inhibits protein synthesis in lymphoblasts resulting in apoptosis. Normal cells on the other hand can synthesize L-asparagine by Asparagine synthetase and therefore tend to resist L-Asparaginase.

The anti-proliferative effects of L-asparaginase from *A. terreus* (PC-1.7 A) was evaluated after 24, 48, 72 and 96 h of incubation on two leukemia cell lines (RS4;11 and HL-60) and PBMC. There was no effect on proliferation of PBMC, whereas 50% reduction in cell viability was observed after 72 hours on the cell line HL-60 and after 96 hours on the cell line RS4; 11 (26). The cytotoxic effect of L-Asparaginase from *Aspergillus flavus* (KFF20) was studied on MCF – 7 cells and the IC₅₀ value were found to be 120.875 μg/ml (27). The incubation of Hep-G2 with gradual doses of *Penicillium brevicompactum* NRC 829 L-asparaginase lead to a gradual inhibition in the cell growth with a low IC₅₀ values of 76.4 μg/ml (28).

The purified L-Asparaginase induces apoptosis in human cancer cell lines (HL-60, MOLT-4, MDA-MB-231 and T47D.) Morphological changes during apoptosis include membrane blebbing, cell shrinkage, chromatin condensation, formation of apoptotic and scattered apoptotic bodies (29). Morphological analysis of our cell lines after L-Asparaginase treatment revealed that the cell population had reduced significantly in number. Cells undergoing apoptosis were characterized by cellular rounding up, shrinkage, membrane blebbing and loss of cell adhesion.

In our studies we observed that the four cell lines showed varied levels of inhibition as well as resistance to L-Asparaginase enzyme. MCF-7 cell lines showed an IC₅₀ value of 310.12(μg/ml), HeLa cell lines 377.66(μg/ml), whereas the HepG2 cell lines were completely resistant. 3T3L1 cell lines which are normal cells also showed...
resistance to L-Asparaginase. Probably the differences might be due to the differences in the level of purity of the enzymes used in the study. Further purification of *Scopulariopsis brevicaulis* L-Asparaginase might prove to be useful. It is not clear why in our study the cell lines appeared to be comparatively resistant to *Scopulariopsis brevicaulis* L-Asparaginase compared to other fungal Asparaginases. This is the first report on the selective cytotoxicity of L-Asparaginase from *Scopulariopsis brevicaulis*.

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REFERENCES


**In vitro studies on antioxidant, α–amylase and α–glucosidase inhibitory activities of ethanol extracts of Syzygium cumini seeds**

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**ABSTRACT**

**Introduction and Aim:** Diabetes mellitus is a metabolic disorder leads to many secondary complications. The drugs used for treatment causes serious side effects. *Syzygium cumini* is used in traditional medicine for treating many diseases. The aim of the present study is to estimate phytochemical contents, antioxidant activity, α–amylase and α–glucosidase inhibitory activities of ethanol extract of seeds of *S. cumini* by *in vitro* methodology.

**Materials and Methods:** The powdered seeds were extracted with ethanol. Quantitative analysis of Total alkaloids, Total phenols, Total flavonoids, Total tannins, Total saponins and Total steroids were carried out. DPPH scavenging activity, α–amylase and α–glucosidase inhibitory activities were measured with varying concentration of extract.

**Results:** Total alkaloids content was observed to be higher followed by Total phenol content. Total saponin was found to be present in lesser amount among the tested phytochemicals. 500 µg/ml and above concentrations of seed extract possess above 90% DPPH scavenging activity; 1000 µg/ml concentration of the extract exhibited 43.20% and 19.80% inhibition activity on α–amylase and α–glucosidase enzymes.

**Conclusion:** The above results indicate a higher antioxidant activity and appreciable inhibitory activities of enzymes responsible for elevated circulation of glucose. These activities are due to the presence of phytochemicals present in the seeds extract of *S. cumini* and can be utilized for the management of Diabetes.

**Keywords:** *Syzygium cumini*; ethanol extract; phytochemicals; antioxidant; α–amylase; α–glucosidase.

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**INTRODUCTION**

Diabetes mellitus is one of the most common chronic endocrinial metabolic disorders. Diabetes mellitus is associated with insufficient production or utilization of insulin in the body resulting elevated level of circulating blood glucose known as hyperglycemia. In India, it is expected that by the year 2025 there would be around 57.2 million people affected with diabetes (1). Oral hypoglycemic agents such as sulfonylureas, glibenclamides and biguanides and insulin are used for the treatment of diabetes. The oral hypoglycemic agents in practice were found to cause unwanted serious side effects (2). Therapeutically it is necessary to reduce the postprandial hyperglycemia to manage diabetes mellitus (3). In carbohydrate metabolism, α–amylase and α–glucosidase are the important enzymes involved in the breakdown and digestion of carbohydrates. Inhibitors of α–amylase and α–glucosidase are potential compounds for the treatment of diabetes (4).

Hence it is desirable to search for a hypoglycemic agent especially plant based having therapeutic efficacy and without any deleterious side effects. One such a plant with edible fruits is *Syzygium cumini* Skeels tree belongs to Myrtaceae family, commonly called as Jamun and it is also known as black plum, Java plum and Indian blackberry (5). In Tamil language, it is called as Naaval tree. The fruit is called as Naaval Pazham. Traditional healers use *S. cumini* for treating diabetes and related complications (5). Vaish et al., (6) reported the therapeutic effect of jamun seeds in alloxan induced diabetes.
The barks, seeds and leaves of *S. cumini* was reported to possess anti diarrheal activity (7). Seed extract of *S. cumini* was found to enhance insulin secretion in the isolated pancreatic islet cells of normal and diabetic animals. The extract was also found to inhibit the insulinase activity in liver and kidney (8, 9). It was reported that the presence of alkaloids, glycoside jambolin and jambosine in seeds of *S. cumini* cease the breakdown of starch to sugar (5). The present study was focused to estimate the phytochemical content, antioxidant and antidiabetic activity of *S. cumini* seeds extract by *in vitro* method.

**MATERIALS AND METHODS**

The fruits of *S. cumini* was collected from in and around the villages of Orathanadu Taluk, Thanjavur District. The pulp of the fruits was removed and the seeds were washed with tap water and shade dried. Dried seeds were powdered using a pulverizer. The powdered seed was extracted with ethanol. After 24 h, the insoluble content present were removed by centrifugation at 3000rpm for 15 minutes. The supernatant was rotary vacuum evaporated at 60°C and lyophilized. The dried extract was stored and used for the analysis.

**Quantitative analysis of phytochemicals**

Quantitative analysis of total alkaloids (10), Total phenols (11), Total flavonoids (12), Total tannins (13), Total saponins (14) and Total steroids (15) were carried out.

2, 2 – diphenyl – 2- picryl hydrazyl (DPPH) radical scavenging activity

The DPPH radical scavenging activity was carried out by the method of Mensor et al., (16). 1.0 ml of 0.3 mM DPPH ethanol solution was added to 2.5 ml of varied concentrations (100 - 1000μg/ml) of ethanol extract of *S. cumini* seeds and the reaction was allowed to take place at room temperature for 30 minutes and the absorbance was measured at 518nm where as 1mM Morin was used as positive control. The results were expressed as % of scavenging activity.

**α- Amylase inhibitory assay**

The α-amylase inhibition assay was carried out by 3, 5-dinitrosalicylic acid (DNSA) method (17). 100 to 1000 μg/ml concentration of seed extract was prepared by dissolving in 10% DMSO and phosphate buffer and sodium chloride at pH 6.9. Equal volumes (200μl) of α-amylase and plant extract was mixed and incubated for 10 minutes at 30°C. Then 200μl of 1% starch solution was added, after 3 minutes incubation, the reaction was terminated by DNSA reagent and the reaction mixture was boiled in a water bath for 10 minutes at 85 - 90°C. After cooling the mixture the absorbance was measured at 540 nm in a spectrophotometer. Acarbose was used as a positive control. α- amylase inhibition activity was expressed as % of inhibition.

**α- Glucosidase inhibitory assay**

It was assayed by the method of Kim et al., (18). 100μl of α glucosidase (1 U/ml) was incubated with 50μl of different concentrations of seed extract of *S. cumini* for 10 minutes. Then 50 μl of substrate p – nitrophenyl glucopyranoside (3.0 mM) was added to initiate the reaction, and the reaction mixture was incubated for 20 minutes at 37°C and terminated by the addition of 2.0ml of 0.1 M sodium carbonate solution and the activity was measured at 405 nm. The results were expressed as % of inhibition of α - glucosidase activity. Acarbose was used as a positive control.

**RESULTS**

One hundred grams of *S. cumini* seed powder was extracted with ethanol and the dry weight of the seed extract was 1.89g. Table 1 presented the Total alkaloids, Total phenol, Total flavonoids, Total tannins, Total saponin and Total steroids. Total alkaloids content was higher in the extract followed by Total phenol content. Total saponin was found to be in lower amount among the tested phytochemicals.

**Table 1. Quantitative analysis of phytochemicals in ethanol extract of seeds of *S. cumini***

<table>
<thead>
<tr>
<th>Phytochemicals</th>
<th>Quantity (mg/g)</th>
</tr>
</thead>
</table>

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Table 2 represented the DPPH scavenging activity, \(\alpha\) – amylase inhibition and \(\alpha\) – glucosidase inhibition activity of ethanol seed extract of S. cumini. 100 – 1000 \(\mu\)g/ml concentration of ethanol seed extract of S. cumini was tested. 500 \(\mu\)g/ml concentration of ethanol extract exhibited more than 90% scavenging activity (98.11%). All the higher concentrations above 500 \(\mu\)g/ml expressed 99% scavenging activity. 1000 \(\mu\)g/ml concentration of seeds extract exhibited 43.20% of \(\alpha\) – amylase inhibition activity and 19.80% of \(\alpha\) – glucosidase inhibition activity whereas positive control acarbose exhibited 99.30% and 93.70% inhibition activity respectively. There was found to be a gradual increase in the inhibition of enzyme activity with the increasing concentration of the seeds extract.

Table 2. DPPH scavenging activity, \(\alpha\) – amylase inhibition and \(\alpha\) – glucosidase inhibition activity of ethanol extracts of seeds of S. cumini.

<table>
<thead>
<tr>
<th>Extract Concentration ((\mu)g/ml)</th>
<th>% of DPPH scavenging activity</th>
<th>% of (\alpha)–amylase inhibition activity</th>
<th>% of (\alpha)–glucosidase inhibition activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>100</td>
<td>49 ± 0.98</td>
<td>3.70 ± 0.20</td>
<td>0.40 ± 0.10</td>
</tr>
<tr>
<td>200</td>
<td>61.45 ± 1.22</td>
<td>8.00 ± 0.30</td>
<td>2.00 ± 0.10</td>
</tr>
<tr>
<td>300</td>
<td>75.78 ± 1.67</td>
<td>13.10 ± 1.1</td>
<td>3.90 ± 0.20</td>
</tr>
<tr>
<td>400</td>
<td>88.61 ± 1.88</td>
<td>17.80 ± 1.4</td>
<td>6.20 ± 0.20</td>
</tr>
<tr>
<td>500</td>
<td>98.11 ± 2.32</td>
<td>22.40 ± 2.10</td>
<td>8.60 ± 0.30</td>
</tr>
<tr>
<td>600</td>
<td>99.65 ± 2.21</td>
<td>28.20 ± 2.30</td>
<td>11.10 ± 0.60</td>
</tr>
<tr>
<td>700</td>
<td>99.61 ± 1.34</td>
<td>33.70 ± 2.90</td>
<td>13.50 ± 0.70</td>
</tr>
<tr>
<td>800</td>
<td>99.59 ± 1.31</td>
<td>36.90 ± 3.0</td>
<td>15.90 ± 0.90</td>
</tr>
<tr>
<td>900</td>
<td>99.80 ± 0.26</td>
<td>36.90 ± 3.1</td>
<td>17.40 ± 1.0</td>
</tr>
<tr>
<td>1000</td>
<td>99.79 ± 0.31</td>
<td>43.20 ± 3.5</td>
<td>19.80 ± 1.1</td>
</tr>
<tr>
<td>Positive control</td>
<td>99 ± 0.98</td>
<td>99.30 ± 1.1</td>
<td>93.70 ± 2.5</td>
</tr>
</tbody>
</table>

(Values are expressed as Mean ± SD of triplicates)

DISCUSSION

Phytochemical analysis plays a greater role to identify a compound of therapeutic efficiency. Earlier qualitative studies in our laboratory on the seeds extract of S. cumini revealed the presence of alkaloid, phenol, flavonoid, tannin, saponin, Steroids, cardiac glycosides, anthraquinone glycosides, oils and fats, lignin, terpenoids, phlobatannins, coumarin, quinine, sugar and amino acids. In diabetic condition an imbalance between free radicals and antioxidant scavenging system occurs result in elevated lipid peroxidation and complications (19, 20). It was reported that the phytochemicals like flavonoids and total phenols present in the seed extract were found to serve as an antioxidant and account for the scavenging effect on free radicals (21, 22). Reddy et al (23) have reported that polyphenol rich plant foods have insulin like effect and also acts as inhibitors of enzymes like \(\alpha\)- amylase and \(\alpha\)- glucosidase. \(\alpha\)- amylase is an enzyme that functions by cleaving the carbohydrates into smaller units of saccharides and \(\alpha\)-glucosidase breaks the 1,4-\(\alpha\)-bonds of starch and disaccharides to glucose(24). \(\alpha\) -glucosidase found in the small intestine brush border cell lining. Drugs serves as Inhibitors of \(\alpha\)- glucosidase inhibit the breakdown of
carbohydrates as a result slows down the absorption of glucose and decrease the level of postprandial hyperglycemia (3, 4). Alpha-glucosidase inhibitors act by inhibiting the enzyme. The drugs which are for treating type II Diabetes includes enzyme inhibitors produces side effects like diarrhea, abdominal bloating and flatulence (25). The observations in the present study have shown 43.20% inhibition of α-amylase and 19.80% inhibition of α-glucosidase enzymes by the ethanol extracts of seeds of S. cumini. The synthetic drugs may be effectively replaced by seeds of S. cumini in the management of diabetes.

CONCLUSION

The results of the present work indicated the antioxidant potential and hypoglycemic activity of seeds of Syzygium cumini by inhibiting the activity of key enzymes in digestion of carbohydrates and thereby support the traditional use of this plant for the treatment and management of diabetes mellitus.

REFERENCES


Taurine supplementation restores antioxidant status and hepatic membrane-bound enzymes in streptozotocin-induced diabetic rats

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ABSTRACT

Introduction and Aim: Chronic hyperglycemia in diabetes causes cellular damage through increased lipid peroxidation and reduced levels of antioxidants. The activities of membrane-bound enzymes are affected by oxidative stress. Taurine, a sulfur containing amino acid is shown to have hypoglycemic activity, antioxidant property and membrane stabilization. The aim of the study is to check the effect of supplementation of taurine on lipid peroxidation, antioxidant status and hepatic membrane-bound enzymes in streptozotocin-induced diabetic rats.

Materials and Methods: Thirty-two Wistar male albino rats of 19±1 weeks of age weighing 200-220 grams were randomly divided into four groups and each group consisted of eight animals. Group I (control) standard chow diet; Group II (chow diet with taurine); Group III (diabetes induced) and Group IV (diabetic receiving taurine). At the end of 45th day, all animals were sacrificed by cervical decapitation after overnight fasting. Blood and liver tissue samples were collected. The levels of glucose in plasma and lipid peroxidation, antioxidants and the activities of Na⁺/K⁺, Ca²⁺ and Mg²⁺-ATPases in liver homogenate were analyzed.

Results: Altered levels of antioxidants and activities of Na⁺/K⁺, Ca²⁺ and Mg²⁺-ATPases were restored to normal by taurine supplementation in diabetic rats.

Conclusion: The present study indicates that supplementation of taurine could protect liver plasma membrane against oxidative damage by acting as antioxidant and restoring the normal activities of Na⁺/K⁺, Ca²⁺ and Mg²⁺-ATPases.

Keywords: Taurine; lipid peroxidation; antioxidants; membrane-bound enzymes; ATPases.

INTRODUCTION

Diabetes mellitus (DM) is a metabolic disorder characterized by chronic hyperglycemia which causes cellular damage through several pathways (1). Free radicals are formed disproportionately in diabetes by auto-oxidation of glucose, non-enzymatic glycation of proteins and subsequent oxidative degradation of glycated proteins (2). The free radicals are neutralized by the antioxidants present in the living system. However, excess production of free radicals exhausts antioxidants, which results in oxidative stress (3). Oxidative stress plays a major role in the development of complications in DM (4). ATPases are membrane-bound enzymes which
regulate movements of many different types of ions or molecules across biological membranes (5). They play a significant role in many metabolic pathways and in a variety of pathological processes. For instance, the activity of Na\(^+\)/ K\(^+\)-ATPase is impaired in the cell membrane of various tissues in diabetic subjects and this defect may play a role in the development of complications (6). Findings of many in vivo and in vitro studies indicated that the activities of these enzymes were altered due to oxidative stress in DM.

Taurine, 2-aminoethanesulfonylic acid is present as a free amino acid in mammalian tissues like liver, heart, brain, and leukocytes. It is hypoglycemic, hypolipidemic and anti-atherosclerotic and an effective antioxidant (7). It plays a major role in the maintenance of various cellular functions like osmoregulation, neuromodulation, detoxification, bile acid conjugation, calcium homeostasis, and membrane stabilization (8). It is considered as semi essential nutrient but cells deficient with taurine show various pathologies (9). Various clinical complications observed due to altered metabolism of taurine in DM showed positive outcomes with taurine supplementation (10). The aim of the present study was to investigate the effect of taurine supplementation on lipid peroxidation and antioxidant status and the hepatic membrane-bound enzymes such as Na\(^+\)/ K\(^+\), Ca\(^{2+}\) and Mg\(^{2+}\)-ATPases in streptozotocin (STZ) - induced diabetic rats.

**MATERIALS AND METHODS**

Wistar strain male albino rats of 19±1 weeks of age weighing 200-220 grams were obtained from the Central Animal House, Rajah Muthiah Medical College and Hospital (RMMC&H), Annamalai University. They were housed in standard conditions and maintained on a standard chow diet and water ad libitum. They were randomly divided into four groups and each group consisted of eight animals. Group I (control) standard chow diet; Group II (chow diet plus taurine) Group III (diabetes induced), Group IV (diabetic receiving taurine). After 24-hour fasting, the animals were injected intraperitoneally with STZ at a dose of 50 mg/kg body weight in 0.1 M citrate buffer (pH 4.5). The control animals received citrate buffer alone. Diabetes was confirmed by measuring the fasting plasma glucose concentration 48 hour after STZ injection. Taurine was administered orally once daily at a dose of 100 mg/kg body weight. At the end of 45\(^{th}\) day, all animals were sacrificed by cervical decapitation after overnight fasting. Blood samples were collected in heparinized tubes and liver specimens were collected in homogenizing buffer (0.1 M Tris-HCl, pH 7.4). The study was approved by the Institutional Animal Ethics Committee (IAEC).

**Biochemical analyses**

Plasma glucose was determined by glucose oxidase-peroxidase (GOD-POD) method by using Boehringer Mannheim reagent kit in Erba Smart Lab analyzer, USA. A portion of the liver was homogenized, and the homogenate was used for the estimation of lipid peroxidation as thiobarbituric acid reactive substances (TBARS), antioxidants, and membrane-bound enzymes. TBARS was estimated by the methods of Yagi (11) and Ohkawa et al., (12). The activities of superoxide dismutase (SOD) by the method of Kakkar et al., (13) Catalase (CAT) by Sinha (14) Glutathione peroxidase (GPX) by Rotruck (15) and the concentration of reduced glutathione (GSH) by Beutler (16). Na\(^+\)/ K\(^+\)-ATPase activity was assayed by the method of Bonting (17), Mg\(^{2+}\) -ATPase by the method of Ohnishi et al. (18) and Ca\(^{2+}\)-ATPase by the method of Hjerten and Pan (19).

**Statistical Analysis**

One-way analysis of variance (ANOVA) test was applied in order to evaluate any significant difference in the mean values. All values used in analysis represent the mean ± SD of eight rats in each group. The results were considered
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statistically significant if the p values were 0.05 or less.

RESULTS
Table 1 shows the status of plasma glucose in control and experimental animals in each group.

Table 1: Food intake, body weight and plasma glucose levels in experimental animals

<table>
<thead>
<tr>
<th>Groups</th>
<th>Food Intake (gm)</th>
<th>Body weight (gm)</th>
<th>Plasma glucose(mg/dl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>18.22 ± 0.05</td>
<td>244.1 ± 8.94</td>
<td>103.75 ± 4.77</td>
</tr>
<tr>
<td>Control + Taurine</td>
<td>18.35 ± 0.06*</td>
<td>241.5 ± 10.86*!</td>
<td>100.25 ± 2.92*</td>
</tr>
<tr>
<td>Diabetic</td>
<td>20.82 ± 0.14**</td>
<td>202.75 ± 13.35**!</td>
<td>344.13 ± 41.71**#</td>
</tr>
<tr>
<td>Diabetic + Taurine</td>
<td>19.53 ± 0.16***</td>
<td>221.00 ± 11.01***#</td>
<td>329.52 ± 7.4***</td>
</tr>
</tbody>
</table>

Values are expressed as mean ± SD. *Group 2 compared with Group 1; ** Group 3 compared with Group 1 ***Group 4 compared with Group 3; !p <0.05; # p<0.001.

Table 2 shows the hepatic lipid peroxidation and antioxidant status. The levels of TBARS were significantly raised in diabetic animals. Supplementation of taurine reduced their levels significantly. The activities of antioxidants-SOD, CAT and concentration of GSH were significantly reduced while the activities of GPX were markedly increased in diabetic rats. Taurine administration restored to their normal status.

Table 2: Hepatic lipid peroxidation and antioxidant status

<table>
<thead>
<tr>
<th>Groups</th>
<th>TBARS</th>
<th>SOD</th>
<th>Catalase</th>
<th>GPX</th>
<th>GSH</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.34 ± 0.40</td>
<td>5.87± 0.13</td>
<td>52.5 ± 0.55</td>
<td>3.66 ± 0.37</td>
<td>5.01± 0.11</td>
</tr>
<tr>
<td>Control + Taurine</td>
<td>0.28 ± 0.42*</td>
<td>5.51 ± 0.06*#</td>
<td>53.29 ± 0.56*!</td>
<td>3.81 ± 0.09*#</td>
<td>5.22 ± 0.14*!</td>
</tr>
<tr>
<td>Diabetic</td>
<td>0.49 ± 0.06**</td>
<td>3.92 ± 0.68**#</td>
<td>34.88 ± 0.55**#</td>
<td>6.8 ± 0.06**#</td>
<td>2.83 ± 0.39**#</td>
</tr>
<tr>
<td>Diabetic + Taurine</td>
<td>0.37 ± 0.02***#</td>
<td>5.78 ± 0.70***#</td>
<td>41.13 ± 1.16***#</td>
<td>4.14 ± 0.08***#</td>
<td>4.28 ± 0.62***#</td>
</tr>
</tbody>
</table>

Values are expressed as mean ± SD. *Group 2 compared with Group 1; ** Group 3 compared with Group 1 ***Group 4 compared with Group 3; !p <0.05; # p<0.001.

TBARS – nmoles /mg of protein; Catalase- μ moles of H₂O₂ utilized/min / mg of protein; GPX – μg of GSH consumed / min/ mg of protein; SOD- 50% inhibition of NBT reduction/min / mg of protein, GSH- mg/ 100g tissue.

Table 3 shows the activities of hepatic membrane-bound enzymes. The activities of Na⁺/ K⁺, and Mg²⁺-ATPases were profoundly reduced while the activities Ca²⁺-ATPase were overexpressed in diabetic rats. These activities were restored to normal with taurine supplementation.

Table 3: Hepatic membrane-bound enzymes

<table>
<thead>
<tr>
<th>Groups</th>
<th>Na⁺/ K⁺-ATPase U/mg of protein</th>
<th>Mg²⁺-ATPase U/mg of protein</th>
<th>Ca²⁺ATPase U/mg of protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>1.845 ± 0.056</td>
<td>2.575 ± 0.406</td>
<td>1.79 ± 0.067</td>
</tr>
<tr>
<td>Control + Taurine</td>
<td>1.25 ± 0.057*</td>
<td>2.297 ± .84*</td>
<td>1.783 ± 0.069*</td>
</tr>
<tr>
<td>Diabetic</td>
<td>0.798 ± 0.05***#</td>
<td>1.537 ± 0.287***!</td>
<td>4.74 ± 0.142***#</td>
</tr>
<tr>
<td>Diabetic + Taurine</td>
<td>1.562 ± 0.424***#</td>
<td>2.187 ± 0.454***!</td>
<td>2.415 ± 0.384***#</td>
</tr>
</tbody>
</table>

Values are expressed as mean ± SD. *Group 2 compared with Group 1; ** Group 3 compared with Group 1 ***Group 4 compared with Group 3; !p <0.05; # p<0.001.

DISCUSSION
The present study found that taurine supplementation did not improve the glycemic status in diabetic rats. This is in contrast to earlier studies which reported that taurine was effective in reducing hyperglycemia (20, 21). This could be
attributed to the low dosage used in the present study. Hyperglycemia is the most important factor in the onset and progress of diabetic complications. Diabetes is always associated with over generation of free radicals and depletion of antioxidants with concomitant oxidative stress (2, 3, 8). In hyperglycemia, the excess polyol pathway causes depletion of NADPH which is required by glutathione reductase for regeneration of GSH from glutathione disulfide (GSSG). This explains the decreased concentration of GSH and overexpression of GPX. This indicates the oxidative stress in the background of hyperglycemia. Many animal studies showed that taurine supplementation was beneficial to diabetes and its complications (20, 21). The present study observed that taurine administration restored the hepatic antioxidant enzyme activities and reduced glutathione. This could be due to the action of taurine by preventing the generation of oxidants (22).

ATPases are very sensitive to oxidative stress which inactivates the enzyme by modifying the active site (7). The present study found that the activities Na+/ K+ and Mg2+-ATPases in liver homogenates were significantly decreased while the activities of Ca2+-ATPase was found to be increased. Taurine supplementation restored the normal activities of these ATPases. The sulfonic acid group of taurine plays a major role in preventing a direct attack by oxidants on cell membranes (23). These calcium pumps are the major targets that are readily affected in diabetes (24). The over expression of Ca2+-ATPase may be due to the energy imbalance because of impairment of Na+/ K+, and Mg2+-ATPases. The present study strengthens the findings of the various animal studies. As some of the findings of the human studies are inconclusive, more studies on human trials will be of highly useful. If an effective dose of taurine for human beings is established, that could be used in the prevention of diabetic complication.

CONCLUSION

The present study found that lowering of glucose induced lipid peroxidation and enhancement of the antioxidant status. The activities of hepatic Na+/ K+, Ca2+ and Mg2+-ATPases are preserved by taurine supplementation, implicating the inhibition of development of complication in diabetes mellitus.

REFERENCES

Correlation of red cell indices and hemoglobin concentration with serum ferritin among iron deficiency anemia patients

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ABSTRACT

Introduction and Aim: Iron deficiency anemia is the commonest cause of anemia in developing country like India in all age groups. It is most easy to prevent as well as to treat. Its diagnosis and treatment are based on serum ferritin levels in developed countries which is not possible in India at primary healthcare setting. This study was undertaken to explore if red cell indices could replace serum ferritin in detecting iron deficiency.

Materials and Methods: Study of association of red cell indices like Mean Corpuscular Volume (MCV), Mean Corpuscular hemoglobin (MCH), Mean Corpuscular hemoglobin Concentration (MCHC), Red cell Distribution Width (RDW) and Hemoglobin concentration (Hb%) with iron deficiency anemia and the correlation of these Red cell indices and Hb% with serum ferritin was done in 220 anemia patients of all age groups with Hb% <12g/dL.

Results: Descriptive data showed skewed distribution of serum ferritin. Statistically significant reduction in all red cell indices values among iron deficiency anemia patients and statistically significant correlation of all red cell indices values except red cell distribution width with serum ferritin was found.

Conclusion: Unlike developed countries we can use simple estimation of hemoglobin concentration along with red cell indices for diagnosing iron deficiency anemia in primary healthcare setting of India.

Keywords: Anemia; iron deficiency; ferritin; RBC indices.

INTRODUCTION

Iron deficiency is one of the commonest nutritional deficiencies in India as well as in the world (1, 2). Most important effect of iron deficiency is anemia. India tops the list of nations with the highest number of anemic women and children (3) and the main cause is iron deficiency. According to NFHS-4 survey 60.9% of children aged 6-59 months and 44.8% of women aged 15-49 years are anemic (3).

Iron deficiency anemia leads to fatigue, low quality of life, breathlessness, recurrent infections, stress, low productivity etc. It is also associated with poor maternal and birth outcomes. A 1 g/dL increase in Hemoglobin has been estimated to decrease the risk of maternal mortality by 29% and perinatal mortality by 28% (4, 5). Anemia was also quantified to account for close to 9% of the total global disability burden from all causes (6).
Iron is also required for neural metabolic activities and for synthesis of neurotransmitters like Dopamine, Serotonin and GABA (7). Iron deficiency leads to decreased attentiveness and impaired cognitive function in children (8, 9). Hair loss is also linked to iron deficiency. Iron is essential for immune function (10, 11), body temperature regulation (12), thyroid function (13) and vascular tone regulation by binding to nitric oxide (14).

Anemia is considered as a late manifestation of iron deficiency (15, 16). So normal hemoglobin levels do not rule out iron deficiency. Bone marrow aspiration to assess iron stores is the definitive test. But it is invasive. Estimation of serum ferritin levels is the gold standard test to assess iron status (17). But it is expensive, and the facilities are not distributed uniformly. Hence this study was undertaken to assess the correlation of Red cell indices and hemoglobin concentration with serum ferritin levels and to know if Red cell indices can be used for detecting iron deficiency at the primary healthcare level.

**Objectives**

1. To estimate hemoglobin concentration (Hb%), Hematocrit (Hct), Mean Corpuscular Volume (MCV), Mean Corpuscular hemoglobin (MCH), Mean Corpuscular hemoglobin Concentration (MCHC), Red cell Distribution Width (RDW) and Serum ferritin in the study population.

2. To study the association of red cell indices and hemoglobin concentration with iron deficiency anemia in the study population.

3. To study the correlation of these Red cell indices and hemoglobin concentration with serum ferritin among study population.

**METHODOLOGY**

**Study design:** Cross sectional study.

**Study setting:** K R hospital attached to Mysore Medical College and research Institute.

**Study population:** Patients seeking care in outpatient departments of Medicine, OBG & Pediatrics. Sample size was estimated with the prevalence of Iron Deficiency Anemia at 70% (3), 5% chance and 20% absolute error. It was found to be 179. Our study included 220 subjects. Convenient sampling methodology was adopted.

**Inclusion criteria:** Subjects of both genders of all age groups, whose Hb% was < 12gm/dL, who were willing to participate in the study. Exclusion criteria: None After obtaining consent, clinically suspected anemia cases were subjected to Hb% estimation and subjects with Hb% <12gm/dL were further evaluated for IDA by complete hemogram and serum ferritin estimation. Subjects were sent to central laboratory attached to K R Hospital for collection of blood sample and estimation of complete hemogram & serum ferritin. Blood samples were collected in EDTA (2ml) and sterile vacutainer (3 ml). EDTA anticoagulated sample was used for estimation of Hb% and Red cell indices on Sysmex semi-automated cell counter. Serum was separated from the sample in sterile tube sample and Ferritin levels were estimated on automated chemiluminescence immune analyzer. The Association of red cell indices and hemoglobin with iron deficiency anemia and correlation between Red cell indices & Hb% with serum ferritin was estimated.

R software was used for analysis of data.

**RESULTS**

The study included 220 subjects of all age group with 178 females and 42 males.

Table 1a gives the descriptive data of age, hemoglobin concentration, hematocrit, and red cell indices of the study population. It shows mean age to be 34.5 years with std. deviation of 12 and mean MCV 69.7fL with std. deviation 7.1. Since serum ferritin distribution was skewed, a better summary
measures of median and quartiles were used for its analysis and tabulated (Table 1b), which shows a median of 43.8 and std. deviation of 43.55.

Table 1a: Descriptive measures of all the indices in the sample (n=220):

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Minimum</th>
<th>Maximum</th>
<th>Mean</th>
<th>Std. Deviation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (yrs)</td>
<td>18</td>
<td>58</td>
<td>34.52</td>
<td>12.089</td>
</tr>
<tr>
<td>Hb% (g/dL)</td>
<td>6.0</td>
<td>11.7</td>
<td>9.651</td>
<td>1.4186</td>
</tr>
<tr>
<td>Hct (%)</td>
<td>22.0</td>
<td>36.0</td>
<td>29.817</td>
<td>3.4278</td>
</tr>
<tr>
<td>MCV (fL)</td>
<td>55.0</td>
<td>85.0</td>
<td>69.709</td>
<td>7.1211</td>
</tr>
<tr>
<td>MCHC (%)</td>
<td>28.0</td>
<td>36.0</td>
<td>31.591</td>
<td>1.6008</td>
</tr>
<tr>
<td>MCH (pg)</td>
<td>16.0</td>
<td>30.0</td>
<td>22.391</td>
<td>3.4511</td>
</tr>
<tr>
<td>RDW (%)</td>
<td>10.6</td>
<td>18.0</td>
<td>14.039</td>
<td>1.8430</td>
</tr>
</tbody>
</table>

Table 1b: Descriptive measure of serum ferritin (ng/ml; n=220)

<table>
<thead>
<tr>
<th>Mean</th>
<th>56.328</th>
</tr>
</thead>
<tbody>
<tr>
<td>Std. Error of Mean</td>
<td>2.9364</td>
</tr>
<tr>
<td>Median</td>
<td>43.850</td>
</tr>
<tr>
<td>Std. Deviation</td>
<td>43.5538</td>
</tr>
<tr>
<td>Minimum</td>
<td>12.6</td>
</tr>
<tr>
<td>Maximum</td>
<td>240.0</td>
</tr>
<tr>
<td>Percentiles</td>
<td></td>
</tr>
<tr>
<td>25</td>
<td>30.900</td>
</tr>
<tr>
<td>50</td>
<td>43.850</td>
</tr>
<tr>
<td>75</td>
<td>70.500</td>
</tr>
</tbody>
</table>

A serum ferritin cutoff of <30ng/ml (18) was used as diagnostic for iron deficiency anemia, and the association of all red cell indices and hemoglobin concentration was done with iron deficiency anemia (Table 2). It shows statistically significant reduction in all the parameters used in iron deficiency anemia with p value <0.001 for hemoglobin concentration, hematocrit, MCV, MCHC and MCH, and 0.015 for RDW.

Table 2: The association of parameters used with iron deficiency anemia

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Iron deficiency anemia</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Present Mean (SD)</td>
<td>Absent Mean (SD)</td>
</tr>
<tr>
<td>Hb%</td>
<td>8.5(1.8)</td>
<td>10(0.97)</td>
</tr>
<tr>
<td>Hct</td>
<td>27.6(4.5)</td>
<td>30.6(2.5)</td>
</tr>
<tr>
<td>MCV</td>
<td>65.9(7.4)</td>
<td>70.9(6.5)</td>
</tr>
<tr>
<td>MCHC</td>
<td>30.4(2.1)</td>
<td>31.9(1.1)</td>
</tr>
<tr>
<td>MCH</td>
<td>20.3(3.8)</td>
<td>23.0(3)</td>
</tr>
<tr>
<td>RDW</td>
<td>14.5(2.1)</td>
<td>13.8(1.7)</td>
</tr>
</tbody>
</table>

Spearman correlation was used to find the correlation of hemoglobin and red cell indices with serum ferritin since serum ferritin did not follow normal distribution. It was tabulated in Table 3 and scatter plots were drawn to look for correlation. It shows there is statistically
significant positive correlation of serum ferritin value <0.001.

Table 3: Correlation of red blood cell indices and hemoglobin with serum ferritin among anemia patients

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Serum ferritin Spearman correlation coefficient</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hb%</td>
<td>0.56</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Hct</td>
<td>0.55</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>MCV</td>
<td>0.48</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>MCHC</td>
<td>0.56</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>MCH</td>
<td>0.53</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>RDW</td>
<td>0.04</td>
<td>0.537</td>
</tr>
</tbody>
</table>

Scatter plot of Hb% and Serum ferritin.

Scatter plot of MCHC and serum ferritin.

Scatter plot of MCV and serum ferritin.

Scatter plot of MCH with serum ferritin.

Scatter plot of Hct and serum ferritin.

Scatter plot of RDW with serum ferritin.
DISCUSSION

This was a cross sectional study done using routine clinical and laboratory data carried out among anemia patients attending the outpatient department.

There was statistically significant association between all the parameters used i.e. Hemoglobin concentration, Hematocrit, Mean Corpuscular Volume, Mean Corpuscular Hemoglobin Concentration, and Mean Corpuscular Hemoglobin (with p value < 0.001) and Red Cell Distribution Width (with p value < 0.05) with Iron Deficiency Anemia. In a study done in Mexico by Hershko et al, it was found that combined use of just MCV and serum ferritin was enough to identify whether individual was normal or β-thalassemia traits or iron deficient with accuracy rate of >95% (19).

As for iron deficiency anemia in general public it was found that there was statistically significant positive correlation of all the parameters used except Red cell distribution width with serum ferritin with p value < 0.001. These findings were similar to the study done by Rigvarthan et al., in pregnant Indian women of 2nd and 3rd trimester, in which the result showed significant association of red cell indices with best cut off of Hemoglobin concentration < 9.6gm/dL (89.5% sensitivity, 83% specificity) and of Mean Corpuscular Volume < 75.6fL (85.7% sensitivity, 80.1% specificity) for detecting IDA (20).

Further to this study results, many studies have found Mean Corpuscular Volume, Mean Corpuscular Hemoglobin and Red Cell Distribution Width as the important parameters for screening as well as for detecting Iron deficiency anemia (21).

Few studies suggest serum ferritin as the most sensitive parameter of iron stores especially in pregnant women against any other hematological parameter as the concentration of serum ferritin is not affected by Hemo-dilution in pregnancy (22).

But India being developing country and many infections and cross infections are common and hence serum ferritin is not a reliable indicator of Iron stores (23) as it is an acute phase reactant which is elevated in infections in spite of iron deficiency.

Since significant correlation of red cell indices and Hemoglobin concentration with serum ferritin was found in the study, use of those parameters alone for detecting Iron deficiency can be taken into consideration. Further studies are required with a larger sample size.

REFERENCES


Effect of ethanolic seed extract of *Caesalpinia bonducella* on Mifepristone induced PCOS rats

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ABSTRACT

**Introduction and Aim:** PCOS is a complicated endocrinopathy of women in reproductive age with unknown etiology causing ovulatory dysfunction and metabolic disturbances. The present study investigated the effects of ethanolic seed extract of *Caesalpinia bonducella* (ESECB) in a mifepristone model of polycystic ovarian syndrome, which has the same clinical and metabolic features as in PCOS women.

**Materials and Methods:** A daily administration of mifepristone (4mg/kg b.w.) to female rats for 8 days induces PCOS by indicating persistent estrous cycle. PCOS induced rats were treated with ESECB 200mg and 400mg/kg b.w. *per oral* for 28 days and at the end, the weight of the body and reproductive organs were determined. Biochemical parameters were also estimated. Metformin was used as a standard drug.

**Results:** A significant increase ($p<0.01$) in the weight of the body and reproductive organs as well as hyperglycemia and dyslipidemia were observed in the PCOS induced animals, which were reduced considerably in the ESECB drug treated animals. Among two concentrations the animals which received 400mg/kg b.w. of ESECB drug showed significant effect when comparable to the animals which received 200mg/kg b.w. of ESECB.

**Conclusion:** Based on these observations the ethanolic seed extract of *Caesalpinia bonducella* (ESECB) can be used as a potential drug in the management of PCOS.

**Keywords:** *Caesalpinia bonducella*; ESECB; PCOS; Mifepristone.

INTRODUCTION

Polycystic Ovary Syndrome (PCOS) is considered as a multifaceted disorder with endocrinological and metabolic complications in women of reproductive age groups leading to the major cause of decrease in fertility (1). The pathophysiological symptoms include oligo menorrhea, amenorrhea, anovulation, numerous antral follicles, hyper androgenemia, and hirsutism (2) with metabolic abnormalities such as obesity, dyslipidemia, and insulin resistance (3). The treatment of PCOS can be done in several ways in spite of the unknown etiology and therefore regarded as an important research topic to the scientific community with main focus on lipid imbalance, oxidative stress, insulin resistance and genetics. Current PCOS treatment mainly concentrates to induce ovulation in the form of fertility enhancing procedures. Since these drugs will have severe side effects such as arthritis, joint or muscle pain and psychological disturbances, an alternative therapeutic approach is needed (4). The treatment of PCOS requires a multi therapeutic approach, and now a day’s worldwide the focus is being laid
on phytotherapy which is constituted with huge number of phytochemicals and having the advantage of minimal or no side effects. The plant *Caesalpinia bonducella* belongs to the family *Caesalpiniaceae* which is commonly called as “Kazharchikai” in Tamil and is found all over India especially in tropical regions of the world (5). The seed is called as “bonduc nut” or “fever nut” and is hard, glossy, and greenish to ash grey in color (6). The seeds possess multiple therapeutic properties like immunomodulatory, antioxidant, anti-inflammatory, antipyretic, analgesic (7, 8) hypoglycemic, anti-hyperglycemic, and hypo-lipidemic (9, 10). Vidyasagar and Prashant Kumar (11) have reported that the leaf powder is used for the gynecological problems like menorrhagia and leucorrhoea. Though the seed possesses several medicinal values, it has not been scientifically evaluated in the treatment of PCOS. Hence the present investigation was undertaken to assess the ameliorative effect of ethanolic seed extract of *Caesalpinia bonducella* (ESECB) on mifepristone induced PCOS in female rats.

**MATERIALS AND METHODS**

The chemical mifepristone was purchased from Merck Millipore. The kits used for the analysis were obtained from Ranbaxy India Ltd. All chemicals used are of analytical grade.

**Collection and preparation of ethanolic seed extract of *Caesalpinia bonducella* (ESECB)**

The seeds of *C. bonducella* were obtained from local market, Chennai, India and were authenticated by Dr. Mythreyi, Professor of Pharmacognosy Department, K.K. College of Pharmacy, Chennai, India. The voucher specimen is deposited in above mentioned venue for future reference. The seeds were crushed into a coarse powder after shade drying and were extracted using 90% ethanol at room temperature by cold maceration process for three days. The ethanolic extract was subjected to filtration and evaporated using rota flash evaporator for solvent evaporation. The yield of the sample was calculated and the extract was preserved in the refrigerator till investigations. The yield was found to be 1.25 %w/w.

**Animals**

Adult albino female rats of Wistar strain were used in the pharmacological studies. The inbred animals were taken from animal house in C. L Baid Mehta College of pharmacy, Chennai, India. The maintenance of animals were carried out with natural 12 ± 1 h day–night cycle, in the propylene cages in a well ventilated room temperature and throughout the experimental period were given balanced rodent diet in the form of pellets and tap water *ad libitum*. Before the experimental procedure, they were sheltered for one week in the laboratory for acclimatization. The Animal Ethics Committee constituted for the purpose as per CPCSEA (Committee for the Purpose of Control and Supervision of Experiments on Animals), approved the experimental protocol.

**Acute toxicity studies**

The acute toxicity studies were already conducted for *Caesalpinia bonducella* ethanolic extract by Kshirsagar *et al.*, (12) as per the OECD guidelines 420, up to the test dose of 2000mg/kg and reported that no mortality was observed and the biochemical and hematological parameters were normal. One tenth of the LD50 dose that is 200mg/kg b. w. was selected for studying the efficacy of the ESECB extract on PCOS treatment and in addition a higher dose of 400mg/kg b. w. was also used in the study.

**Induction of PCOS by Mifepristone in female rats**

A group of four-day cyclic Wistar female rats, were injected subcutaneously (sc) with the Mifepristone RU486, (4 mg RU486/0.2 ml oil) over eight consecutive days starting from the estrous cycle by the method of Sanchez-Criado.
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(13). After the injection, daily vaginal smear test was prepared to observe and monitor variation of estrous cycle and its irregularity. Once persistent vaginal cornification (PVC) stage is achieved, an indicator of follicular cysts in ovaries the animal grouping was done for further treatment.

Animal grouping and Treatment pattern

Thirty adult female rats were randomly divided into five groups of six animals each.

Group I- (Normal control) was administered only with the vehicle (2% CMC suspension).

Group II- Served as negative control received Mifepristone RU486 alone.

Group III- PCOD induced rats treated with ESECB200 mg/kg per oral for 28 days.

Group IV - PCOD induced rats treated with ESECB 400 mg/kg per oral for 28 days.

Group V- Served as standard and treated with Metformin 20mg/kg per oral for 28 days.

Blood sample and organ collection

After the last dosing of ESECB, all the animals were sacrificed by employing euthanasia procedure and the ovaries and uterus were identified, dissected out, blotted free of blood and cleared of connective tissue or fat. The weight of the reproductive organs was immediately taken. Blood samples were collected by retro-orbital puncture into anticoagulant pre-coated plain sterile Eppendorf tubes as well as plain sterile Eppendorf tubes and allowed to clot at room temperature. Serum samples was separated by centrifugation at 3000 rpm for 10 min and stored at -20°C for hormone assay. Anticoagulant added blood samples were used for the studying hematological parameters.

Estimation of biochemical parameters

The Biochemical parameters such as Serum glucose, triglyceride, total cholesterol, HDL, total protein, urea and triglycerides were determined using diagnostic kits (Ranbaxy India Ltd). The estimation of hemoglobin was in carried out using Cyanomet-hemoglobin method.

Statistical analysis

The values reported are Mean±SE. The statistical analysis was carried out using analysis of variance (ANOVA) followed by Dunnet’s ‘t’ test. The p values < 0.05 were considered as significant.

RESULTS

Estrous cycle by vaginal smear

As a result, all rats from Mifepristone group were completely acyclic and remained in constant estrus while control rats had normal cycle. The rats from Mifepristone plus ESECB treated group have cycle but less than controls. All of the rats (6 of 6, 100%) in the control group showed normal estrous cyclicity while (4 of 6, 80%) in the PCOS and ESECB treated rats were recovered from the disrupted estrous cycle induced by Mifepristone.

Effect of ESECB on body weight and reproductive organs

Table- I shows the effect of body weight and reproductive organs weight in different groups of experimental animals. In the present study there is a significant increase in the body weight, ovary and uterus weight is observed in the mifepristone treated PCOS rats when comparable (p< 0.01) to the control rats. The 28 day oral administration of 200 mg and 400mg /kg b. w. ESECB drug treatment reduced the weight of the body and the reproductive organs in these PCOS rats. There was a remarkable decrease of ovary weight in the group IV rats which received 400mg /kg b. w. of the ESECB drug which was lower than the positive drug Metformin used in the present study.
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Table-1 Effect of ESECB on weight of body and reproductive organs in different experimental rats.

<table>
<thead>
<tr>
<th>Treatment groups</th>
<th>Body weight in gm</th>
<th>Ovary weight in mg</th>
<th>Uterus weight in mg</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group-I (control)</td>
<td>112.0 ± 2.36a**</td>
<td>0.155 ± 0.08a**</td>
<td>0.194 ±1.07a**</td>
</tr>
<tr>
<td>Group-II (Mifepristone 4mg/kg b. w treated)</td>
<td>202.5 ± 2.21</td>
<td>0.200 ± 0.06</td>
<td>0.165 ± 0.02</td>
</tr>
<tr>
<td>Group-III (ESECB 200mg/kg b. w treated)</td>
<td>151.73 ±1.44 b**</td>
<td>0.171 ± 0.02b**</td>
<td>0.180 ± 2.01b**</td>
</tr>
<tr>
<td>Group-IV (ESECB (400mg/kg b. w treated)</td>
<td>145.8 ± 1.724c **</td>
<td>0.143 ± 1.04c**</td>
<td>0.190 ± 3.04c**</td>
</tr>
<tr>
<td>Group-V (Metformin 20 mg/kg b. w treated)</td>
<td>121.1 ± 1.8d**</td>
<td>0.150 ± 2.04d**</td>
<td>0.174 ± 0.03d**</td>
</tr>
</tbody>
</table>

Values are mean ± SEM of 6 animals in each group statistical significance test for comparison was done by ANOVA followed by Dunnet’s ‘t” test. Comparison between a-Group I Vs Group II, b-Group IVs Group III, c-Group II Vs Group IV and d - Group II Vs Group V. P values*p<0.05, **p<0.01, ***p<0.001, NS–Not Significant.

Estimation of biochemical parameters
The effect of the ESECB extract on the biochemical parameters such as hemoglobin, glucose, total proteins, urea and creatinine of PCOS induced rats were shown in the Table-2. It is observed that there is a significant increase in the glucose level in the PCOS induced group II rats comparable to control rats. The group III and group IV mifepristone induced PCOS rats which received the ESECB drug treatment showed a decrease in the blood glucose level indicating the hypoglycemic potential of the drug. There is no significant variation found in the other biochemical parameters such as hemoglobin, total proteins, urea and Creatinine in the present study.

Table-2 Effect of ESECB on Biochemical parameters in different experimental rats

<table>
<thead>
<tr>
<th>Treatment groups</th>
<th>Hemoglobin (g/dL)</th>
<th>Glucose (mg/dL)</th>
<th>Total protein (g/dL)</th>
<th>Urea (mg/dL)</th>
<th>Creatinine (mg/dL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group-I (control)</td>
<td>13.08 ± 0.8a**</td>
<td>84.55±1.09a</td>
<td>6.33± 0.35a ns</td>
<td>30.57 ± 1.55a ns</td>
<td>0.65±0.18a**</td>
</tr>
<tr>
<td>Group-II (Mifepristone 4mg/kg b. w. treated)</td>
<td>11.15 ± 0.8</td>
<td>152.0±1.07</td>
<td>6.43 ± 0.52</td>
<td>32.18 ± 1.52</td>
<td>0.97 ± 0.14</td>
</tr>
<tr>
<td>Group-III (ESECB 200mg/kg b. w. treated)</td>
<td>11.16± 0.9 bns</td>
<td>124.5 ± 1.67b**</td>
<td>6.70 ± 0.44b NS</td>
<td>31.35 ± 1.13 b,NS</td>
<td>0.95 ± 0.18bNS</td>
</tr>
</tbody>
</table>
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<table>
<thead>
<tr>
<th>Treatment groups</th>
<th>Total Cholesterol (mg/dl)</th>
<th>Triglycerides (mg/dl)</th>
<th>HDL-Cholesterol (mg/dl)</th>
<th>LDL-Cholesterol (mg/dl)</th>
<th>VLDL-Cholesterol (mg/dl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group-IV (ESECb (400mg/kg b. w. treated)</td>
<td>11.51 ± 1.2c NS</td>
<td>105.4± 0.97c**</td>
<td>6.98 ± 0.23c NS</td>
<td>31.53 ± 1.36c NS</td>
<td>0.86 ± 0.09c NS</td>
</tr>
<tr>
<td>Group-V (Metformin 20 mg/kg b. w. treated)</td>
<td>14.6 ± 1.1d**</td>
<td>114.76 ± 0.98d**</td>
<td>6.50 ± 0.38d NS</td>
<td>35.05 ± 1.10d **</td>
<td>0.90 ± 0.14d NS</td>
</tr>
</tbody>
</table>

Values are mean ± SEM of 6 animals in each group; statistical significance test for comparison was done by ANOVA followed by Dunnet’s t-test. Comparison between a - Group I Vs Group II, b - Group II Vs Group III, c - Group II Vs Group IV and d- Group II Vs Group V. P values *p<0.05, **p<0.01, ***p<0.001, NS- Not significant.

Study of lipid profile

There is a significant increase in the total cholesterol, triglycerides LDL-cholesterol and VLDL-cholesterol as well as decrease in HDL-cholesterol level in the group-II PCOS Induced rats when compared (p< 0.01) to the normal control group as shown in the Table-3. These levels were reverted back significantly in the PCOS induced and ESECb drug treated group III and group IV rats when comparable to (p< 0.01) group II PCOS induced animals. The group IV animals which received 400mg/kg b. w. of ESECb extract showed a significant protection against the dyslipidemia when comparable to the group III animals which received 200mg/kg b. w. of ESECb extract in the present study.

Table-3 Effect of ESECb on Lipid profile in different experimental rats

<table>
<thead>
<tr>
<th>Treatment groups</th>
<th>Total Cholesterol (mg/dl)</th>
<th>Triglycerides (mg/dl)</th>
<th>HDL-Cholesterol (mg/dl)</th>
<th>LDL-Cholesterol (mg/dl)</th>
<th>VLDL-Cholesterol (mg/dl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group-I (control)</td>
<td>94.1± 1.068a **</td>
<td>88.31 ± 1.39a**</td>
<td>42.55 ± 1.62a **</td>
<td>49.4 ± 1.19a**</td>
<td>17.35±1.47a**</td>
</tr>
<tr>
<td>Group-II (Mifepristone 4mg/kg b. w treated)</td>
<td>184.5 ± 1.698</td>
<td>173.5 ± 1.180</td>
<td>24.65 ± 1.36</td>
<td>126.13 ± 1.05</td>
<td>44.53±1.66</td>
</tr>
<tr>
<td>Group-III (ESECb 200mg/kg b. w treated)</td>
<td>153.8 ± 1.171b**</td>
<td>165.4 ± 1.42 b**</td>
<td>24.51 ± 1.44 b**</td>
<td>96.48 ± 1.27b**</td>
<td>33.48±1.35b**</td>
</tr>
<tr>
<td>Group-IV (ESECb 400mg/kg b. w treated)</td>
<td>104.7 ± 1.314c**</td>
<td>145.3 ± 1.40c**</td>
<td>32.26 ± 0.99 c**</td>
<td>50.57 ± 1.13c**</td>
<td>29.80±3.92c**</td>
</tr>
<tr>
<td>Group-V (Metformin 20 mg/kg b. w treated)</td>
<td>122.3 ±1.512d**</td>
<td>81.49 ± 1.43d**</td>
<td>39.31 ± 1.32d**</td>
<td>76.30 ± 1.15d**</td>
<td>16.31±1.39d**</td>
</tr>
</tbody>
</table>

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DISCUSSION

Polycystic ovary syndrome (PCOS), an endocrine disorder most common in women during their reproductive years, is characterized by at least two of the following: hyper-androgenism, oligo or anovulation, hirsutism, and polycystic ovaries which may further develops in to ovarian cancer (14). PCOS is known to be a major cause of female infertility. RU486 (mifepristone), a progesterone receptor antagonist, is one the most common drugs used for emergency contraception can be used to generate rat models of PCOS (15). Evidence from clinical studies indicates that RU486 suppresses follicle development, ovulation, and Corpus luteum formation, by disrupting the negative feedback of P4 to the hypothalamus (16). In the present investigation RU486 (mifepristone) was used for inducing PCOS in rats and the ameliorative effect of ethanolic seed extract of Caesalpinia bonducella (ESECB) on PCOS induced rats were evaluated.

The clinical features developed in adult rats treated for 8 days with the drug mifepristone were quite similar to the PCOS women. The vaginal smear histology depicts the physiological function of ovaries. The PCOS rats were observed with uterus ballooning and remarkable increase in body weight and ovary weight as like in other models. Nearly 50% of the PCOS women population are obese and are found to have increased visceral fat distribution. Adipose tissue, particularly visceral adipose tissue is considered as an active endocrine organ affecting the metabolic and normal reproductive functions. The administration of ESECB plant extract considerably decreased the body as well as the weight of reproductive organs indicating the protective nature of the drug against PCOS. Several phytochemicals, two phytosterols namely heptasane and sitosterol and a few fatty acids such as lignoceric acid, oleic acid, palmitic acid, linolenic, stearic acid present in seeds may account for the reduction in the weight of both body and reproductive organs by correcting the impaired metabolic functions (17).

It is well documented that the occurrence of type 2 diabetes mellitus and impaired glucose tolerance increases due to visceral obesity in PCOS condition due to worse metabolic profile (18). These above complications may lead to insulin resistance gradually. So it is very crucial to reduce the hyperglycemic condition in the mifepristone induced PCOS rats to reduce the metabolic complications. In our study the ESECB treated PCOS induced rats showed a reduction in the blood glucose level which may be due to the presence of flavonoids, saponins, tannins, glycosides and triterpenoids (19). Kannur et al., (20) also have reported the anti-hyperglycemic action of the plant extract by blocking glucose absorption.

One of the well-recognized metabolic abnormalities in PCOS is dyslipidemia due to insulin resistance where decreased levels of high-density lipoprotein-cholesterol (HDL-C) and increased levels of triglycerides (TG), low density lipoprotein –cholesterol (LDL-C) and very low-density lipoprotein (VLDL-C) is observed (21). In our present investigation also there is a dyslipidemia condition with increased triglycerides, LDL and VLDL cholesterol and decreased level HDL cholesterol in PCOS induced rats. The ethanolic seed extract of Caesalpinia bonducella (ESECB) altered this dyslipidemia condition and there is an increase in HDL-C and decrease in triglycerides, LDL and VLDL cholesterol. Williamson (22) have reported the

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presence of several phyto constituents such as β – carotene, phytosterin, aspartic acid, β-sitosterol, citrulline, flavonoids, bonducellin, furano diterpenes and arginine in the ethanolic seed extract of Caesalpinia bonducella and Lilaramet al., (23) have showed that the plant has got significant cardio protective effect by lowering the levels of cholesterol, triglycerides, LDL and increasing the levels of HDL. These findings supported our findings of lipid lowering activity observed in the present study.

CONCLUSION

It can be concluded that ethanolic seed extract of Caesalpinia bonducella can be successfully used in the management of the PCOS which needs a multifaceted therapy in controlling endocrine and metabolic complications. The ESECB restored the altered morphology, glucose level and lipid profile in mifepristone induced PCOS animals similar to Metformin, indicating that this drug can be used individually or as an adjuvant to Metformin in treating the complications of PCOS and thereby regaining the fertility in women by inducing ovulation.

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Meera et al: Effect of ethanolic seed ..........rats

Evaluation of antioxidant and anti-cancer functions of *Hopea ponga* leaf extract and its manganese nanoparticles: *In-vitro*

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ABSTRACT

Introduction and Aim: The green-synthesized Manganese nanoparticles (MnNPs) were produced rapidly by treating Manganese ions with an aqueous extract of *Hopea ponga* leaf. The reaction process was monitored using UV-visible spectroscopy. FTIR spectra of plant extract and MnNPs are illustrated. The aim of the present study is to evaluate *in-vitro* antioxidant and anticancer activity of aqueous leaf extract of *H. ponga* and its synthesized MnNPs.

Materials and Methods: Herbal extraction was carried out by Soxhlet extraction method with water as a solvent. The antioxidant potential of plant extracts and MnNPs were evaluated by FRAP, H$_2$O$_2$, DPPH and PM assays. The effect of aqueous extract of *H. ponga* along with its synthesized MnNPs on the viability of non-small cell lung cancer (A549) cells was determined by MTT assay.

Results: The synthesized MnNPs showed significant antioxidant activity in all performed assays. The aqueous extract from leaves of *H. ponga* has strong dose dependent anticancer activity against non-small cell lung cancer cells A549.

Conclusion: The newly synthesized MnNPs showed significant antioxidant activity in all performed assays. Further, aqueous extract from leaves of *H. ponga* have a strong dose dependent anticancer activity against non-small cell lung cancer cells A549.

Keywords: *Hopea ponga*; manganese nanoparticles; green synthesis; anti-oxidant; anti-cancer.

INTRODUCTION

Nano-biotechnology is an emerging field that utilizes nano-based-systems for various biomedical applications. This rapidly developing field has raised the possibility of using therapeutic nanoparticles in the diagnosis and treatment of human diseases (1). Metallic nanoparticles are most promising as they contain remarkable antibacterial properties due to oligodynamic nature of metal to inhibit the microbes as well as due to their large surface area to volume ratio (2). The research from past few years shows that utilization of biological organisms is a novel method for the synthesizing metal nanoparticles, which can be preferred over the existing chemical and physical methods (3). Plant extracts and bioactive compounds which are isolated from medicinal plants are used for antibacterial, antifungal and antiviral therapy (4). Some major categories of plant derived products include personal care products, phyto-cosmetics, herbal medicines and phyto-pharmaceuticals (5).

*Hopea ponga* is an endemic tree belonging to *Dipterocapaceae* family found in tropical evergreen forest of Western Ghats of Karnataka (6). This plant was reported to be used as traditional medicine in the treatment of piles and snake bite (7) and acts as an astringent (8). Methanolic extract of seed wings of *H. ponga* exhibits antioxidant and
antibacterial activity (9). The literature survey indicates that there are only few pharmacological studies are reported on this plant, revealing the scope for our venture in unmasking its anti-inflammatory property. Metal nanoparticles have a high specific surface area and are studied extensively because of their unique physicochemical characteristics such as catalytic, electronic, magnetic properties and antimicrobial activities (10) and (11). The physical and chemical principles to synthesize metal nanoparticles are not environmentally friendly because of few drawbacks such as the production of hazardous by-products, intermediary compounds, presence of toxic organic solvents and these strategies consume high energy during the process of synthesizing nanoparticles (12). Hence, the present study focuses on green synthesis of metal nanoparticles from plant source.

MATERIALS AND METHODS

Collection of plant material

Fresh leaves of *H. ponga* were collected from Anashi forest range of Western Ghats, Uttar Kannada District, Karnataka, India during the period of September 2016. After the identification and authentication leaves were washed under running tap water, shade dried and homogenized to coarse powder with mechanical grinder. It was stored in airtight containers at -20°C for further analysis.

Preparation of plant extract

About 25 g of coarse powder of leaves were extracted with 250 ml of distilled water in Erlenmeyer flask using Soxhlet apparatus for 24 hours at 40-50°C and kept in room temperature for 24 hours under dark condition. The extract was filtered and stored in air tight bottle at 4°C until use. The solvent was evaporated with rota evaporator and the resultant crude yield was weighed.

Synthesis of Manganese nanoparticles (MnNPs)

The *H. ponga* leaf extract was extracted by cold extraction method. 0.05 M of Potassium permanganate (KMnO₄) was prepared and used for the synthesis of manganese nanoparticles. 10 ml of *H. ponga* leaf extract was added into 90 ml of aqueous solution of 0.05 M Potassium permanganate for reduction into Mn⁺ ions and kept at room temperature for 30 minutes. After the reaction time, the colorless reaction mixture changed to dark pink color indicating the oxidation / reduction reaction. Further this mixture with MnNPs was centrifuged at 10,000 rpm for 10 minutes, repeated centrifugation and re-dispersion in double distilled water was carried out to avoid the traces of aqueous extract in the synthesized MnNPs, which were allowed to dry to form powder (9).

Characterization of newly synthesized MnNPs

UV–Visible spectroscopy-based Analysis

The reduction of Manganese ions in the colloidal solution was confirmed and analyzed by UV–Visible spectroscopy and observed wavelength was between 200-1100 nm with distilled water as a reference and 0.05 M KMnO₄ as a blank (13), (Model: HITACHI 2J1-0004) at Karnataka University Dharwad. Addition of aqueous leaf extract of *H. ponga* to the aqueous solution of 0.05 M KMnO₄ leads to color change in the reaction mixture from dark pink to brownish color within 15 minutes.

Fourier transform infrared spectroscopy analysis

FTIR is used to recognize the function groups which are bound to Manganese surface and involved in the formation of MnNPs. FTIR spectroscopy of dried MnNPs was carried out by potassium bromide (KBr) pellet (FTIR grade) method in 1:100 ratio and spectrum was recorded using (Model: NICOLET 6700) at USIC, Karnataka University Dharwad, India.

Scanning Electron Microscopy (SEM) analysis

Surface morphology of nanoparticles was determined by SEM. The sample was prepared by
centrifuging the colloidal solution at 10,000 rpm for 5 minutes. The pellet was re-dispersed in deionized water and re-centrifuged then dried to get pellet. Then the drop of MnNPs suspension was placed on carbon coated grid. The sample was dried under lamp and was subjected to SEM (Model - ZEISS) at Manipal Institute of technology, Manipal, Karnataka, India.

**Determination of antioxidant activity by in-vitro methods**

Antioxidant mechanisms in biological tissues are extremely complex and by a single method it is difficult to decide the antioxidant capacity of crude extracts (14). Hence in the present study, four in-vitro assays viz., FRAP, PM, H$_2$O$_2$ and DPPH assay are employed.

**Ferric ion reducing antioxidant power assay**

Ferric ions reducing power was measured according to the method of Oyaizu (15). Aqueous extract of *H. ponga* and its MnNPs in different concentrations ranging from 100 µl to 500 µl were mixed with 2.5 ml of 20mM phosphate buffer and 2.5 ml 1% w/v potassium ferri-cyanide and the mixture was incubated at 50°C for 30 min. After incubation, 2.5 ml of 10 % w/v tri-chloro acetic acid and 0.5 ml 0.1%, w/v ferric chloride were added to the mixture and incubated for 10 min. The absorbance was measured at 700 nm using a UV-Visible spectrophotometer. Ascorbic acid was used as reference standard for all assays.

**Hydrogen peroxide (H$_2$O$_2$) scavenging assay**

The antioxidant activity of aqueous leaf extract and MnNPs were assessed based on their ability to scavenge the H$_2$O$_2$ (16). 0.6 ml of 4 mM H$_2$O$_2$ solution in phosphate buffer (pH-7.4) was added to 0.5 ml of known concentration of standard ascorbic acid and to tubes with different concentrations ranging from 100 µl to 500 µl of plant extracts in phosphate buffer. Absorbance was measured at 230 nm after 10 minutes against the blank solution containing phosphate buffer without H$_2$O$_2$.

**DPPH free radical-scavenging ability assay**

Radical scavenging activities of aqueous leaf extract and MnNPs were determined using the DPPH radical as a reagent, according to the methods of Rice-Evans (17). 100 µL of a DPPH radical solution in ethanol (60 µM) was mixed with 100 µL of sample solution in ethanol. The mixture was incubated for 30 min in the dark at room temperature and then absorbance was measured at 517 nm using a UV-Visible Spectrophotometer. The DPPH scavenging activity was calculated using the following equation:

Percentage of inhibition = \( \frac{A_c-A_t}{A_c} \times 100 \)

Where, \( A_c \) is the absorbance of the control reaction (100 µl of ethanol with 100 µl of the DPPH solution) and \( A_t \) is the absorbance of the test sample. The IC$_{50}$ value was calculated for all the samples used. Lower absorbance of the reaction mixture indicated higher free radical activity.

**Phospho-molybdenum (PM) assay**

Total antioxidant activity was estimated by PM assay using standard procedure of Prieto et al. (18). Aqueous leaf extract of *H. ponga* and its MnNPs in different concentration ranging from 100 µl to 500 µl were added to each test tube containing 3 ml of distilled water and 1 ml of molybdate reagent solution. These tubes were incubated at 95°C for 90 min. After incubation, these tubes were normalized to room temperature and the absorbance of the reaction mixture was measured at 695 nm.

**Determination of cell viability by MTT Assay**

The effect of aqueous leaf extract of *H. ponga* along with its MnNPs on the viability of non-small cell lung cancer (A549) cells was determined using the standard colorimetric MTT assay using the 3-(4,5-dimethylthiazol- 2-yl)-2,5-dimethyl tetrazolium bromide dye (Sigma, St. Louis, MO, USA), according to Carmichael et al., The percentage growth inhibition was calculated using the following formula and concentration of test drug needed to inhibit cell growth by 50% (IC$_{50}$) values is generated from the dose-response curves for each
cell line. This assay is based on the reduction of MTT by the mitochondrial dehydrogenase of intact cells to a purple formazan product (19).

\[
\text{Inhibition Percentage} = \frac{\text{OD of Test sample}}{\text{OD of control}} \times 100
\]

**Statistical analysis**

All experiments were performed in triplicates (n=3) and the data are represented as the mean ± standard deviation and standard error. Differences between the means of the individual groups were analyzed using the one-way ANOVA analysis SPSS software 20 Version (IBM).

**RESULTS**

**UV-Visible characterization**

Formation of MnNPs was confirmed by the UV-Visible spectral analysis of colloidal solution for wavelength scanning between 200-1100 nm. The analysis reveals maximum absorption peak (λ max) at 340 nm. (Fig.1)

**Fourier transform infrared spectroscopy analysis**

FTIR spectroscopy was used to identify the functional groups responsible for bio-reduction of Mn\(^+\) into Mn\(^0\) nanoparticles. FTIR spectra showed shifts in some peaks of leaf extract of *H. ponga* viz., 3390 cm\(^{-1}\), 1718 cm\(^{-1}\), 1611 cm\(^{-1}\), 1513 cm\(^{-1}\), 1450.86 cm\(^{-1}\) and 1371.17 cm\(^{-1}\) with functional groups nitro-carbon compounds, alkanes, alkyl halides (Fig. 2). Some functional groups were present in both FTIR spectra of the plant extract and MnNPs viz., phenols, aromatics, alcohol and alkyl halides with peaks at 3699.75 cm\(^{-1}\), 3344.77 cm\(^{-1}\), 2928 cm\(^{-1}\), 1632.85 cm\(^{-1}\), 1540.31 cm\(^{-1}\), 1384.26 cm\(^{-1}\) and 1315.45 cm\(^{-1}\) which may be acting as capping and reducing agents for synthesis of MnNPs (Fig. 1).

![Fig. 1: The peaks of Manganese NPs synthesized from aqueous leaf extract of *Hopea ponga*](image1)

![Fig. 2. The peaks of aqueous leaf extract of *Hopea ponga*](image2)
Shettar and Vedamurthy: Evaluation of ………nanoparticles in-vitro

**Scanning Electron Microscopy**

Surface morphology of the synthesized MnNPs was confirmed by the SEM studies. SEM images showed irregular shaped MnNPs and size in diameter range of 30-150 nm. (Fig. 3)

![SEM images of MnNPs](image)

**Fig. 3:** Showing SEM images of irregular shaped MnNPs and size in diameter range of 45-100 nm

**In-Vitro antioxidant activity**

**FRAP Assay**

In the present study aqueous leaf extract of *H. ponga* and its MnNPs were subjected to FRAP assay along with ascorbic acid as standard. Increase in the concentration of samples lead to increase in antioxidant activity along with absorbance. The antioxidant activity of MnNPs was less on comparison with standard and higher than the aqueous leaf extract with absorbance of 0.939±0.048 but whereas standard and aqueous leaf extract exhibited higher absorbance 1.465±0.003 and 0.643±0.005 respectively (Fig. 4)

![FRAP assay results](image)

**Table 2. H_{2}O_{2} assay of aqueous leaf extract of Hopea ponga and its synthesized Manganese nanoparticles**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Concentration</th>
<th>Percentage of Inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Std. Ascorbic acid</td>
<td>100 μg/mL</td>
<td>74.46 ± 0.130</td>
</tr>
<tr>
<td>Aqueous extract of Hopea ponga</td>
<td>100 μg/mL</td>
<td>54.44 ± 0.212</td>
</tr>
<tr>
<td>Manganese NPs</td>
<td>100 μg/mL</td>
<td>67.43 ± 0.512</td>
</tr>
</tbody>
</table>

Results are expressed as Mean ± SD (n=3)

**Hydrogen peroxide scavenging assay**

Hydrogen peroxide radical scavenging assay revealed that MnNPs showed appreciable...
scavenging activity with inhibition percentage 67.04±0.519 which was less than that of standard i.e. 74.46±0.13 and aqueous leaf extract exhibited 54.44±0.213 % inhibition (Fig. 5).

<table>
<thead>
<tr>
<th>Table 3. Percentage inhibition: DPPH assay of aqueous leaf extract of <em>Hoepe ponga</em> and its synthesized Manganese nanoparticles</th>
</tr>
</thead>
<tbody>
<tr>
<td>Concentration</td>
</tr>
<tr>
<td>----------------</td>
</tr>
<tr>
<td>10 µg</td>
</tr>
<tr>
<td>20 µg</td>
</tr>
<tr>
<td>30 µg</td>
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<tr>
<td>40 µg</td>
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<td>50 µg</td>
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</table>

**Figure 6. Percentage inhibition: DPPH assay of aqueous leaf extract of *Hoepe ponga* and its synthesized Manganese nanoparticles**

The antioxidant activity of aqueous leaf extract of *H. ponga* and its synthesized MnNPs was compared with ascorbic acid as standard (20). The results revealed that the antioxidant activity of MnNPs was less than both standard and aqueous leaf extract with percentage of inhibition 69.30±0.654 whereas standard and aqueous extract showed significant percentage of inhibition i.e. 83.91±0.35 and 74.82±0.46 respectively (Fig. 6).

<table>
<thead>
<tr>
<th>Table 4. PM assay of aqueous leaf extract of <em>Hoepe ponga</em> and its synthesized Manganese nanoparticles</th>
</tr>
</thead>
<tbody>
<tr>
<td>Concentration</td>
</tr>
<tr>
<td>----------------</td>
</tr>
<tr>
<td>100 µl</td>
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<tr>
<td>200 µl</td>
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<tr>
<td>300 µl</td>
</tr>
<tr>
<td>400 µl</td>
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<tr>
<td>500 µl</td>
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</tbody>
</table>

**Figure 7. PM assay of aqueous leaf extract of *Hoepe ponga* and its synthesized Manganese nanoparticles**

In the present study known concentrations of aqueous leaf extract and MnNPs were subjected to PM assay along with ascorbic acid as standard. Synthesized MnNPs showed higher activity than the aqueous leaf extract with absorbance 0.744±0.021 whereas aqueous leaf extract showed less absorbance than standard as well as MnNPs i.e. 0.44±0.005. Standard showed the highest activity among tested samples with absorbance value 1.131±0.007 (Fig.7)
Anticancer activity of aqueous extract from leaves of *H. ponga* and its synthesized Manganese nanoparticles against non-small cell lung cancer cells (A549)

In the present study, the untreated A549 cell line was taken as control group, which was treated with standard drug Epotoside considered as positive control group whereas aqueous leaf extract of *H. ponga* (Table 5) and its MnNPs treated A549 cell line was taken as treated group. A549 cell lines were treated with different concentrations viz., 50 µg, 100 µg, 150 µg, 200 µg and 250 µg of aqueous leaf extract(Table 5) and that of MnNPs was 5 µg, 10 µg, 20 µg, 40 µg and 80 µg and standard drug Epotoside (50 µM and 100 µM) (Table 6).
DISCUSSION

Cellular oxidants, called reactive oxygen species (ROS), are constantly produced in animal and human cells. Excessive ROS can induce oxidative damage in cell constituents and promote a number of degenerative diseases and aging. Cellular antioxidants protect against the damaging effects of ROS. However, in moderate concentrations, ROS are necessary for a number of protective reactions (21). Antioxidant mechanisms in biological tissues are complex and by single method it is difficult to decide the antioxidant capacity of crude extracts (14). In the present study, four in-vitro assays viz., FRAP, H₂O₂, DPPH and PM assays were employed. FRAP assay includes use of ferri-cyanide and ferric ions as chromogenic oxidants. In the present study, the antioxidant activity of MnNPs was less on comparison with standard but standard showed higher absorbance (Table 1). The H₂O₂ scavenging activity of extracts was compared with ascorbic acid which is a standard. In this assay the MnNPs showed appreciable scavenging activity which was higher than aqueous leaf extract (Table 2) (22). DPPH assay is widely accepted method for evaluating antioxidant activity of plant-based drugs and crude extracts. The scavenging ability of leaf extracts of H. ponga is found to be higher compared to other genus of Dipterocarpaceae family (22). Here the antioxidant activity of MnNPs was less than standard and aqueous leaf extract (Table 3). PM assay revealed that aqueous leaf extract of H. ponga showed the higher activity as compared to MnNPs (Table 4). Both aqueous extract and standard drug increase in concentration above 50 µg and 50 µM respectively and causes the decreases in cell count and division of cell compared to untreated control group. In case of aqueous leaf extract 50 % of cell viability was observed to be 438.59 µg for A549 and for standard drug Epotoside it was observed to be 89.285 µM for A549. IC₅₀ values for standard and extract were calculated and depicted in Table 5. At initial concentration 50 µg there was no noticeable inhibition of cells growth was observed for the cell lines. Morphological studies revealed that compared with control group, treated group and positive control group showed
significant increase in detached cells in culture medium. The cells displayed as turgid and shrunken in shape compared to untreated control cells. Morphological changes in nucleus represented apoptosis. Whereas normal cells appeared normal and regular in shape, in case of treated group chromatin condensation, elongation of cells and decrease in cell count and density were observed which are the characteristic features of apoptosis. Microscopic examination revealed the morphological changes and shrinkage of cells leading to cell apoptosis were induced by the leaf extract of *H. ponga* and it’s MnNPs (Figures 10 and 11). MnNPs exhibited prominent anticancer activity on A549 with IC\textsubscript{50} value 101.01 µM. IC\textsubscript{50} values for standard and extract were calculated and depicted in Table 6. In all treated samples increase in cell viability was observed at minimum concentration only. As the concentration increases viability of cells was decreased.

**CONCLUSION**

The present study successfully demonstrated the bio reduction of Manganese ions into MnNPs by aqueous leaf extract of *H. ponga*. This process was completely undertaken through green synthesis route in which synthesis of MnNPs is rapid and simple. The newly synthesized MnNPs showed significant antioxidant activity in all performed assays. Overall studies revealed that, aqueous extract from leaves of *H. ponga* have a strong dose dependent anticancer activity against non-small cell lung cancer cells A549. Further studies are needed for detailed characterization of the toxicity and mechanism involved with antioxidant activity of these particles. In future these MnNPs can have promising potential applications in drug formulation and biomedical application.

**ACKNOWLEDGEMENT**

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The efficacy of two-year yogic practice on selected pulmonary function test in postmenopausal women
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ABSTRACT

Introduction and Aim: Menopausal transition has been well associated with a series of hormonal changes that has been linked to impairment of respiratory function. The present study was designed to evaluate the cumulative effect of practicing yoga on certain respiratory parameters in postmenopausal women.

Materials and Methods: Sixty postmenopausal women were divided into two groups (n=30 each). Based on the duration of yoga, they were grouped into Group I – (Regularly doing yoga for one year) and Group II (Regularly doing yoga for the two years). The women not doing any yogic exercises were taken as the control group; Group Ia (for One year) and Group II a (for two years). The respiratory parameters were measured with the help of vitalograph.

Results: All the observed respiratory parameters such as vital capacity (VC), Forced vital capacity (FVC), FEV1 (Forced expiratory volume during the 1st second.), FEV1 ratio, PEFR (Peak expiratory flow rate). FEF50 (Forced Expiratory Flow at 50%), showed a significant (P<0.0001) improvement in Group II when compared to the Group I.

Conclusion: Yoga practice can be advocated to improve pulmonary function tests in post-menopausal women which might help in preventing respiratory diseases during aging process. Optimum benefit of yoga was observed during the two years of yoga practice in the postmenopausal women. Continued practice of yoga might be also considered as a preventive exercise to impair age related morbidity and improve the quality of life.

Keywords: Post- menopausal; yoga; pranayama; pulmonary function test.

INTRODUCTION

Menopause is the phase where the ovarian function declines and the reproductive ability of the women decreases (1). Various menopausal symptoms will be present for a prolonged period of menopausal period (2). In the recent years the increased incidence of cardiovascular diseases in women after menopause is the leading cause of mortality and morbidity. The physiologic function of the respiratory function declines (3). Literature survey shows that with the advancing age the stiffening of the thoracic cage increasing the work of breathing (4, 5). Drastic variations in the respiratory parameters has been observed as the age advances (6, 7). Menopausal period has been linked with the hormonal variations causing weakening of respiratory function (8). Female hormones play an important role in overall lung health. Increased deposition of body fat and central obesity might be related to impairment of lung function via several mechanisms (9, 10). Reduced lung function has
been well associated within increased metabolic risk factors.

The beneficial effect of hormonal replacement therapy has long been controversially discussed. Complementary therapies have been used by the menopausal women to cope with their symptoms and yoga is one among them (11, 12). Yoga is an ancient Indian science as well as the way of life, which includes practice of asanas in specific posture and pranayama which includes the regulated breathing techniques. Breathing maintains the dynamic bridge between body and mind and pranayama is the important yogic practices, which can produce different physiological responses in healthy individuals. The regular practice of yoga has been shown to increase the strength of muscles, flexibility of the body and also improvement in the cardiovascular functions. Overall regular yoga practice indirectly improves the quality of life (13, 14). Simple yoga postures can be easily adopted by physically active and inactive people and also practiced at any time with empty stomach. The outdoor physical activities are restricted by the environmental barriers such as extreme rain, heat or cold. Yogic exercises can be done in the limited space and also in any weather conditions (11, 12). There is an increased need for a safe and effective intervention programmes in postmenopausal women decreasing the adverse effects on pulmonary functions as the age advances. The present study was designed to evaluate the complete and accumulative effect of practicing yoga on certain respiratory parameters in postmenopausal women.

MATERIALS AND METHODS

This study is a randomized controlled trial in postmenopausal women recruited in one center in Mangalore between November 2015 and Aug 2018. The present study was done after obtaining the consent from the Institutional Ethical Committee. The purpose of the study was explained, and written approval of each individual was obtained. Post-menopausal women (48-60 years) who joined for the yoga class were randomly selected. Post-menopausal women having the history of cardiovascular vascular and respiratory diseases were excluded from the study. The respiratory parameters such as Vital capacity, FVC, FEV1, FEV1 ratio, PEFR, FEF 50, was measured with the help of vitalograph (Pneumotrac; 11). The women included in the study group who failed to achieve 80% attendance during the yoga schedule were excluded from the study. They practiced yoga regularly as per the yoga therapy schedule. Yoga was strictly under the supervision of yoga teacher.

**Yoga intervention**

All the subjects had to practice yoga for 1 hour, 20 minutes daily, five days a week between 4:00 pm to 6:00 pm. Procedure of daily yoga sessions are as follows. The subjects were informed about the procedures in brief and were asked to relax physically and mentally for 10 minutes. The yoga practice (1 hour-20 minutes) schedule starting from prayer and followed by warm up exercises, Surya namaskara, Asanas, Pranayama and concluded by meditation/laughing exercise as follows:

<table>
<thead>
<tr>
<th>Yoga</th>
<th>Time in minutes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Prayer</td>
<td>1</td>
</tr>
<tr>
<td>Mild warm up exercise</td>
<td>10</td>
</tr>
<tr>
<td>Suryanamaskara</td>
<td>15</td>
</tr>
<tr>
<td>Asanas: Shavasana, Naukasana, Halasana,</td>
<td>35</td>
</tr>
</tbody>
</table>

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Instructions to do yoga was done by certified yoga instructors who had at least 5 years of yoga teaching experience. The intensity of yoga exercise was determined by the instructors in order to provide a progressive level of challenge to the subjects. The performance of the subject was continuously supervised by the attending yoga instructor. All the yogic exercises were stated to be suitable for the post-menopausal yoga beginners and senior individuals included in this study. The effect of yoga was studied in two groups of 30 each. The study group were categorized into Group I – (Regularly doing yoga for one year) and Group II (Regularly doing yoga for the two years). The respiratory parameters such as Vital capacity (VC), FVC (Forced Vital capacity), FEV1 (forced expired volume in 1 second), FEV1 ratio, PEFR (Peak expiratory flow rate), (FEF) 50% (Forced expiratory flow measured at the mid-flow, were measured with the help of computerized vitalometer (Pneumotrac). The women not doing yoga were taken as the control group for the Group Ia (for One year) and Group IIa (for two Years).

RESULTS

Group I showed significant (P<0.001) improvement in VC and FVC but did not show any significant changes in the other parameters such as FEV1, FEV1 ratio, PEFR, FEF50 (Table 1).Women belonging to the group II showed a significant improvement (P<0.0001) in all the respiratory parameters when compared to respective control group (Table 2).Comparatively group II showed a significant improvement in the respiratory parameters while compared to group I (Table 3).

Table 1: Effect of one-year yoga therapy on respiratory parameters

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Group I (n=30)</th>
<th>Group I A (n=30)</th>
</tr>
</thead>
<tbody>
<tr>
<td>VC</td>
<td>2.40 ± 0.3</td>
<td>2.21±0.3**</td>
</tr>
<tr>
<td>FVC</td>
<td>2.51±0.4</td>
<td>2.30±0.3**</td>
</tr>
<tr>
<td>FEV1</td>
<td>2.09±0.4</td>
<td>1.94±0.3NS</td>
</tr>
<tr>
<td>FEV1 ratio</td>
<td>0.78±0.13</td>
<td>0.77±0.03 NS</td>
</tr>
<tr>
<td>PEFR/m</td>
<td>359.04±41.5</td>
<td>346.7±21.9 NS</td>
</tr>
<tr>
<td>FEF 50 L/S</td>
<td>3.61±0.52</td>
<td>3.54±0.3 NS</td>
</tr>
</tbody>
</table>

**P<0.001; Group I versus Group I A. NS (Not significant); Group I versus Group I A

Table 2: Effect of two-year yoga therapy on respiratory parameters
Bhat et al: The efficacy ……postmenopausal women

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Group II (n=30)</th>
<th>Group II a (n=30)</th>
</tr>
</thead>
<tbody>
<tr>
<td>VC</td>
<td>2.91±0.5</td>
<td>2.38±0.3***</td>
</tr>
<tr>
<td>FVC</td>
<td>2.83±0.5</td>
<td>2.40±0.30***</td>
</tr>
<tr>
<td>FEV1</td>
<td>2.38±0.4</td>
<td>2.03±0.3***</td>
</tr>
<tr>
<td>FEV1 ratio</td>
<td>0.91±0.07</td>
<td>0.78±0.03***</td>
</tr>
<tr>
<td>PEF/m</td>
<td>398.7±64.5</td>
<td>351.8±21.5***</td>
</tr>
<tr>
<td>FEF 50 L/S</td>
<td>4.05±0.66</td>
<td>3.58±0.32***</td>
</tr>
</tbody>
</table>

***P<0.0001; Group II versus Group IIa

Table 3: Comparative effect of one year and two-year yoga therapy on respiratory parameters

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Group I</th>
<th>Group II</th>
</tr>
</thead>
<tbody>
<tr>
<td>VC</td>
<td>2.91±0.5</td>
<td>2.40 ± 0.3***</td>
</tr>
<tr>
<td>FVC</td>
<td>2.83±0.5</td>
<td>2.51±0.4***</td>
</tr>
<tr>
<td>FEV1</td>
<td>2.38±0.4</td>
<td>2.09±0.4***</td>
</tr>
<tr>
<td>FEV1 ratio</td>
<td>0.91±0.07</td>
<td>0.78±0.13***</td>
</tr>
<tr>
<td>PEF/m</td>
<td>398.7±64.5</td>
<td>359.04±41.5***</td>
</tr>
<tr>
<td>FEF50 L/S</td>
<td>4.05±0.66</td>
<td>3.61±0.52***</td>
</tr>
</tbody>
</table>

***P<0.0001; Group I versus Group II

Statistical analysis

Statistical analysis was done using one-way ANOVA following which post hoc test to compare mean between the groups using SPSS version 18.0 (SPSS, Chicago, IL, USA). Data were expressed in mean ± standard deviation. Significant level was set as P < 0.05.

DISCUSSION

The autonomic function that can be consciously controlled harmonizing the sympathetic and parasympathetic nervous system together is breathing (15, 16). The involuntary nervous system is influenced by various breathing mechanisms. Evaluation of lung function test is a valued tool for assessing the respiratory system. It is a simple screening method which can be performed by using standardized equipment to measure pulmonary functions. This study was planned to determine the impact of one year and two-year regular yoga practice on certain respiratory parameters in postmenopausal women. Literature survey shows that yogic practice will cause progress in the overall respiratory functions (17-20). Yogic breathing moves have impressive valuable effects on respiratory muscle efficiency (20). Yogic practice improves lungs and chest expansion to the greatest conceivable degree. Yogic form of breathing exercise is pranayama. It is an art of prolongation and control of breath.
which helps to bring the conscious awareness in breathing; to reshape breathing habits and patterns. The persistent conditioning of breathing pattern of pranayama increases the pulmonary function in healthy individuals. Prolonged breath holding Pranayama like Kapalbhati and Nadisodhan included in our present study schedule involves powerful strokes of exhalation, which trains the subject to make full use of diaphragm and abdominal muscles (21). Slow, deep and full inhalation and exhalation as in Anulom-vilom and Bhashrika pranayama also improves respiratory muscle efficiency. The results observed in our present study are in accordance with the other reports. Maximum inflation and deflation during the breathing exercise is an important physiological stimulus for the release of surfactants and prostaglandins into the alveolar spaces causing the drastic rise in the lung compliance (22). Activation of the stretch receptors reflex decrease the tracheobronchial smooth muscle tone leading to the diminished air flow resistance and augmented airway caliber profoundly influencing the pulmonary function test.

CONCLUSION

Optimum benefit of yoga was observed during the two years of yoga practice in the postmenopausal women. Continuing practice of yoga in postmenopausal women might be considered as a preventive exercise to reduce the age-related complications in respiratory diseases preventing mortality and morbidity, thus improving the quality of life. More studies are required for further understanding the genuine adjustments in the respiratory muscles and changes in execution in the larger group. The results of the present study might gain attention in to the possible importance of yoga practice in the Post-menopausal women as a preventive exercise to impair age associated illness and improve the quality of life by creating awareness.

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Contraceptive preferences for birth spacing among married women of reproductive age group in a semi urban area near Chennai- A cross-sectional study

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ABSTRACT

Introduction and Aim: Adequate birth spacing has proved to be an important factor in reducing both Maternal and Childhood mortality and morbidity. According to NFHS 4 data currently in India only 2/3rd of the births are having spacing of more than 2 years. Therefore this study was planned to find out the awareness and practice of birth spacing among Married women residing in a semiurban area near Chennai.

Methods: Cross-sectional study was conducted among 115 married women of Reproductive age group. Pretested and predesigned semi structured Questionnaire was used to collect data on Awareness and practice regarding birth spacing. Data was analysed using proportions.

Results: 96.5% women considered birth spacing as a good practice, 71.3% women had knowledge regarding birth spacing methods. 31.6% women practiced spacing after their first child. IUCDs was the most preferred method adopted. Lack of awareness regarding contraceptives followed by fear of complications was the major reason for not adopting spacing methods.

Conclusion: Attitude regarding spacing was found to be good but knowledge and practice were less. It is high time that New contraceptives like injectable DMPA, POP and Centchroman be introduced in all the health facilities and people should be made aware of all the available methods.

Keywords: Birth spacing; contraceptive; spacing methods; terminal methods.

INTRODUCTION

Birth spacing is defined as the time interval between two births. WHO and other International organizations recommend a time interval of 2-3 years between 2 children. If the gap between 2 children is less than 18 months, it is said to be inadequate birth spacing, which reflects on maternal, peri-natal, neonatal, post neonatal, infantile and childhood outcomes (1).

Adequate birth spacing reduces the maternal risk of anaemia during pregnancy. It reduces the maternal morbidity and mortality. There has been reduction in preterm birth, low birth weight, small size for gestational age and foetal death due to increase in adequate birth spacing. It also reduces neonatal, infantile and childhood mortality. It also reduces childhood malnutrition (1). India has an average birth spacing of 32 months (2) according to National Family Health Survey (NFHS-4). However one-third of births have occurred in less than 2 years since the preceding birth. Spacing between two childbirths with duration more than 2 years is found to be 70.2% at National level (SRS Statistical Report 2013) (3).

Birth Spacing Campaigns under National Health Mission aims at educating women to maintain 3
year gap between first and second child as one of its objectives (4). National family welfare program has launched a scheme for spacing at birth by utilizing services of ASHAs who are paid cash incentives for counselling newly married couples to ensure spacing of 2 years after marriage and couples with 1 child to have spacing of 3 years after the birth of 1st child. The scheme was operational in 18 States (EAG, North Eastern and Gujarat and Haryana), but has now rolled out to few other states of India (5).

This study was undertaken to evaluate the awareness and practice of birth spacing among married women in the reproductive age group in Urban field practice area of a private medical college.

**METHODOLOGY**

**Study design and population**

The present study was conducted in the Urban field practice area of Private Medical College, Thirumazhisai, a Town Panchayat belonging to Thiruvallur District of Tamil Nadu. The study population included married women of reproductive age group who were willing to participate in the study.

**Sample size and sampling technique**

Sample size calculated was 115 married women belonging to the reproductive age group, residing at Thirumazhisai. They were selected by simple random technique. The sample size calculation was based on the formula: \( N = \frac{4pq}{l^2} \). The prevalence was considered to be 83%, knowledge about the need for birth spacing from a study on spacing done in rural Haryana (6) and the error was taken as 7%.

**Data collection and analysis**

A questionnaire was prepared in English based on various previous similar studies conducted (6-12) and it was validated by the Professors of OBG and Community Medicine. A pilot study with 20 subjects in ‘Nazarthpeth’ a semi urban area, was conducted to verify the feasibility of the same. Questionnaire was translated into Tamil and then back to English. Approval from the Institutional ethics committee was obtained before starting the study. Informed consent was obtained from the selected females after explaining them about the study in detail. The data collection was done by interview method using vernacular language (Tamil). Data was entered into MS Excel sheet and analysed using the Statistical Package for Social Sciences (SPSS) Software (version 19).

**RESULTS**

**Socio-demographic of the participants**

The total number of 115 married women belonging to reproductive age group participated in the study. The age of the respondents ranged between 18 and 45 years. The average age of the respondents was 31.6 years.

Out of the 115 respondents, majority (80.8%) were homemakers. Most of them (86%) got married between 15 and 25 years. The average age at marriage of the respondents was 20.6 years.

Among the total respondents, 109 had children and the remaining 6 did not have any children. Out of the respondents who had children, 37 had one child and the remaining 72 had more than one child.

**Respondents’ knowledge on birth spacing and its methods**

In the present study 73.9% women had heard about birth spacing and 71.3% had knowledge regarding spacing methods (Table 1).
Table 1: Distribution of respondents according to their knowledge on birth spacing (n= 115)

<table>
<thead>
<tr>
<th>Variable</th>
<th>Yes (73.9%)</th>
<th>No (26.1%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ever heard of birth spacing</td>
<td>85</td>
<td>30</td>
</tr>
<tr>
<td>Knowledge of birth spacing methods</td>
<td>82</td>
<td>33</td>
</tr>
</tbody>
</table>

Table 2: Respondents’ source of information regarding birth spacing

<table>
<thead>
<tr>
<th>Source of information</th>
<th>Number</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Health facilities</td>
<td>43 (50.59%)</td>
<td></td>
</tr>
<tr>
<td>Family and friends</td>
<td>35 (41.18%)</td>
<td></td>
</tr>
<tr>
<td>Mass media</td>
<td>9 (10.59%)</td>
<td></td>
</tr>
<tr>
<td>Base*</td>
<td>85</td>
<td></td>
</tr>
</tbody>
</table>

* multiple responses.

When asked about the source of information regarding birth spacing, half of the women told health facility was the source of information followed by family and friends (41.18%) (Table 2).

Birth spacing methods known by respondents

82 of total respondents knew about various birth spacing methods. Among them majority women (85.37%) knew about IUCDs followed by OCP (41.46%) as spacing methods (Table 3).

Table 3: Knowledge about various birth spacing methods among respondents (n=82)

<table>
<thead>
<tr>
<th>Contraceptive method</th>
<th>Number</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>IUD</td>
<td>70 (85.4%)</td>
<td></td>
</tr>
<tr>
<td>OCP</td>
<td>34 (41.5%)</td>
<td></td>
</tr>
<tr>
<td>Condom</td>
<td>22 (26.8%)</td>
<td></td>
</tr>
<tr>
<td>Calendar method</td>
<td>9 (10.9%)</td>
<td></td>
</tr>
<tr>
<td>Contraceptive injection</td>
<td>1 (1.2%)</td>
<td></td>
</tr>
<tr>
<td>Exclusive breast feeding</td>
<td>1(1.2%)</td>
<td></td>
</tr>
<tr>
<td>Base*</td>
<td>82</td>
<td></td>
</tr>
</tbody>
</table>

*multiple responses

Respondents’ attitude on birth spacing intervals

In the present study 111 respondents (96.5%) considered Birth spacing as a good practice. When asked whether there was the need to space birth even if one had all the needed resources, 105 respondents (91.3%) indicated that they thought it was necessary to space birth despite the availability of resources. However, 10 respondents (8.7%) did not think it was necessary to practice birth spacing if one had all the resources to cater for their children.

Respondents’ practice of various methods of birth spacing

Among the 109 respondents who had children, 41 (37.6%) followed and the rest 68 (62.4%) did not follow any birth spacing method. Among 41 women who followed birth spacing, most of them used Copper-T (46.3%) followed by Calendar method (43.9% ; Table 4)
Jain et al: Contraceptive preferences ..........Chennai- A cross- sectional study

Table 4: Various contraceptive methods used by the respondents who followed birth spacing methods after their first child

<table>
<thead>
<tr>
<th>Contraceptive method used</th>
<th>Number</th>
</tr>
</thead>
<tbody>
<tr>
<td>Copper-T</td>
<td>19(46.3%)</td>
</tr>
<tr>
<td>Condom</td>
<td>3 (7.3%)</td>
</tr>
<tr>
<td>Calendar method</td>
<td>18 (43.9%)</td>
</tr>
<tr>
<td>OCP</td>
<td>1 (2.4%)</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>41</strong></td>
</tr>
</tbody>
</table>

None of the subjects used any contraceptive method before first child. In the present study among 68 respondents who did not practiced any contraceptive method after 1st child birth, lack of awareness of contraceptives was found to be the major reason (66.2%) followed by fear of contraceptives (26.5%) for not adopting any method of contraception after first child birth (Table 5).

Table 5: Various reasons given by respondents who had more than one child who did not use any temporary contraceptive methods

<table>
<thead>
<tr>
<th>Reason for not using any contraceptive method</th>
<th>Number</th>
</tr>
</thead>
<tbody>
<tr>
<td>Did not know about contraceptive methods</td>
<td>45 (66.2%)</td>
</tr>
<tr>
<td>Fear of contraceptives</td>
<td>18 (26.5%)</td>
</tr>
<tr>
<td>Conceived during lactation period</td>
<td>3 (4.4%)</td>
</tr>
<tr>
<td>Not allowed</td>
<td>2 (2.9%)</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>68</strong></td>
</tr>
</tbody>
</table>

Birth spacing intervals

In the present study among 72 respondents who had more than one child the average birth spacing interval was found to be 2.7 years (31 months). 69.4% women had 2-3 years of spacing between two children (Table 5).

Table 6: Birth spacing duration followed by respondents who had more than one child

<table>
<thead>
<tr>
<th>Birth spacing interval</th>
<th>Number</th>
</tr>
</thead>
<tbody>
<tr>
<td>&lt; 2 years</td>
<td>15 (20.8%)</td>
</tr>
<tr>
<td>2-3 years</td>
<td>50 (69.4%)</td>
</tr>
<tr>
<td>2-5 years</td>
<td>6 (8.3%)</td>
</tr>
<tr>
<td>&gt;5 years</td>
<td>1 (1.2%)</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>72</strong></td>
</tr>
</tbody>
</table>

Partners’ involvement in birth spacing

Among 109 respondents who had children, 64 females (58.7%) discussed birth spacing with their husbands while the remaining 45 females (41.3%) did not discuss birth spacing with their partners. Among the 64 females who discussed regarding birth spacing with their husbands, 31 (48.4%) followed contraceptive methods. Among the 45 females who did not discuss birth spacing...
with their husbands, only 10 (22.2%) followed contraceptive methods the difference was found to be statistically significant (Table 7).

Table 7: Association of adoption of spacing methods with partners’ involvement

<table>
<thead>
<tr>
<th>Partners involvement in birth spacing</th>
<th>Spacing method followed</th>
<th>Spacing not followed</th>
<th>Total (109)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Yes</td>
<td>31 (48.4%)</td>
<td>33 (51.6%)</td>
<td>64 (58.7%)</td>
</tr>
<tr>
<td>No</td>
<td>10 (22.2%)</td>
<td>35 (77.8%)</td>
<td>45 (41.3%)</td>
</tr>
</tbody>
</table>

\[X^2 = 7.7381, \ p = 0.005407\]

Respondents’ practice of contraceptive methods after their last child

Out of the 71 who followed contraception after the last child, 70 (98.6%) of them had undergone tubectomy. 68 of which preferred this method since it is a permanent method and their family was complete and 2 of them because of their poverty. One of them used condom as the couple found it to be easy with no side effects, however there was a failure within 3 months following which the respondent had to undergo medical termination of pregnancy.

DISCUSSION

This study was conducted among women of reproductive age group at Thirumazhisai a suburban area near Chennai. In this study 73.9% of the women had knowledge about birth spacing which was found to be less than reported by Yadav et al., (82.6%) in Haryana (6).

In the present study, the major sources of information regarding birth spacing was health facility followed by family and friends. Mass media played a very meger role in spreading birth spacing information. Study on birth spacing by Christina et al., conducted at Ghana, reported health facility (38.9%) followed by mass media (32.6%) and family and friends (28.5%) were the important source of information (11). Both the studies suggest that health facility have contributed as a significant source of information to the general public regarding contraceptives.

In the present study 71.3% of the women had knowledge about contraceptive. This was very much similar to study conducted at Kerala where the knowledge about contraceptive among women was found to be 70% (12).

In this study birth spacing was considered as a good practice among 96.5% of the subjects. This was found to be higher than reported by Yadav et al., in Haryana where birth spacing was considered as a good practice among 90.6% of the subjects (6).

In the present study 39.4% of the total participants practiced birth spacing this was found to be similar to that reported in Kerala where 40% of the total participants practiced birth spacing (12).

Lack of information regarding contraceptives was found to be the major reason for non adoption of spacing contraceptive methods followed by fear of complications. Fear of complications and lack of information was found to be the major factors in the study by Peethambar and Devii Kerela (12). In this study the average birth spacing was found to be 30 months. Whereas India has average birth spacing of 31 months (2) according to National Family Health Survey (NFHS-4). This indicates that Thrumazhisai has birth spacing slightly less than the National average.

In the present study women who discussed birth spacing with their spouses adopted spacing methods more then women who could not discuss it with their spouses and the difference was found to be statistically significant. Men are the decision
makers in Developing countries, involvement of Men in family planning and practicing birth spacing is essential.

Government of India under the new initiatives in national family welfare program has expanded the basket of choice of contraceptives. New contraceptives like injectable DMPA, Progesterone Only Pills (POP) and Centchroman (Chaya) have been introduced in the program (5). But in the present study except for one participant none had knowledge regarding these new contraceptives.

In the present study, choice of method of contraception after completion of family is found to be tubectomy (98.6%). None of the couples have followed vasectomy. This is similar to low level of vasectomy as reported by various studies throughout India (13).

CONCLUSION
Attitude regarding birth spacing was found to be good among the study population. Most of the women considered birth spacing as a good practice but lacked the knowledge regarding contraception methods. This problem can only be tapered by giving proper health education and counseling to the mother as well the family. Women should be given prenatal counseling about birth spacing, the need, importance and benefits of spacing their children. They should be educated about the various methods available and given the choice to choose the best among them. The husbands should also be encouraged to participate in the same.

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Jain et al: Contraceptive preferences …….Chennai- A cross-sectional study

Effect of mobilization on ankle mobility and balance performance in diabetic patients
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ABSTRACT

Introduction and Aim: Diabetic Cheiroarthropathy is defined as the condition of restriction joint mobility due to pseudo-sclerodermatous hand, the fibrosis of the elastin connective tissues over the skin. It limits joint mobility especially around Tibia fibular mobility that are interrelated with the flexibility of ankle motion that results in the balance disorder in diabetic population. The aim of the study was to determine the effects of the tibia fibular mobilization technique on ankle joint in diabetes mellitus patients.

Materials and Methods: 60 diabetic subjects were randomly assigned into experimental group and control, in which experimental group received Tibia fibular mobilization technique and conventional treatment whereas control group received only conventional treatment 1 time a week for 3 weeks. Results: There is no significant difference in Ankle dorsi flexion range of motion in both extremities but there is significant difference in ankle plantar flexion range of motion in both extremities and functional reach test in both extremities.

Conclusion: There is a significant difference in the ankle plantar flexion range of motion and balance factor in the diabetic population.

Keywords: Diabetic cheiroarthropathy; diabetes; tibia fibular mobilization technique; functional reach test.

INTRODUCTION

Diabetic cheiroarthropathy is defined as the condition of restriction joint mobility due to pseudo-sclerodermatous hand, the fibrosis of the elastin connective tissues over the skin. Basically pain-free restricted proximal metacarpo-phalangeal or interphalangeal joints extension right after the full bend of fingers can be seen in diabetic cheiroarthropathy (DCA). In severer condition, tight waxy skin surface, unbearable pain and stiffness might incorporate. There were several clinical features are commonly observed in DCA, such as tight waxy skin over the extremities because of the replacement of fibro tissues with collagen tissues. Basic cause of rheumatic complications in DM is obesity and low physical activity, more incidence of OA in DM is due to adipocytes. According to British Cohort poor blood glucose control leads to rheumatic complications. Many of the scientific studies showed that there was a large amount of the diabetes population having a high risk of fall because of the restricted range of motion and poor foot sensation feedback. This threatening problem shall be paid attention and resolved. Even though diabetic population did have a high awareness for preventing the vital complication, but ankle mobility and balance were often left out. In case of mobilization, physician or therapist are likely to study regarding the subtalar and talocrural joint, but less attention paid over tibia fibular joint (1).
Apart from that, the majority of the research excessively focused on studies of modalities, and manual or hands-on technique was getting less attention. So, by the intention to turn out the effects of manual technique, research regarding Maitland mobilization had chosen. The purpose of this study is to rule out the effect of the mobilization technique on ankle mobility and balance performance in diabetes patients. Maitland joint mobilization technique used to mobilize the tibiofibular joint to a diagnosed case of the diabetic patient for more than 5 years. Studies had found out the mobility of tibiofibular joint are significantly interconnected i.e. ankle mobility as the gear and wheel mechanism. In addition, the mobility of the ankle joint is directly affecting the quality of the ankle strategy as fall prevention.

There are many studies performed to prove the stretching exercise of calf muscle to improve ankle range of motion, the patients were given during 3 squat tasks, static and dynamic postural control, strength of the ankle musculature, joint position sense, and self-reported function with the Foot and Ankle Ability Measure subscales, the patient were improved (2). But still, there is less attention over the tibiofibular joint mobilization. Hence the aimed objective of our study was focusing on a diagnosed diabetic patient group that more than 5 years is because the diabetic population has a high risk of developing DCA and LJM. Hypothesis: Controlled motion of the Achilles tendon, during the optimal healing condition, is achieved by early controlled motion of the ankle joint. This will result in a strong tendon with a shorter length than for immobilized ankle joints. The patient-allowed motion of the ankle will experience a better functional outcome and a better patient-reported health in comparison with immobilization (3).

Accumulation of glycosylated end products on collagen and other connective tissues which is said to be responsible for LJMS. In the course of disease small joints of hands and feet will be impaired with the advanced stages ankle, shoulder, spine, hip will involve and will lead to risks of fall.

LJMS is a broad term under which Achilles tendon pathologies, trigger finger, Dupytrens contractures, carpal tunnel syndrome, frozen shoulder plant arafacies occurs more in diabetics than non-diabetics. LJMS is associated with micro and macro vascular complications, daily stretching exercises will reduce the risk of fall and maintains the quality of life (8).

**MATERIALS AND METHODS**

Patients with both genders aged 40 to 70 years who had chronic diabetes diagnosed more than 5 years were included. Patients with pain around the lower extremities, recent surgery around knee and ankle, tumor, non-cooperative patient and patients having neuropathy were excluded from study.

The subjects from various physiotherapy center and any volunteers from the patient family who fulfilled the inclusion criteria of the study were selected. Informed consent was taken from the patients before starting the study. This study was a simple randomized single-blind controlled study. The subject who got the odd number in sequences were allocated in the treatment group and for those who were the even numbers were assigned the control group. The data analysis was done using SPSS version 22. The descriptive data were expressed as mean ± standard deviation (SD). The data has a normally distributed pattern and the parametric paired “t” test was used to measure statistically with the value of P < 0.05 is considered statistically significant. Experimental group was given hot pack for 10 minutes over the calf, self-stretching of calf muscle-hold for 15 sec; 10 reps per set for 2 sets, strengthening exercise with calf raises of10 repetitions, 3 set, proximal and distal tibiofibular Grade 4, 1 minute per set, 5 sets of mobilization. Control group was given -Hot pack for 10 minutes over the calf ,self-stretching of calf muscle; hold for 15 sec; 10 reps per set for 2 sets, Strengthening exercise – calf raises; 10 repetitions, 3 sets. Both of the groups were given approximately 45 minutes to complete for a single
session of the treatment. The participation duration for each subject is 3 weeks.

RESULTS

Table 1: Comparison of dorsiflexion of the control group and experimental group

<table>
<thead>
<tr>
<th>Groups</th>
<th>Left Dorsiflexion</th>
<th>Right Dorsiflexion</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Number</td>
<td>Median</td>
</tr>
<tr>
<td>Control</td>
<td>30</td>
<td>26.3667</td>
</tr>
</tbody>
</table>

Inf: There is no significant difference in left and right dorsiflexion among experimental group and control group.

Table 2: Comparison of plantarflexion of the control group and experimental group

<table>
<thead>
<tr>
<th>Groups</th>
<th>Left Plantarflexion</th>
<th>Right Plantarflexion</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Number</td>
<td>Median</td>
</tr>
<tr>
<td>Control</td>
<td>30</td>
<td>60.8333</td>
</tr>
<tr>
<td>Experimental</td>
<td>30</td>
<td>35.5667</td>
</tr>
</tbody>
</table>

Inf: There is significant difference in the left and right dorsiflexion among experimental group and control group.

Table 3: Comparison of functional reach test of the control group and experimental group

<table>
<thead>
<tr>
<th>Groups</th>
<th>Number</th>
<th>Median</th>
<th>SD</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>30</td>
<td>39.4000</td>
<td>8.13507</td>
<td>.000</td>
</tr>
<tr>
<td>Experimental</td>
<td>30</td>
<td>32.0800</td>
<td>5.90847</td>
<td>.000</td>
</tr>
</tbody>
</table>

Inf: There is a significant difference between functional reach test among the experimental group and the control group. As mean and standard deviation of Control and Experimental group are 39.4000 and 32.0800, And 8.13507 and 5.90847. The Experimental group shows significant improvement in functional reach test.

DISCUSSION

Many studies observed that the Maitland joint mobilization technique on diabetic’s population. Cherqoui et al., stated that the diabetic population tended to suffer limited joint mobility syndrome over the ankle joint which will directly lead to poor balancing ability. Thus, by application of joint mobilization were intended to prevent the LJMS and improvise better balancing mechanism.

The current study was conducted to evaluate the effects of mobilization technique on ankle mobility and balance performance in diabetes patients. Combination of Maitland mobilization technique on distal and proximal tibia fibular with standard treatment statistically significantly improved ankle dorsiflexion, plantarflexion, and functional reach test and the hypothesis was accepted tibia-fibula (proximal and distal) mobilization is effective to increase the ankle joint mobility in the group of chronic DM patient.

Rheumatic complications with DM Associated formation of glycosylated end products which leads to vasculopathy and neuropathy. More the duration of DM more will be the rheumatic complications in DM. Especially if diagnosed early systematic rheumatologic exam to screen any musculoskeletal complications, which can be potentially treatable.

There should be multidisciplinary collaboration between the rheumatologist, endocrinologist, and physiotherapist (1). A similar review observed by Gerrits, stated that the limited joint mobility syndrome is mainly caused by the long term of macro/microvascular complication and LJMS are unlikely reversible and lack of curable treatment option. Gerrits further mentioned the best way to cure the LJMS is to prevent it before the development of chieoroarthropathy. Hence, active joint exercise along with sustained stretching and good glycemic control was suggested to the diabetic population to maintain their healthy
joints. A related study conducted by Zimny et al., stated that the limited ankle mobility was one of the factors that leads to elevated forefoot peak plantar pressure which will leads to foot ulceration, therefore plantar pressure measurement is recommended to include in future study to evaluate the application of joint mobilization in order to maintain or improve the ankle range of motion and to control the elevated plantar pressure in advance.

Another experimental study performed by Beazell et al., found to be similar to the current study which is stated that proximal and distal manipulation of the tibia fibular joint would be altered and improve ankle range of motion (4). According to some authors (1, 2, 15), treadmill walk with ankle stretching orthosis (ASO) will significantly improve ankle dorsiflexion range (5). Study done by Cockayne et al., the falls in elderly was the incidence rate of falls per participant in the 12 months following randomization. The secondary outcomes included the proportion of fallers and multiple fallers, time to first fall, fear of falling, fracture rate, health-related quality of life (HRQoL) and cost-effectiveness. They found that the interventions were potentially effective (6). Deussen and Alfuth studied the effect of sensorimotor training on balance strength, joint function on and planter foot sensitivity in chronic ankle sprain in athletes found there would be no difference in experimental and control group(7). Daily range of motion exercises and maintenance of good glycemic control are the frame work for prevention of LJMS (8). Hoch et al., studied two-week joint mobilization intervention on dynamic balance and range of motion in adults with (CAI) concluded that joint mobilization targeted to talar glide improves the overall functional independence in adults with (CAI) within 1 week (9). Hoch and McKeon studied the effect of grade III mobilization of talar would improve dorsiflexion range of motion (10).

Jeon et al., studied to compare the effects of 2 ankle-stretching techniques on ankle DFROM and concluded that as the talus was stabilized with a strap and the anterior glide of distal tibia at talocrual joint was facilitated, it would to improve the ankle dorsiflexion range, the active dorsiflexion and planter would improve with 3 weeks of self-stretching intervention (11).

Scaddan et al., studied that joint mobilization is the way of treating joint arthritis (12). Apart from that, Kalten born et al., mentioned that range of motion of the affected joint will improve after a joint mobilization session because of greater extensibility of the ligament structures and non- contractile capsular and the increased activity of the joint sensation and conduction of afferent impulses (13). The study carried out by Grindstaff et al., found to be similar to the current study stating that there was a presence of facilitation spinal excitability of the calf muscle group while manipulating distal tibiofibular joint in CAI patients (14). Hence the presence of the stimulation of sensory receptors during the joint mobilization and facilitation of the afferent activity combine with the amplification of neuromuscular activation of the muscles structure that stabilized the joint, eventually resulting in the improvement of dynamic postural control during the functional reach test among the diabetic subjects. So the study concludes that there is a significant difference in the ankle range of motion and functional reach test in the diabetic population (15). Terada et al., studied static stretching which would improve ankle dorsiflexion after acute ankle sprain, they perform various interventions such as stretching, manual therapy, electrotherapy, ultrasound, and exercises, to increase ankle dorsiflexion but the combined interventions are more effective than one intervention (16). Cheiroarthopathy is common in people with type 1 diabetes and related to high level of glycaemia. A routine examination of musculoskeletal system examination is required to rule out arthritic complications with type 1 diabetes (17).
CONCLUSION

Mobilization of tibiofibular joint will be beneficial in improving the ankle range of motion and balance factor in the diabetic population.

REFERENCES

Promoting hemiplegic gait

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ABSTRACT

Introduction and Aim: Balance synergy includes a number of postural response that enable an individual to arise and remain erect during standing and locomotion. Balance deficits causes an insufficient coordination, postural instability and impaired gait. Maintaining the independence in activities of activities of daily living is an important factor for the quality of life. Hence this study is done to promote Hemiplegic Gait in stroke patients.

Materials and Methods: 150 community dwelling hemiparetic subjects from Chennai aged <65 both male and female subjects were allocated in three groups (n=50) using convenient sampling method and were followed up for a period of 12 weeks with intervention duration of 45 mins daily and were assessed with POMA, FALL RISK, TUG and 6 minute walk test. Paired ‘t’ test was used for assessing pre and post-test values.

Results: There was significant difference with p < 0.001 at both Tinetti Performance oriented mobility assessment, Time up and Go Test, Fall Risk and 6 minute walk test minimal significant difference in chair stand test in both the groups.

Conclusion: There was significant difference in POMA, FALL RISK, TUG and 6 minute walk test p <.005 in Group C & Group B.

Keywords: Stroke; balance; gait; fall risk.

INTRODUCTION

Cerebrovascular accidents leads to inability of muscles to generate the appropriate forces, decrease in motor unit firing rates, decreased multisensory integration, consequently leads to poor balance control and force deficits depends on the muscle length (1) and it is commonly associated with a decrease in balance ability.

Balance synergies include a number of postural responses that enable an individual to arise and remain erect during standing and locomotion. Standing is an active process in which the sway of the body is kept within the limits of the base of support provided by the feet. Anticipatory postural responses are changes in postural muscle groups that precede voluntary movements made to offset disturbances in balance that would result from the voluntary control (2).

Balance deficits causes an insufficient coordination, instability and also compromise the reduced postural stability during standing and less coordinated responses to both self induced and external balance perturbations (3) and sensory information for postural control are somatosensory, visual, vestibular symptoms (4) and motor responses that affects the joint range of movement, abnormal muscle tone, loss of motor coordination and reduction in muscle strength affects the person ability to transfer, ambulate within home environment and community and it deteriorates balance function which consequently increases their fall risk and
reduces the independence in activities of daily living.

Maintaining the independence in activities of daily living is an important factor for the quality of life. Stroke survivors who need assistance for activities of daily living always feel socially isolated, overwhelmed, and abandoned. Gillespie et al., stated that the hemiplegic patient also place burden to their family caregivers, therefore, affecting their family relationships. Jia et al., stated that age, education, care givers, history of past illness, smoking and muscle strength are influencing factors of activities of daily living for stroke subjects(5).

Fear of falling is concerned with falling due to loss of self efficacy sense in body balance and mobility which results in restricted activities of daily living and social deprivation(3). Weedersteyn et al., found the incidence of falls is higher in community dwelling stroke individuals than in the general healthy elderly population. Forster et al, found the majority of falls occur during walking which suggests that dynamic balance control during gait is an important issue (2). Hyndman et al., found no significant difference between the characteristics of their community based faller and non-faller groups in stroke. Mackintosh et al, also reported that reduced mobility and poor balance among recurrent fallers in community stroke peoples (6).

Walking is one of the most important activities for enabling community participation (7). Initial walking function is impaired in two – thirds of the stroke population and this impairment is the greatest contributor to post stroke functional disability (2). Characteristics walking pattern of stroke includes a slow walking cycle, a short stance phase on the affected side and relatively long swing phase (8). Subjects with hemiplegia displays increasing recovery of their independent walking ability however they lack the ability to walk fast or far enough, which reduces their ability to walk outside (7) and the walking adaptability is the ability to adjust walking to behavioural task goals and environmental circumstances and it is a complex of gait adaptability that is obstacles, clearance, negotiation and locomotor adaptation and it is one of the component to generate stepping and maintain the postural equilibrium (9) so the individuals walk with lesser distances with higher oxygen consumption. Thus, indicating they walk with higher oxygen demand (2).

Walking exercise has diverse effects and to improve gait and balance ability. So, that feet are called the second heart. It may enhance cardiovascular endurance as well as developing leg muscles. The effect of walking exercise aimed at improving balance sense and motor control ability with elderly people, patient with a chronic disease. Backward walking improves the movement components to stimulate the muscles in lower limbs more than the forward walking (10). Nadeau et al, reported that backward walking and forward walking had different exercise physiology. The temporal spatial characteristics of backward walking could increase the frequency and endurance for walking (11). Osugi et al., reported that walking speed, step length, and cadence were lower in backward walking than in forward walking. Winter et al, reported that joint movement patterns are similar for backward walking and forward walking (12). Grasso et al., suggested that the patients trained for backward walking requires a greater level of energy expenditure than in the patients trained for forward walking and it increases the cardiorespiratory and metabolic responses and oxygen consumption (7).

Balance plays one of the major role in one’s life to do their activities of daily living independently. Muscle weakness, imbalance is one of the major factor leading to reduced mobility and reducing the independency level in hemiplegic subjects. The effect of progressive body weight supported treadmill training in forward and backward gait training and the differences between progressive body weights supported treadmill training in forward and backward gait training in hemiplegic subjects showed that the gait performance of the subjects
improved in progressive body weight supported treadmill training in forward and backward gait training. Hence this study aims to know the effect of dynamic balance exercise in forward and backward walking in hemiplegic subjects.

MATERIALS AND METHODS

150 chronic hemiplegic subjects from Vadallur and Chennai were included in the study based on inclusion criteria: Age 50-70 years, both male and female subjects, Brunnstrom grading 4 and 5 in paretic lower limb, subjects who were able to walk with support or without support, Subjects with MMSE score 24-30. Patients with open wounds in leg, impaired sensory loss in leg, any recent surgeries in lower limb, Deep Vein Thrombosis, any disability other than stroke, like spinal cord lesion, polyneuropathy, peripheral nerve lesion, recent fractures in lower limb were excluded. Then informed consent was obtained from them and Subjects were allotted in three groups. Group A (Control group) n = 50, Group B n = 50 and Group C n = 50 (Experimental group) using randomized controlled trial method. The randomization was done by using lot method. Group A (Balance Exercises and Conventional Physiotherapy Exercises. Group B (Balance exercises and forward walking with and without support) and Group C (Balance exercise and backward walking with and without support).

PROCEDURE

The hemiplegic subjects who fulfil the inclusion criteria were included and informed Consent was obtained from them. Subject’s demographic data, onset duration and hemiplegic side and assessment was noted. The pre test was taken and the exercise was demonstrated to the subjects involved in the study and was informed that they have to do the exercise regularly and can withdraw from the study if they have any discomfort or difficulty. Group therapy was given under supervision. The subjects were asked to wear comfortable clothing and they have to do warm up and cool down exercise before and after the exercise. Group A: Balance exercises (20 minutes) and Conventional Physiotherapy (15 minutes). Group B: Balance exercises (20 minutes) and forward walking with and without support (15 minutes). Group C: Balance exercise (20 minutes) and backward walking with and without support (15 minutes).

Warm up exercises - 5 minutes

<table>
<thead>
<tr>
<th>S. No.</th>
<th>Exercise</th>
<th>Repetition</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Hip Flexion</td>
<td>5 Times</td>
</tr>
<tr>
<td>2.</td>
<td>Knee Extension</td>
<td>5 Times</td>
</tr>
<tr>
<td>3.</td>
<td>Seated Marching</td>
<td>30 Seconds</td>
</tr>
</tbody>
</table>

Cool down exercises - 5 Minutes

<table>
<thead>
<tr>
<th>S. No.</th>
<th>Exercise</th>
<th>Repetitions</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Leg Press</td>
<td>5 times</td>
</tr>
<tr>
<td>2.</td>
<td>Seated Kick</td>
<td>5 times</td>
</tr>
<tr>
<td>3.</td>
<td>Wide Half Stands</td>
<td>30 seconds</td>
</tr>
</tbody>
</table>

Dynamic balance exercises

Wobble board exercise: The subjects were asked to stand on the wobble board with eyes open; the subjects’ feet and shoulder width apart. Asked to hold a rail for support and stand for 2 to 3 minutes. The subjects were asked to stand on the wobble board with eyes open. The subjects’ feet and shoulder width apart and stand without support for 2 to 3 minutes.

<table>
<thead>
<tr>
<th>S. No.</th>
<th>Exercise</th>
<th>Repetitions</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Stepping</td>
<td>2 Minutes</td>
</tr>
</tbody>
</table>
2. Step Up & Step Down 10 Repetitions
3. Cross Walking 2 Minutes
4. Tandem Walking 2 Minutes
5. Side Walking 2 Minutes
6. Step Back 2 Minutes
7. Trunk Rotation With Medicinal Ball 10 Repetitions

If subjects feel any discomfort they can take rest for 5 minutes between the sessions. Subjects were followed up for a period of 3 months with intervention duration of 35 minutes for 4 days per week for 3 months and will be assessed pre and post-test with Morse Fall Risk Scale, POMA (Tinetti Performance Oriented Mobility Assessment), Time Up and Go Test, and 6 Minute Walk Test.

Data analysis and interpretation

All statistical analysis were performed on IBM compatible micro computer using Statistical Package for the Social Sciences (SPSS 17.0).

The significance was set at alpha=0.005 level. Paired t Test was used to compare the pre and post values of Morse Fall Risk Scale, POMA (Tinetti Performance Oriented Mobility Assessment), Time Up and Go Test, and 6 Minute Walk Test in chronic hemiplegic subjects.

<table>
<thead>
<tr>
<th>Table 1: Morse Fall Risk Score of Group A, Group B and Group C</th>
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<tbody>
<tr>
<td>FALL RISK PRE TEST - FALL RISK POST TEST</td>
</tr>
<tr>
<td>GROUP – A</td>
</tr>
<tr>
<td>GROUP – B</td>
</tr>
<tr>
<td>GROUP - C</td>
</tr>
</tbody>
</table>

Graph 1: Morse Fall Risk Score of Group A, Group B and Group C

![Graph 1](image)

Table 2: POMA of Group A, Group B and Group C

<table>
<thead>
<tr>
<th>POMA PRE TEST - POMA POST TEST</th>
<th>Mean</th>
<th>Std.Dev</th>
<th>Std.Error Mean</th>
<th>t- value</th>
<th>df</th>
<th>Sig. (2-tailed)</th>
</tr>
</thead>
<tbody>
<tr>
<td>GROUP – A</td>
<td>2.06</td>
<td>1.48</td>
<td>0.21</td>
<td>9.87</td>
<td>49</td>
<td>.000</td>
</tr>
<tr>
<td>GROUP – B</td>
<td>3.02</td>
<td>1.57</td>
<td>0.22</td>
<td>13.59</td>
<td>49</td>
<td>.000</td>
</tr>
<tr>
<td>GROUP - C</td>
<td>4.42</td>
<td>1.01</td>
<td>0.38</td>
<td>30.88</td>
<td>49</td>
<td>.000</td>
</tr>
</tbody>
</table>

Graph 2: POMA of Group A, Group B and Group C

![Graph 2](image)
Table 3: Time Up and Go Test of Group A, Group B and Group C

<table>
<thead>
<tr>
<th>TUG PRE TEST - TUG POST TEST</th>
<th>Mean</th>
<th>Std.Dev</th>
<th>Std.Error Mean</th>
<th>t- value</th>
<th>df</th>
<th>Sig. (2-tailed)</th>
</tr>
</thead>
<tbody>
<tr>
<td>GROUP – A</td>
<td>-0.46</td>
<td>0.3</td>
<td>0.04</td>
<td>-11.51</td>
<td>49</td>
<td>.000</td>
</tr>
<tr>
<td>GROUP – B</td>
<td>-0.61</td>
<td>0.24</td>
<td>0.03</td>
<td>-16.96</td>
<td>49</td>
<td>.000</td>
</tr>
<tr>
<td>GROUP - C</td>
<td>-0.73</td>
<td>0.17</td>
<td>0.06</td>
<td>-29</td>
<td>49</td>
<td>.000</td>
</tr>
</tbody>
</table>

Graph 3: Time Up and Go Test of Group A, Group B and Group C

Table 4: 6 Minute Walk Test Laps of Group A, Group B and Group C

<table>
<thead>
<tr>
<th>LAPS PRE TEST - LAPS POST TEST</th>
<th>Mean</th>
<th>Std.Dev</th>
<th>Std.Error Mean</th>
<th>t- value</th>
<th>df</th>
<th>Sig. (2-tailed)</th>
</tr>
</thead>
<tbody>
<tr>
<td>GROUP – A</td>
<td>1.19</td>
<td>0.69</td>
<td>0.10</td>
<td>12.16</td>
<td>49</td>
<td>.000</td>
</tr>
<tr>
<td>GROUP – B</td>
<td>2.3</td>
<td>0.99</td>
<td>0.14</td>
<td>16</td>
<td>49</td>
<td>.000</td>
</tr>
<tr>
<td>GROUP - C</td>
<td>2.72</td>
<td>0.88</td>
<td>0.33</td>
<td>21.81</td>
<td>49</td>
<td>.000</td>
</tr>
</tbody>
</table>

Graph 4: 6 Minute Walk Test Laps of Group A, Group B and Group C
RESULTS

- Table 1 Shows the Group A (Control Group) mean value of Morse Fall Risk score: 32±1.14, Tinetti Performance Oriented Mobility Assessment (POMA): 22.4±0.38, Time Up and Go Test: 1.49±0.07, Six Minute Walk Test: Laps: 5.75±0.16, Steps: 174.9±5.13 and Meters: 46±1.28.

- Table 2 shows the Group B (Experimental Group 1) mean value of Morse Fall Risk score: 37.75±1.25, Tinetti Performance Oriented Mobility Assessment (POMA): 23.1±0.28, Time Up and Go Test: 0.98±0.05, Six Minute Walk Test: Laps : 7.55±0.21, Steps: 214.75±6.85 and Meters : 58.35±1.7

- Table 3 shows the Group C (Experimental Group 2) mean value of Morse Fall Risk score: 25.75±1.41, Tinetti Performance Oriented Mobility Assessment (POMA) : 25±0.21, Time Up and Go Test: 0.71±0.04, Six Minute Walk Test: Laps: 8±0.20, Steps: 229.55±6.75, and Meters: 63.55±1.7

DISCUSSION

Balance deficits causes an insufficient coordination, instability and also compromise the reduced postural stability during standing and less coordinated responses to both self induced and external balance perturbations\(^3\) and sensory information for postural control are somatosensory, visual, vestibular symptoms\(^4\) and motor responses that affects the joint range of movement, abnormal muscle tone, loss of motor coordination and reduction in muscle strength affects the person ability to transfer, ambulate within home environment and community and it deteriorates balance function...
which consequently increases their fall risk and reduces the independence in activities of daily living.

Fear of falling is concerned with falling due to loss of self efficacy sense in body balance and mobility which results in restricted activities of daily living and social deprivation(3).

The walking adaptability is the ability to adjust walking to behavioural task goals and environmental circumstances and it is a complex of gait adaptability that is obstacles, clearance, negotiation and locomotor adaptation and it is one of the component to generate stepping and maintain the postural equilibrium(9) so the individuals walk with lesser distances with higher oxygen consumption. Thus, indicating they walk with higher oxygen demand (2).

Walking is one of the most important activities for enabling community participation (7). Walking exercise has diverse effects and to improve gait and balance ability. So, that feet are called the second heart. It may enhance cardiovascular endurance as well as developing leg muscles. The effect of walking exercise aimed at improving balance sense and motor control ability with elderly people, patient with a chronic diseases. Backward walking improves the movement components to stimulate the muscles in lower limbs more than the forward walking (10).

Backward walking on a treadmill challenges the stability and thus it is possible that step shortening can be partially attributed to the subject’s uncertainty and attempt to maintain stability. If this is the case, proprioceptive information from lower limbs may be important for proper correction of step length. Backward walking activates the patella femoral joint and femoral muscles in a more stabilized manner (10). According to this result, three groups were statistically significant in Morse Fall Risk, POMA (Tinetti Performance Oriented Mobility Assessment), Time up and Go Test, and Six Minute Walk Test (p=0.0001).

Monitoring the Fall Risk over post stroke period is important even when there is a improved baseline gait/balance function in post stroke. Hence this study was measuring the Morse Fall Risk Score in three groups. According to this result, Fall Risk was reduced more in Group C-Balance Exercise and Backward Walking, when compared to other two groups, (Group A-Conventional Physiotherapy and Balance Exercise, Group B-Balance Exercise and Forward Walking).

POMA (Tinetti Performance Oriented Mobility Assessment) was used to assess the Balance and Gait. According to this result, Balance and Gait was more improved in Group C-Balance Exercise and Backward Walking, when compared to other two groups, (Group A-Conventional Physiotherapy and Balance Exercise, Group B-Balance Exercise and Forward Walking).

The Time Up and Go Test is commonly used to measure the balance and walking ability in which arises from the chair and back to the chair. The outcome of Time Up and Go Test is Six Meters were found to be more increased in Group C-Balance Exercise and Backward Walking, than the other two groups, (Group A-Conventional Physiotherapy and Balance Exercise, Group B-Balance Exercise and Forward Walking).

The Six Minute Walk Test is commonly used to measure the level of walking ability following stroke and subsequent recovery during the rehabilitation process (7). The outcome of the Six Minute Walk Test are Laps (Rounds), Steps (counts) and Meters were found to be more increased in Group C-Balance Exercise and Backward Walking, than the other two groups, (Group A-Conventional Physiotherapy and Balance Exercise, Group B-Balance Exercise and Forward Walking).

The present study concluded that Walking factors such as Morse Fall Risk Score, Tinetti Performance Oriented Mobility Assessment (POMA), Time up and Go Test, and Six Minute Walk Test were compared between pre and post exercise training. Based on those factors, I found
that backward gait training has improved their walking ability in chronic hemiplegic subjects.

Limitations

Specific type of lesion was not included, Gender Bias, Body Mass Index (BMI) was not included, Lesion onset was not same duration, Functional activity level was not considered, and Motivation and stress level was not considered.

Recommendations

Sub-acute stroke patients can be included in further studies, Patient’s activity level can be considered, and Patient’s motivation and stress level can be considered, to increase different training principles, For example: the combination of Neuromuscular Electrical Stimulation and Robotics or Neuro-modulating.

CONCLUSION

The present study concluded that the dynamic balance exercise along with backward gait training group improved significantly than Group A (Conventional Physiotherapy and Balance Exercises) and Group B (Balance Exercise and Forward Walking) in Morse Fall Risk Score, POMA (Tinetti Performance Oriented Mobility Assessment), Time Up and Go Test and Six Minute Walk Test and were able to gain confidence and had better performance in their activities of daily living.

REFERENCES


Comparison of Jaffe and enzymatic methods for creatinine estimation and their effect in GFR calculation in a tertiary care hospital

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ABSTRACT

Introduction and Aim: Serum creatinine (SCr) and estimated glomerular filtration rate (eGFR) gives the idea of overall functional status of kidney. Measurement of SCr by different method and discordant result between them can misclassify different stages of chronic kidney disease (CKD). Many of the tertiary care health centre, SCr measured by Jaffe method is still the method of choice because of its cost effectiveness even if it is more susceptible for interference. Our aim was to measure SCr level by both Jaffe’s & enzymatic method and analysing the discordance rate of eGFR and their effect in staging of kidney disease in CKD patients.

Materials and Methods: In this observational study 330 serum sample were analysed for creatinine by Jaffe’s and enzymatic methods in a fully automated analyser using commercially available kits. Modification of Diet in Renal Disease (MDRD) formula was used for estimating GFR.

Results: In 330 subjects eGFR values calculated on incorporating SCr were found as 58.84 ± 68.34 (median (IQR) = 26.67(15.09-90.84) and 44.49 ± 41.18 (Median (IQR) = 26.86 (14.69-67.69)) ml/min/1.73 m² respectively by Jaffe’s and enzymatic method. Concordance correlation coefficient between two methods for SCr was statistically significant. Bland-Altman plot showed with increasing value of SCr, the difference between the SCr values given by these two methods increased. Jaffe’s creatinine has lower value in comparison to enzymatic Cr. There was a significant difference between eGFR obtained from SCr estimated by Jaffe’s and enzymatic method.

Conclusion: All laboratory should use uniform method for creatinine estimation. Enzymatic method for creatinine estimation should not be compromised over cost of test, so that less variability in creatinine result can lead to more accurate staging of the disease.

Keywords: Analytical variation; glomerular filtration rate; method comparison; renal dysfunction.

INTRODUCTION

Renal function can be assessed by estimation of glomerular filtration rate (GFR). Measurement of serum creatinine (SCr) has been used as a cost effective and endogenous marker, to assess glomerular function since decades. Indeed, SCr measurement is incorporated into the equation designed to estimate GFR (eGFR) (1). Calculated GFR value is superior to SCr alone, as the later might be influenced by many biological factors like age, gender, ethnicity, muscle mass, nutritional status of the individual and the analytical method it is being estimated (2-4). Primarily eGFR calculation is done to identify the presence of renal diseases and is been reported to be useful for determination of different stages of CKD and also to monitor response to treatment (5). SCr is commonly
measured on automated analyser using either Jaffé’s method or enzymatic assay. Indeed, Jaffé’s method is more susceptible to interferences like bilirubin, ketone bodies, protein etc. Whereas enzymatic assay for SCr exhibits comparatively less interference, both in frequency and degree of interference (6). Such type of interference, that either increase or decrease the creatinine value, will consequently affect eGFR calculation and misclassification of CKD. Therefore, several authors have also suggested to abandon Jaffé’s assay and are in favour of enzymatic assay (7). Though Jaffé’s method is subjected to bias due to interfering substances and loss of analytical specificity, still it is most commonly used in clinical practice, as it is less expensive compared to enzymatic method.

**eGFR and Creatinine relationship**

Analytical bias in SCr measurement gives unreliable renal functional status, incorrect staging and inappropriate treatment modality. Inter-laboratory variations in SCr measurement was observed worldwide because of different method of estimation. Two methods are most widely used by the lab professionals for measuring SCr and urinary creatinine. Jaffé’s alkaline picrate method was initially developed in the year of 1886. This method is interfered positively and negatively by number of chromogens such as bilirubin, cephalosporin, ketone bodies, glucose, protein, etc. (14), but still remain as most commonly used method. Over the year for overcoming these interference this method has undergone many modifications. Now compensated Jaffé (adjusted for protein interference) is used. In Enzymatic creatinine assay overall influence of interfering substance is less but is not used by clinical laboratory because of its high cost (15). Accuracy in the test result, cost of the test, ability to differentiate between the states of diseases, decision in treatment modality should be taken care while evaluating the method comparison studies.

Therefore, the present study was attempted to analyse the discordance rate in eGFR value based on serum creatinine measurement by both Jaffe’s method and enzymatic method. To compare the influence of creatinine methodologies on eGFR calculation and to see whether low cost Jaffé’s creatinine assay still can be used safely in renal diseases or should be discarded.

**MATERIALS AND METHODS**

The present observational study was conducted in central diagnostic laboratory (Biochemistry section) of a tertiary care institute, over a period of six months (1st August 2018 to 31st January 2019). 330 participants who came for renal profile analysis were explained about their leftover serum sample analysis for SCr by different method in the same laboratory. Informed consent was obtained from them and explained about less than minimal risk is
involved. Pregnant women were excluded from the study.
Left over serum samples were processed on the same day of collection for SCr. It was analysed by both Jaffe’s alkaline picrate and enzymatic method in fully automated clinical chemistry analyser, using commercially available kit as per manufacturer’s protocol. Internal Quality control (IQC) and External Quality Assurance Service (EQAS) was monitored for SCr regularly.
Kinetic Jaffe reaction without de-proteinization compensated for serum creatinine based on the principle of yellow-red coloured alkaline picrate formation. The rate of dye formation is directly proportional to creatinine concentration in the sample.
In enzymatic assay creatinine undergoes series of reactions. Initially it is converted to sarcosine and urea by creatine amidinohydrolase. Sarcosine is oxidised to glycine, formaldehyde and hydrogen peroxide by sarcosine oxidase. The final reaction involves peroxidise- catalysed oxidation of a leuco dye to produce coloured product and absorbance is measured.
Information regarding age, gender, height, weight was recorded and GFR was calculated by using 4 variables MDRD formula as follows = 175 × [S Cr]^{-1.154} × [age]^{-0.023} ×0.742 (If female) × 1.212 (If black) (16)

**Statistical analysis**

The data was analysed by Statistical Package for the Social Sciences (SPSS) software version 15.1. Quantitative data was described using mean and standard deviation as well as median (Inter Quartile range) for the variables having skewed distribution. Bland-Altman plot (17) was used to assess the agreement in Serum Cr and eGFR values obtained using Jaffe and enzymatic methods. Concordance correlation was also performed to assess the degree of concordance between these two methods. A p-value less than 0.05 was considered as statistically significant.

**RESULTS**

The present study included 330 subjects with a mean age of 57±18 years. Among these 108(32.7%) were female. Average value of SCr obtained by Jaffe and enzymatic method for 330 patients were 2.93±2.28 (Median (IQR)= 2.3 (1.1- 4.0) and 2.83±2.41 (Median (IQR)=2.19 (0.82-3.99) respectively. eGFR calculated on the basis of SCr values were found as 58.84±68.34 (Median (IQR)= 26.67 (15.09-90.84)) and 44.49±41.18 [Median (IQR)= 26.86 (14.69-67.69)] respectively by Jaffe and enzymatic methods.
The concordance correlation coefficient between Jaffe and enzymatic method for creatinine was very high i.e. 0.987(0.984-0.989) which was statistically significant.

The Bland-Altman plot (Fig.1) showed that with increasing value of serum Cr, the difference between the serum Cr values given by these two method increased. In this case Jaffe’s creatinine has lower values in comparison to enzymatic Cr. However, for lower Serum Cr level, it is reversed. Overall difference between SCr given by these two method is 0.097(-0.631-0.825).The similar results i.e. slightly higher value for SCr in Jaffe’s method was depicted for lower SCr and slightly lower value was depicted in Jaffe’s method in comparison to enzymatic method for higher SCr level in concordance graph (Fig.2). Such exploration indicated that though the magnitude of concordance correlation was very high, there is a poor agreement between the two methods.
Table 1 showed that difference of Jaffe to enzymatic SCr which is significantly higher (Jaffe > Enzymatic) for lower S Cr i.e. SCr≤ 1.5 mg/dL. This difference decreased with increasing SCr value i.e. for group II & III but it was still statistically significant. However, for group IV (SCr> 4 mg/dl), on an average enzymatic values were slightly higher than Jaffe value but not statistically significant.

eGFR values were calculated on the basis of SCr value obtained by two methods.

Table 2 showed the distribution of CKD staging. A total of 267 out of 330 subjects, 80.9% were classified into same stage by both the methods. Both methods have classified 54 patients as normal. However, by Jaffe method 55 patients were classified as normal and by enzymatic method 84 patients were classified as normal. But out of these 84 patients, 28 patients were classified as stage I and 2 patients were classified as stage II by Jaffe’s method.

Among the 330 patient, only 7(2%) were classified to lower staging by Jaffe in comparison to enzymatic. However, 56 (7%) were classified to higher staging by Jaffe in comparison to enzymatic especially for higher eGFR. Concordance correlation for eGFR observed between these two methods is 0.808(95% CI: 0.785-0.830) (Fig.3).

**Table 1:** Mean Difference between Jaffe and Enzymatic method of SCr estimation in four different creatinine range.

<table>
<thead>
<tr>
<th>Groups</th>
<th>No. of observations (n)</th>
<th>Mean difference of two methods ±SD (mg/dL)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group I (SCr ≤ 1.5mg/dL)</td>
<td>120</td>
<td>0.18±0.09</td>
<td>&lt;0.001*</td>
</tr>
<tr>
<td>Group II (1.5-2.5 mg/dL)</td>
<td>66</td>
<td>0.14±0.22</td>
<td>&lt;0.001*</td>
</tr>
<tr>
<td>Group III (2.5-4 mg/dL)</td>
<td>62</td>
<td>0.07±0.24</td>
<td>0.018</td>
</tr>
<tr>
<td>Group IV (≥ 4 mg/dL)</td>
<td>82</td>
<td>-0.05±0.65</td>
<td>0.542</td>
</tr>
<tr>
<td>Total</td>
<td>330</td>
<td>0.10±0.37</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

**Foot notes:** SCr- Serum creatinine, *- P <0.001 indicates that significant difference between Jaffe and Enzymatic method of SCr estimation, when level is <1.5 and 1.5-2.5 mg/dL.
Bland-Altman plot (Fig.4) to assess the agreement between Jaffe and enzymatic Cr for eGFR values showed the mean, lower and upper limits for Jaffe-enzymatic Cr, are -14.35, -78.10 and 49.41 respectively. The observed correlation between difference of eGFR values by two methods and mean eGFR was -0.846 which was statistically significant. This happened mainly as previously explained that the mean SCr level with Jaffe method was significantly higher compared to enzymatic method for lower creatinine values but lower for high creatinine values (Table1). Such negative correlation indicates about the poor agreement between Jaffe and enzymatic method, though there is a good concordance correlation coefficient between Jaffe and enzymatic method for creatinine.

DISCUSSION

CKD is one of the public health problems worldwide. GFR, being estimated by using different formula is the best index to evaluate renal function and CKD staging instead of SCr alone. MDRD formula has been widely used and validated in CKD patients to predict GFR by incorporating SCr value (18). In clinical laboratory Jaffe’s and enzymatic method are the two most common methods used for measuring SCr. Till date, several authors have attempted the comparison study between the above two methods in specialized populations (2, 19) and it was limited to only analytical performances. Information regarding in accuracy of SCr measured by different methods and impact of bias in clinical staging of the disease is our source of concern. The purpose of this study was to evaluate and compare the analytical performance of the SCr measured by Jaffe’s and enzymatic method in a tertiary care health institute, where in spite of large sample load, still Jaffe’s method preferred because of its economy. As analytical performance is the most fundamental criteria for comparison study, we have analyzed Bland-Altman plot to assess the agreement for eGFR calculation derived from both methods. We observed a significant difference between calculated values of eGFR by two different methods (Fig.4), which was significantly correlated to the mean eGFR. This agrees to a similar kind of study by Qiu et al., (20).Thus the magnitude of difference between two methods on a single sample was significant relative to analytical variation, which can lead to misclassification. The most frequent incidence of significant misclassification in our data could be attributed to use of Jaffe’s test (7%) in comparison to enzymatic method, especially for higher eGFR. However, the risk of misclassification may vary depending on the relative proportion of patient to be screened & probability of patient outcome. So it is not sufficient to evaluate the risk on the basis of misclassification, as it might not affect outcome. Looking at the concordance correlation between the two methods, 0.808 (0.785-0.830) in our study, it can be assumed that Jaffe’s method could be cost effective in a population where clinical risk associated renal status is low. Several data have been published giving a significant difference in magnitude relative to biological variation (21). There are still concerns for misclassifying patients of renal failure due to
method non-specificity (22). The overall risk associated with Jaffe’s method depends on probability and consequence of misclassification. Significant higher result was documented (Jaffe’s method > enzymatic method) for low SCr in Table1, which agrees to evaluation by Kume et al., (23). This suggests that the use of method based on enzymatic reaction could improve the specificity of measurement (11). We have also found the negative correlation between differences of eGFR by 2 methods and mean GFR. It was found to be statistically significant (- 0.84). So it is important to note that eGFR has an uncertainty, which varies with the level of SCr (24). This biological variation definitely will have an impact on the interpretation of CKD staging (6).

CONCLUSION

GFR calculation is widely used to diagnose & monitor CKD. It is subject to variation because of analytical error of SCr measurement and biological variation. Present comparison study was an attempt to analyse the discordance rate in eGFR based on serum creatinine measurement obtained by both Jaffe’s and enzymatic methods. Existing data observed a considerable overestimation of eGFR in compensated Jaffe’s method in individuals with low SCr than enzymatic method. In enzymatic method more subjects falling under normal (84 subjects) category were more and at the same time by Jaffe’s method 55 subjects were normal. In our study concordance correlation for SCr, and eGFR by both the methods was good but agreement between the two was poorly observed. Both Jaffe’s method and enzymatic method could meet requirement in routine use so far as analytical performance is concerned. As we know, bias due to measurement difference is the most important factor that affects misclassification, so this problem could be overcome choosing correct method of analysis. Both biological variations as well as analytical imprecision should be taken into account to avoid uncertainty of measurement of eGFR. Such exploration will help in decision making of universal adoption of a particular method to get more accurate staging and accordingly management of the disease process. Such finding will help to evaluate clinical effectiveness and associated clinical risk in CKD patients. Simultaneously outlay can be reduced if bulk will be tested and all the laboratories may have one method for estimation.

Limitations

Our study was limited only to analytical performance. Effect of interference and different clinical conditions other than CKD was not included in this study. A study on larger group must be warranted.

References


Correlation between ocular perfusion pressure and estimated translamina cribrosa pressure difference in healthy young adults

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ABSTRACT

Introduction and Aim: Glaucoma is a chronic optic neuropathy which is the leading cause of irreversible blindness across the globe. It is known that Intraocular pressure is responsible for these altered changes but recent experimental and clinical studies have reported that cerebrospinal fluid pressure (CSFP) and trans-lamina cribrosa pressure difference (TLCPD), may be an important cause of glaucomatous optic nerve damage, especially in subjects with normal-tension glaucoma. To assess the correlation between ocular perfusion pressure (OPP) and TLCPD in healthy young adults.

Materials and Methods: One hundred healthy young adult volunteers comprising of 50 obese (25 males and 25 females) and 50 non-obese (25 males and 25 females) in the age group of 18-19 years among MBBS Phase I students of JSS Medical College, JSSU, Mysore were the subjects for the study. Blood Pressure and Intra Ocular Pressure were recorded. Mean arterial pressure (MAP), OPP, CSFP and TLCPD were calculated.

Results: There exists a positive correlation (0.50) between OPP and TLCPD. There was a statistically significant difference in OPP, CSFP and TLCPD between obese and non-obese groups.

Conclusion: The present study provides information on the relationship of TLCPD with CSFP and OPP that are higher in obese individuals.

Keywords: Ocular perfusion pressure; trans-lamina cribrosa pressure difference; body mass index; glaucoma.

INTRODUCTION

The trans-lamina cribrosa pressure difference (TLCPD) is defined as the difference between cerebrospinal fluid pressure (CSFP) minus intraocular pressure (IOP). There is growing evidence that TLCPD as compared with the IOP may be a more important factor in the pathophysiology of the optic nerve head disorders including the development of glaucomatous optic neuropathy (1).

The optic nerve is said to be exposed not only to IOP in the eye, but also to Intra-Cranial Pressure (ICP), as it is surrounded by cerebrospinal fluid (CSF) in the subarachnoid space. The lamina cribrosa demarcates (2) these two pressurized zones and the pressure difference between them is called translaminar pressure difference (TPD) (TPD= IOP – ICP).

It has been assumed that both the TLCPD and the translamina cribrosa pressure gradient may be of importance in pressure-related optic nerve head diseases (3).

The CSFP is the counter-pressure against the IOP across the lamina cribrosa and is part of the equation of the TLCPD as IOP – CSFP (4).

Recent experimental and clinical studies have reported that CSFP may play an important role in the pathogenesis of glaucomatous optic neuropathy.
Moreover, several studies have demonstrated that glaucomatous optic nerve damage may develop in subjects with normal IOP due to abnormally low CSFP (5).

OPP is defined as arterial blood pressure (BP) minus IOP. Mean ocular perfusion pressure is generally calculated as two-thirds of mean arterial pressure minus IOP. Occasionally, OPP is further divided into systolic perfusion pressure (SBP minus IOP) and diastolic perfusion pressure (DBP minus IOP). Large population-based studies have determined that reduced OPP is strongly associated with increased prevalence of glaucoma (6). Low DPP has the strongest correlation with the development of glaucoma.

**MATERIALS AND METHODS**

The study was conducted at department of Physiology, JSS Medical College (JSSMC), Mysuru. The study was done after obtaining an ethical clearance from ethical committee of JSSMC. MBBS phase-I students were requested to complete a questionnaire that included inclusion and exclusion criteria and 100 subjects were recruited for the study after obtaining informed consent. The weight, height, Waist Circumference (WC) and Hip Circumference (HC) were recorded. BMI was calculated by dividing weight in kgs by the square of height in meter and Waist Hip Ratio (WHR) was computed. IOP and BP were measured and OPP was calculated for each subject. Subjects with pre-existing refractive errors, acute or chronic conjunctivitis, glaucoma, migraine, and diabetes mellitus were excluded from the study. As per WHO guidelines, those with BMI 18-22.9 were grouped as control and those with BMI >25 as study group. The study was carried out in a quiet room, by a single examiner between 3pm to 5pm to minimize the bias of examiners and diurnal variations of IOP. Subjects were briefed about the study procedure before the test was done and was assured that the procedure was painless. Subjects were asked to relax for 15 minutes in supine position. Resting BP was measured using Mercury Sphygmanometer and mean arterial pressure was calculated using the formula,

\[ \text{MAP} = \text{DBP} + 1/3 \text{ PP} \] (PP=pulse pressure).

The IOP was recorded using Schiotz indentation tonometer. The instrument was calibrated so as the scale reading was made to coincide at zero before recording the IOP. Ciprofloxacin eye drop was instilled prophylactically in both the eyes to prevent any ocular infections after the procedure. OPP was calculated using the formula,

\[ \text{OPP} = 2/3(\text{MAP}-\text{IOP}) \].

**Estimation of CSFP and TLCPD**

The gold standard for ICP evaluation is an invasive measurement of the pressure in the CSF via lumbar puncture or via implantation of a pressure sensor into a cerebral ventricle(7,8). Importantly, this invasiveness includes the potential risk for intracranial haemorrhages and infection.(9).

To overcome these invasive limitations, several approaches have been proposed. Xie and his colleague’s estimated mathematical ICP formula based on three parameters: The algorithm for determining CSFP was constructed based on three parameters, diastolic BP, BMI, and age (10).

Estimated CSFP [mmHg] = 0.44 × BMI [kg/m2] + 0.16 × diastolic blood pressure [mmHg]—0.18 × age [years]—1.91. This formula was applied to groups of subjects by comparing the estimated CSFP with the direct CSFP measurements. The formula was then tested in the independent test group, in which the measured lumbar cerebrospinal fluid pressure (12.6 ± 4.8 mm Hg) then did not differ significantly (p=0.29) from the calculated cerebrospinal fluid pressure (13.3 ± 3.2 mm Hg) and Bland–Altman analysis revealed that 40 of 42 measurements were within the 95% limits of agreement(11). Using the calculated CSFP, TLCPD was calculated as CSFP-IOP.

**Statistical analysis**

Microsoft Excel and SPSS version 19 software were used for data entry and statistical analyses respectively. Mean and standard deviation were worked out to assess the estimate of various
parameters under study. Paired t-test was applied to test the significance of difference between the groups. Pearson’s Correlation test was applied to determine the relationship between the parameters.

RESULTS

Table 1: Physiological characteristics of the study and control groups

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Study Group (obese, n = 50)</th>
<th>Control Group (non-obese, n = 50)</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (Years)</td>
<td>18.26 ± 0.44</td>
<td>18.26 ± 0.44</td>
<td>(≤)1.000</td>
</tr>
<tr>
<td>Weight (Kg)</td>
<td>73.56 ± 8.38</td>
<td>56.12 ± 7.61</td>
<td>(≤)0.001*</td>
</tr>
<tr>
<td>Height (Cm)</td>
<td>162.72 ± 7.44</td>
<td>162.34 ± 10.83</td>
<td>(≤)0.838</td>
</tr>
<tr>
<td>BMI</td>
<td>27.76 ± 2.27</td>
<td>21.28 ± 1.13</td>
<td>(≤)0.001*</td>
</tr>
</tbody>
</table>

*Statistically significant (p<0.05)

Table 2: Mean± SD BP, IOP, OPP, CSFP and TLCPD of study and control groups

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Study Group (obese, n = 50)</th>
<th>Control Group (non-obese, n = 50)</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>SBP (mm Hg)</td>
<td>124.44 ± 6.06</td>
<td>118.44 ± 4.44</td>
<td>(≤)0.001*</td>
</tr>
<tr>
<td>DBP (mm Hg)</td>
<td>78.680± 4.94 2</td>
<td>77.840 ± 2.881</td>
<td>(≤)0.302</td>
</tr>
<tr>
<td>MAP (mm Hg)</td>
<td>93.93±4.75</td>
<td>91.37±2.71</td>
<td>(≤)0.001*</td>
</tr>
<tr>
<td>IOP (mm Hg)</td>
<td>16.71±1.07</td>
<td>16.170± 0.969</td>
<td>(≤)0.010*</td>
</tr>
<tr>
<td>SOPP (mm Hg)</td>
<td>105.73±5.20</td>
<td>100.38±4.26</td>
<td>(≤)0.05*</td>
</tr>
<tr>
<td>DOPP (mm Hg)</td>
<td>59.97±4.10</td>
<td>61.26±3.37</td>
<td>(≤)0.61</td>
</tr>
<tr>
<td>MOPP (mm Hg)</td>
<td>51.48±3.227</td>
<td>50.14 ±1.574</td>
<td>(≤)0.010*</td>
</tr>
<tr>
<td>CSFP (mm Hg)</td>
<td>18.97±1.22</td>
<td>16.56±0.90</td>
<td>(≤)0.0001*</td>
</tr>
<tr>
<td>TLCPD (mm Hg)</td>
<td>3.26±1.52</td>
<td>0.45±1.10</td>
<td>(≤)0.0001*</td>
</tr>
</tbody>
</table>

*Statistically significant (p<0.05)
DISCUSSION

The aim of this work was to study the relationship between OPP and TLCPD in healthy young adults. This study involved 100 young adults aged between 18-19 years. Study group allocation was done based on WHO guidelines, depending on their BMI as obese and non-obese.

There were no significant differences between the two groups in terms of age, height and DBP. The mean SBP, CSFP and TLCPD was significantly higher in the obese and there exists a positive correlation between TLCPD and OPP ($r = 0.501$). Humans evolved with gravity, and gravity affects human physiology—CSF pools in the caudal spinal canal and CSF pressure at eye level is much lower than CSF pressure in the caudal spinal column in the upright position (12, 13). However in microgravity environment, CSF is distributed throughout the sub-arachnoid space tending to equalize pressure in all compartments and negate any posture-induced flow, resulting in higher than normal CSF pressure at eye level (14). The standard body position for ICP measurement is said to be lateral decubitus/supine (15).
Given that humans sleep in the supine or prone position but are upright during the day, it is important to note that IOP and ICP are dynamic parameters and vary according to changes in body position or individual activities (16). Therefore we assessed these parameters in the standard ICP measuring state—a supine position.

However, all these variations are insignificant to young healthy adults as they have intact homeostasis and ability of a vascular bed to maintain its blood flow despite changes in perfusion pressure. The association between higher cerebrospinal fluid pressure and younger age, higher body mass index, and higher blood pressure had also been reported in other studies (17, 18).

Compared with previous population-based studies that used the same estimation formula, the weighted mean TLCPD value in the normal population (2.31 ± 0.06 mmHg), as well as the weighted mean CSFP value, was also very similar. Moreover, the ranges from minimum to maximum value for the CSFP and TLCPD were much greater (-1.80–23.91 mmHg and 14.61–19.11 mmHg, respectively) than that of IOP (6.00–21.00 mmHg) (10).

Fleischman et al. analysed CSFP in five different age groups and found that BMI was positively correlated with CSFP in every age group (19). Positive BMI and CSF pressure associations were found by various prospective and retrospective studies (20).

In our study we analysed young healthy adults and found that mean CSFP was about 2.01 mmHg. Several studies that have examined CSF pressure and age failed to find a relationship of significance (21, 22), while Fleischman et al., in their retrospective analysis of 33,922 patients who had lumbar puncture revealed that CSF pressure decreases with older age. This study found that CSF pressure was stable for the first 50 years of life (2.8 mmHg) after which there was a steady decline by 2.5% at age 50-54 and by 26.9% at age 90-95.

In the Meiktila Eye Study from Myanmar, body height and weight were significantly correlated with age, gender, corneal curvature, axial length, anterior chamber depth, and vitreous chamber length (23).

In this study, it was found that there was a significant difference in baseline MAP and OPP between two genders and these values were higher in males to that of females. A study comprising 72 women and 68 men, showed higher values for ocular blood flow in men compared to women. While estrogen had positive effects on ocular blood flow, the opposite was the case with testosterone (24). These findings were statistically significant only in the younger age group (< 40Yrs). A study investigating choroidal blood flow in men and women also found significant differences. While age had no effect on choroidal blood flow in men, choroidal blood flow was significantly higher in women younger than 40 years compared to women older than 55 years. Although little data is available, oestrogen, progesterone and testosterone are most likely important regulators of blood flow in the retina and choroid, because they are key regulators of vascular tone in other organs. Oestrogen seems to play a protective role since it decreases vascular resistance in large ocular vessels. Some studies indicate that hormone therapy is beneficial for ocular vascular disease in post-menopausal women.

Lee in his landmark study on 6828 healthy Korean population concluded that mean IOP was significantly higher in males when compared with females (25). They also concluded IOP was increased significantly with increasing SBP, DBP and BMI.

**CONCLUSION**

The present study provides information on the relationship of TLCPD with CSFP and OPP, which are higher in obese individuals. Further prospective studies have to be carried out to investigate these parameters in glaucoma patients to understand if fluctuations in CSFP, OPP and TLCPD can contribute to the disease
process. Estimated CSFP and TLCPD may play a significant role in pathogenesis of glaucoma, which may be deemed a misbalance between IOP, CSFP and blood pressure.

REFERENCES


The effect of vitamin C on Endosulfan-induced oxidative stress parameters in prepubertal rats

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ABSTRACT

Introduction and Aim: Oxidative stress plays a very important role in endosulfan-induced toxic effects on reproductive organs. Vitamin C is a potent antioxidant which plays an important role in decreasing oxidative stress. The present study was aimed to investigate the protective role of vitamin C against endosulfan-induced testicular toxicity in Wistar rats. To investigate a protective effect of vitamin C against endosulfan induced toxicity on biochemical changes.

Materials and Methods: Seventy male neonatal Wistar rats were divided into seven groups. The group I was taken as the control group, the endosulfan-treated were grouped into II (3 mg/kg body weight (BW) and group III (6 mg/kg BW), Group IV (9 mg/kg BW) and Group V (12 mg/kg BW). Group VI (9 mg/kg BW) and group VII (12 mg/kg BW) were pretreated with vitamin C (20 mg/kg BW) for 60 days. After the experimental procedures, the testicular weight, lactate dehydrogenase (LDH) enzyme and testosterone in plasma, LDH, steroidogenic enzymes 3β-HSD and 17β-HSD in testis were evaluated. One-way ANOVA was used to determine the statistical significance.

Results: Significant improvement in the testicular weight (P<0.05) , LDH (P<0.05) levels both in plasma and testis, increase in testosterone(P<0.001) and steroidogenic enzyme levels(P<0.001)  was observed in the group pretreated with vitamin C treated group when compared to the endosulfan treated group.

Conclusion: Vitamin C decreases the toxic effect of endosulfan on testis. The present action might be due to its antioxidative properties.

Keywords: Endosulfan, testis; vitamin C; lactate dehydrogenase; testosterone; steroidogenic enzymes.

INTRODUCTION

Endosulfan is an organochlorine pesticide used in the agriculture with its existing toxic effects. Based on the World Health Organization endosulfan has been classified in the category of terminal products and hazardous (1). Reproductive toxicity of endosulfan has been shown to induce degenerative changes in seminiferous epithelium and testicular atrophy. Estrogentic action affects the differentiation and function reducing the reproductive organ weight (2, 3). Furthermore, it has been shown to affect the sperm count and specific testicular marker enzymes and hormones (4, 5). Several studies document the effect of endosulfan on the testis (6-8). Antimutagens are the chemicals preventing genetic damage caused by toxic ants interfering with free radical generation and the formation of toxic metabolites (9, 10). Among them, Vitamin C (L-ascorbic acid) is one of these bio-antimutagens (10-12) with potential health benefits. Both high and low doses of vitamin C do not cause any cytotoxic and genotoxic (13-
The present study aimed to know the protective role of vitamin C against endosulfan-induced testicular toxicity in neonatal rats.

MATERIALS AND METHODS

Animals

Healthy one-week neonatal albino rats (8-12 grams) of Wistar strain were selected for this experiment. The animals were procured and maintained in the Central animal facility, Kasturba Medical College, Manipal Academy of Higher Education. These animals were maintained under controlled conditions of temperature (temperature 28 ± 1°C temperature and 50±5% humidity) and light in animal house having free access to water and standard pallet diet. All the experiments were performed in accordance with the approval and guidelines of Animal Ethics Committee of Kasturba Medical College Manipal, Manipal Academy of Higher Education.

Experimental design

The animals were grouped into seven groups (n=10 in each group). Group I served as control (treated with groundnut oil), Endosulfan was purchased from Meerut Agro Chemicals Industries Ltd., Meerut, India. Groups II-V received 3, 6, 9 and 12 mg /kg doses of endosulfan and Group VI and VII received endosulfan at 9 and 12 mg /kg dose, respectively along with 20 mg/kg dose of Vitamin C procured from Loba Chemicals (P) Ltd., Mumbai, India. All the agents were administered orally for 60 days.

Dose selection and treatment

The dose range was designated based on the reference value close to the predictable, normal to occupational exposure to the human population. The dose calculation was based on the formula that suggestively converses chemical exposure to several species by considering body surface area and metabolism (17). During the treatment period, the body weight of each rat was recorded daily. At the end of treatment, the rats were sacrificed under ether anesthesia, wet weights of testis were noted

Estimation of Lactate Dehydrogenase (LDH) and Testosterone Level in plasma and testicular LDH levels

Blood was collected by cardiac puncture. Plasma was separated by centrifugation method and used for LDH estimation. The right testis was minced, homogenized and centrifuged. The supernatant was collected and used for LDH estimation by spectrophotometric method using the method of Decker and Matthes (18). The testosterone level was estimated using EIAgen kit as described by the manufacturers.

Estimation of steroidogenic enzymes in testis

The testis was weighed and homogenized in PBS (pH 7.2) to make 20% tissue homogenate and filtered through cheesecloth. The homogenate centrifuged, and the pellet was collected, washed once with 0.01 M phosphate buffer containing 0.15 M KCL (pH 7.4) and centrifuged with 0.15M KCL. The supernatant was obtained for the enzyme assay. 3β-hydroxysteroid dehydrogenase estimated by using the method of Miyauchi et al., (19) and 17β-hydroxysteroid dehydrogenase activity was determined by the method of Jaraback et al., (20).

Statistical analysis

Quantitative data were compared among the low and high exposure groups against the control group by the use of Levene's test of homogeneity of variances. If Levene's test indicates lack of homogeneity (p<0.001) Welch ANOVA used otherwise ANOVA was applied to selected measures from this study. When a significant (p<0.05) the main effect occurred, Tukey HSD pairwise comparison test used. Quantitative variables described using Mean ± S.E.M. The data were tabulated and analyzed using SPSS version 15.0 for Windows. p<0.05 was considered significant.
Manokaran et al: The Effect of Vitamin ………Prepubertal Rats

RESULTS

Effect on body weight and testis weight

Significant decrease (p<0.05) in the body weight was observed in the endosulfan exposure group (group VI and Group VII) when compared to control group. Endosulfan treated groups induced a significant decrease (p<0.001) in testis weight in a dose-dependent manner. The administration of vitamin C significantly (p<0.001) increased the testicular weight (Table1).

Table1: Body weight and relative wet testis weight of Wistar rats exposed to various treatments

<table>
<thead>
<tr>
<th>Groups</th>
<th>Body weight (g)</th>
<th>Relative wet left testis weight (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group I- Control (Groundnut Oil)</td>
<td>238±2.90</td>
<td>0.69±0.01</td>
</tr>
<tr>
<td>Group II, III, IV &amp; V Endosulfan</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3 mg/kg</td>
<td>180±3.33*</td>
<td>0.54±0.018*</td>
</tr>
<tr>
<td>6 mg/kg</td>
<td>159±3.14*</td>
<td>0.51±0.008*</td>
</tr>
<tr>
<td>9 mg/kg</td>
<td>148±3.88*</td>
<td>0.46±0.016*</td>
</tr>
<tr>
<td>12 mg/kg</td>
<td>127±3.01*</td>
<td>0.44±0.021*</td>
</tr>
<tr>
<td>Group VI &amp; VII Endosulfan +Vitamin C</td>
<td></td>
<td></td>
</tr>
<tr>
<td>9 mg/kg + 20mg/kg</td>
<td>181±3.66</td>
<td>0.57±0.018</td>
</tr>
<tr>
<td>12 mg/kg + 20mg/kg</td>
<td>166±3.39</td>
<td>0.54±0.015</td>
</tr>
</tbody>
</table>

*p<0.05, significant compared to control; *#p<0.05, significant compared to 9 mg/kg Endosulfan alone, *@p<0.05 significant compared to 12 mg/kg Endosulfan alone. All values are expressed as mean+S.E. from 10 animals.

Effect on lactate dehydrogenase levels

The levels of lactate dehydrogenase (LDH) was significantly increased (p<0.001) in both plasma and testicular tissue. Pretreatment with Vitamin C significantly reduced the LDH level LDH plasma (F<sub>6,63</sub>= 46.900, p<0.001), LDH testis (F<sub>6,63</sub>= 34.913, p<0.001) Table/Fig-2.

Estimation of plasma testosterone level

Endosulfan treated groups showed a significant decrease of a plasma testosterone concentration in a dose-dependent manner compared to control (p<0.05). Administration of vitamin C significantly elevated the plasma testosterone concentration (Table/Fig-2).

Effect on Steroidogenic Enzyme Levels:

Endosulfan at all doses induced a significant (p<0.05) decrease in 3β-HSD and 17β-HSD level compared to control. Administration of vitamin C along with endosulfan produced a significant increase (p<0.001) in the testicular steroidogenic enzyme levels compared to endosulfan treated rats (Table2).
Table 2: Biochemical changes in the plasma and testicular tissue of Wistar rats exposed to various treatments

<table>
<thead>
<tr>
<th>Groups</th>
<th>LDH Level (U/L)</th>
<th>Plasma Testosterone level (ng/L)</th>
<th>3β-HSD (U/g tissue)</th>
<th>17β-HSD (U/g tissue)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Plasma</td>
<td>Testes</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Group I- Control (Groundnut Oil)</td>
<td>27.70±1.39</td>
<td>19.32±2.31</td>
<td>4.43±0.05</td>
<td>168.33±1.88</td>
</tr>
<tr>
<td>Group II, III, IV &amp; V Endosulfan</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3 mg/kg</td>
<td>36.82±2.16</td>
<td>27.94±2.64</td>
<td>3.79±0.05</td>
<td>158.33±1.14</td>
</tr>
<tr>
<td>6 mg/kg</td>
<td>41.95±0.92</td>
<td>40.18±0.87</td>
<td>2.16±0.13</td>
<td>144.83±1.93</td>
</tr>
<tr>
<td>9 mg/kg</td>
<td>47.82±0.90</td>
<td>45.32±2.85</td>
<td>2.05±0.12</td>
<td>127.33±3.29</td>
</tr>
<tr>
<td>12 mg/kg</td>
<td>59.87±1.67</td>
<td>55.93±1.11</td>
<td>1.71±0.06</td>
<td>103.51±4.71</td>
</tr>
<tr>
<td>Group VI &amp; VII Endosulfan +Vitamin C</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>9 mg/kg + 12 mg/kg</td>
<td>33.39±1.20</td>
<td>31.59±1.84</td>
<td>3.96±0.05</td>
<td>154.33±4.49</td>
</tr>
<tr>
<td>12 mg/kg + 20mg/kg</td>
<td>37.62±2.01</td>
<td>35.88±1.70</td>
<td>3.01±0.08</td>
<td>135.33±3.14</td>
</tr>
</tbody>
</table>

LDH=Lactate dehydrogenase *p<0.05, significant compared to control; *#p<0.05, significant compared to 9 mg/kg Endosulfan alone, *@p<0.05 significant compared to 12 mg/kg Endosulfan alone. All values are expressed as mean+S.E. from 10 animals.

DISCUSSION

Body weight is important non-specific indicator systematically replicating the toxicity of substances (21) and can be used to assess the effect of toxicity on the growth status of rats. In the present study the body weight and testicular weight was significantly decreased in dose dependent manner in Endosulfan treated group. Vitamin C pretreatment with endosulfan decreased its toxic effect on body weight and testicular weight.

The estimation of LDH levels in plasma provides a quantitative basis for the loss of cell viability. In our study, endosulfan increased the plasma LDH level, and pretreatment of vitamin C has decreased the LDH levels (22). Our study showed dose-dependent increase in LDH level in plasma which could be due to the cytotoxic effect of the endosulfan. LDH converts pyruvate to lactate and continues the glycolysis in anaerobic conditions. Elevated plasma levels of LDH have been markedly correlated with chronic oxidative stress, cardiotoxicity, testis carcinoma, and many inflammatory conditions. The increase in LDH activity level has a direct effect on testicular functions such as spermatid count, sperm production and morphology (56) and free radical formation (23). Free radical production is known to alter testicular function (23). A marked decrease in the LDH level in the presence of Vitamin C shows its protective effect in endosulfan toxicity.

A variety of pesticides are known to cause atrophy of testis, inhibition of spermatogenesis, histological changes, and a decrease in hormone and steroid level (24).

In the present study, the effect of endosulfan on the gonado-toxicity was studied in prepubertal...
rats by observing the changes in the testosterone and steroidogenic enzymes level. The results show that treatment with endosulfan (3, 6, 9 and 12mg/kg) significantly decreased the levels of 3β - and 17β- hydroxysteroid dehydrogenase in the testis and the plasma testosterone concentration. These observations demonstrate that endosulfan, like other chlorinated pesticides, has inhibitory effects on testosterone biosynthesis (25). The endosulfan at 7.5 and 10 mg/kg dose reported significantly decreased levels of two major steroidogenic enzymes in the testis (4). This supports the above observations on the considerable depletion in the levels of testosterone. Thus the toxic effects of endosulfan on spermatogenesis were probably mediated by decreasing the enzymes of testosterone biosynthesis and hence the circulating testosterone decreased. The pretreatment of vitamin C with endosulfan high doses was an intoxicating antioxidant, decreasing the toxicity of endosulfan concerning to body weight, testis weight, LDH levels.

CONCLUSION

Vitamin C can inhibit oxidative damage depends upon its ability to scavenge the free radicals, its local concentration and its ability to interfere with the initiation and progression of lipid peroxidation. vitamin C with endosulfan can significantly decrease the damage induced by endosulfan to the testicular tissue.

ACKNOWLEDGMENT

Authors thankfully acknowledge Prof. P. Uma Devi, former Head, Department of Radiobiology, Kasturba Medical College, Manipal Academy of Higher Education, Manipal for providing the facility to perform the experiments.

REFERENCES


Design, analysis, fabrication and testing of PC porous scaffolds using rapid prototyping in clinical applications

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ABSTRACT

Introduction and Aim: Rapid prototyping is an advanced fabricating method, where three dimensional objects are built precisely from their three-dimensional computer aided design models in a very short duration. In contrast to traditional machining methods, most of the rapid prototyping techniques tend to fabricate parts based on additive manufacturing process. Fabrication of biomaterial into 3-D scaffold structures is the next vital step in the development of bone implants depending on bone injuries of individual patients, and it is highly demanding among the Indian orthopedic surgeons for treating those bone related defects. Therefore, the need for reliable and economically feasible design, better biomaterials, and efficient fabrication method for scaffold to treat musculoskeletal defects has increased in recent years.

Materials and Methods: Investigation of scaffold for porous structured bone implant is a recently emerging field in medicine and is involved in developing artificial bones like structure using materials like Tri Calcium Phosphate (TCP), Polyether ether ketone (PEEK), Hydroxyapatite (HA), Polycaprolactone, polycarbonate (PC), poly (l-lactide) PLLA or Polyamide (PA) etc., by incorporating pores in the scaffold. In this research, the samples of the scaffold specimens were designed and fabricated using Stereo lithography technique with biocompatible PC resin and the strength of each sample were analyzed.

Results: The porous scaffold models are structured with different designs utilizing the CAD software. The porous scaffold with various porosity and pore shape is analyzed through Finite Element Analysis (FEA). StereolithographyViperSi2 method was utilized to manufacture the polycarbonate scaffold. The manufactured rhombus pore model shows the stress value esteems around 200 MPa, which is nearest to the compressive strength of human bone. Subsequently the rhombus pore model gives better mechanical load bearing capacity when implanted for tissue recovery in bones.

Conclusion: Bisphenol-A Polycarbonate material give better surface completion, 100% pore interconnectivity and new tissue arrangement of the fabricated porous scaffold. The SLA technique offers the more noteworthy load bearing quality and great exactness of the fabricated scaffold.

Keywords: Rapid prototyping; porous scaffold; bio-compatible; pore size; porosity.

INTRODUCTION

Present additive manufacturing enterprises ceaselessly endeavor to improve the advancement cycles with high caliber and cost-effective items to keep up market aggressiveness. Along these lines, the requirement for Rapid Prototyping Techniques (RPT) has begun to assume significant job in fast item improvement cycle for complex item. Dimensional exactness and surface completion are the strengths
of Rapid Prototyping (RP), particularly in the event that they are utilized for shape improvement (1-3). This research work deals with designing, fabrication and testing the load bearing ability of scaffold specimens with different pore geometry and porosity.

MATERIALS AND METHODS
Polycarbonate fills a vital specialty as a standout amongst the most prominent liquid material in the clinical applications (4, 5). Bisphenol-A polycarbonate has been monetarily accessible since the 1970s, and its utilization in medicinal gadgets dates from around that time. Having a wide scope of physical properties polycarbonate offers good load bearing capacity (6-9), unbending nature, and durability that counteracts conceivably hazardous material disappointments (10-12). The biocompatibility is fundamental for any material utilized in immediate or roundabout contact with patients, polycarbonate (PC) grades are accessible that agree to biocompatibility testing benchmarks (13-15).

Design of porous scaffold
The design of porous scaffold model plays an important role in keeping the load bearing of the porous structure. CAD models of porous scaffold designs with different pore geometry and porosities ranges are modeling. The porosity of the structure is modified by differing the spacing between the pores. The scaffold porosity is computed as the proportion of void volume relative to the total volume within the design. The following three different pore configurations were used for this research (11, 16).

Scaffold requirements
The scaffold material is one of the main design factors to be considered in TE of scaffold. The material chosen for the scaffold should meet the following criteria:
- Biocompatibility
- Mechanical strength
- Cell viability
- Pore size
- Porosity

The mechanical properties of the material are especially vital because of the higher load bearing nature of the objective. All the more unequivocally, a porous scaffold material must keep up its basic surface design without surface defects during manufacturing, clinical dealing with, and obsession at the implantation. The implanted material framing new cells from hurtful mechanical forces and withstands the load bearing condition until the new cells can expect the load bearing properties (12, 13).

Types of design and their properties
The porous scaffolds are designed with various configurations using the CAD modeling software. The scaffold with different porosity and pore shape is analyzed through Finite Element Analysis (FEA). All the models were analyzed using the FEA and to identify the best model of hexagonal, cubical and Rhombus with suitable porosity. The scaffold pore size studied is in the range of 400µm to 1905µm and the porosity ranges from 50% to 60% for the purpose of quick new bone tissue formation. The following three porous scaffold models with different cell configurations were selected for fabrication.
- Hexagonal pore model
- Cubical pore model
- Rhombus pore model

Porosity is one of the important properties to be considered while designing a scaffold. In the above-mentioned scaffold models, the porosity ranges from 55% to 60%. The pore size is maintained as 400µm-1905µm for all the three models as shown in Table 1. The porosity and the strength are dependent on the pore size. Hence, appropriate pore size was assigned in order to achieve the expected strength.
Table 1: Porous scaffold details

<table>
<thead>
<tr>
<th>Scaffold Design</th>
<th>Porosity in %</th>
<th>Pore size in μm</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hexagonal pore model</td>
<td>59.08</td>
<td>1905</td>
</tr>
<tr>
<td>Cubical pore model</td>
<td>57.02</td>
<td>1500</td>
</tr>
<tr>
<td>Rhombus pore model</td>
<td>55.40</td>
<td>424</td>
</tr>
</tbody>
</table>

CAD Model of porous scaffold

The CAD models of the porous scaffolds were created using the above-mentioned parameters.

Hexagonal pore model

The scaffold was of the size 20x20x4 mm cuboids as shown in Fig. 1. The hexagonal pore size was 1900μm and these pores were interconnected to each other along the row and column. The porosity of the scaffold was identified as 59.08%. The gaps between the pores were provided in order obtain the required porosity. The hexagonal pores were of thickness 1mm. The scaffold was constructed with drifted pores in order to improve the strength of the bone tissues.

Cubical pore model

The scaffold was of the size 20x20x4 mm cuboid as shown in Fig. 2. The cubical pore size was 1500 μm and these pores were interconnected to each other along the row and column. The porosity of
the scaffold was identified as 57.02%. The gaps between the pores were provided in order to obtain the required porosity. The cubical pores were of thickness 4mm.

**Rhombus pore model** The scaffold was of the size 20x20x4 mm cuboids Fig. 3. The rhombus pore size was 424μm, these pores were interconnected to each other along the row and column. The porosity of the scaffold was determined as 55.4%. The gaps between the pores were provided in order to obtain the required porosity. The rhombus pores were of thickness 1mm. The scaffold was constructed with drifted pores in order to enhance the strength of the bone tissues.

![Fig.3: CAD model of Rhombus pore scaffold](image)

**Fabrication of polycarbonate porous scaffold**

Manufacture of the scaffold specimens involves optimization of process parameters. Fabrication of porous scaffold procedure must produce a porous structure with a reproducible design, which can work as intended for a particular timeframe in the load carrying in the injured portion. The selection of manufacturing method can impact distinctive qualities of the porous scaffold, including required design, strength, biocompatibility, and biological properties.

**Polycarbonate porous scaffold fabrication using SLA:** Stereo lithography ViperSi2 system was used to fabricate the polycarbonate porous scaffold as shown in Fig. 4.

![Fig. 4: Stereo lithography Viper Si2 System](image)

It employed a vat of liquid ultraviolet curable photopolymer resin and an ultraviolet laser to build layers of parts one at a time. The resin used was polycarbonate material. For each layer, the laser beam traced a cross-section of the part pattern on the surface of the liquid resin. Exposure
to the ultraviolet laser light cured and solidified the pattern traced on the resin and joins it to the layer below and finally the required scaffolds are obtained. The support structures were also used in order to act as a mounting device holding the part in position as it is built. To support overhanging cross section and to support unattached islands, supporting material which is same as built material is used. The fabricated scaffolds are shown Fig. 5.

![Fabricated porous scaffold](image)

**Fig. 5:** Fabricated porous scaffold

The SLA method provides the best results and has good surface finish among the other rapid prototyping technology. The only drawback is that the strength of the fabricated component is less.

**Testing of polycarbonate porous scaffold**

The compression test was carried out on the scaffold in order to find the various properties of each scaffold, to compare with the properties of the human bone and to identify which model of the scaffold functions similar to the human bone. All scaffolds were mechanically tested in order to determine their mechanical properties. Axial compression tests with mechanical failure were carried out using a universal testing machine (Z50; Zwick Roell, Ulm, Germany) with a traverse velocity of 1.0 mm/min for all scaffolds. Values of applied load and displacement were continuously recorded during testing. The five samples of scaffolds fabricated were used to determine the mechanical property of the scaffolds using the compression test and the following values were obtained as shown in Table 2.

**RESULTS**

**Table 2: Compressive stress of various scaffold designs**

<table>
<thead>
<tr>
<th>Specimen</th>
<th>Hexagonal pore model</th>
<th>Cubical pore model</th>
<th>Rhombus pore model</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>83.1</td>
<td>184.1</td>
<td>207.1</td>
</tr>
<tr>
<td>2</td>
<td>93.8</td>
<td>172.0</td>
<td>198.6</td>
</tr>
<tr>
<td>3</td>
<td>86.7</td>
<td>175.1</td>
<td>196.4</td>
</tr>
<tr>
<td>4</td>
<td>89.4</td>
<td>179.4</td>
<td>209.4</td>
</tr>
<tr>
<td>5</td>
<td>92.1</td>
<td>180.2</td>
<td>216.1</td>
</tr>
</tbody>
</table>

From the obtained values the three scaffold designs were compared. The graphical comparison of the compressive stress of the scaffolds along the percentage of deformation is shown Fig. 6.
The above graph indicates that the rhombus pore model exhibits the stress values around 200MPa, which is closest to the compressive strength of human bone. Hence the rhombus pore model provides excellent mechanical load bearing strength when implanted for tissue regeneration in bones. Thus, the rhombus pore model is more efficient when compared among the three models of the scaffolds fabricated and so it is chosen for bone regeneration.

**DISCUSSION**

Rapid prototyping helps to facilitate bone formation in an injured human body portion in a lot quicker rate than any time in recent memory. The requirement for Rapid Prototyping Techniques (RPT) has begun to assume crucial job in fast item improvement cycle for complex shaped porous scaffold models. In this research work three types’ unique models of porous scaffolds were designed, manufactured utilizing stereo lithography and tested. The research also proves that the SLA method provide the feasibility of fabricating porous structured components with complex architectures with good quality. Bisphenol-A Polycarbonate material provide better surface finish, 100% pore interconnectivity and new tissue formation of the fabricated scaffold. The SLA method offers the greater load bearing strength and good accuracy of porous scaffold. The investigation on porous structured scaffold manufacturing would be very much helpful in orthopedic applications.

**CONCLUSION**

In this research work polycarbonate was used as the resin to fabricate the scaffolds. Polycarbonate possessed clarity, high strength and impact resistance, good heat resistance, low water absorption, and biocompatibility. In future while fabrication of the scaffolds, Hydroxyl apatite (HA) has been planned to be mixed with the polycarbonate resin. Hydroxyl apatite can be found in teeth and bones within the human body. In this way, it is commonly utilized as a filler to replace the damaged bones.

**REFERENCES**

Mycosynthesis of biocompatible gold nanoparticles using *Penicillium* sp for bromothymol blue degradation

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ABSTRACT

**Introduction and Aim:** The Biosynthesis of Gold nanoparticles (Au NPs) is an eco-friendly, cost effective and nontoxic alternative to chemical and physical methods. In the present study synthesis of Au NPs was performed by using a fungi *Penicillium* sp. isolated from agriculture soil.

**Materials and Methods:** Fungi was isolated from the agricultural field and inoculated into Sabouraud Dextrose broth and incubated at 28º C in a shaker at 180 rpm for 96 to 120 hours. After incubation, the fungal culture was filtered and centrifuged, obtained fungal cell free extracts treated with 1mM gold salt (HAuCl₄). The synthesis of Au NPs was confirmed by UV–visible spectroscopy and particles size was measured using Dynamic Light Scattering (DLS). Haemolytic assay of Au NPs was carried out using Chicken RBCs and results measured at 540 nm in UV-visible spectrophotometer. To study catalytic activity, Bromothymol blue (BB) was subjected to reduction by using sodium borohydride (NaBH₄, 5.28X10⁻² M) in the presence of Au NPs. Then the color change was monitored by visual observation.

**Results:** The synthesis of Au NPs was preliminary observed by a color change from yellow to purple and confirmed by a peak at 560 nm using a UV–visible spectroscopy. The DLS analysis showed that the Au NPs were poly-dispersed and size ranges from 130 to 150 nm. The biosynthesised Au NPs was studied for their biocompatibility and dye degradation properties.

**Conclusion:** The obtained results revealed that biosynthesized Au NPs shows a minimum level of toxicity to chicken erythrocytes and good catalytic activity towards the degradation of hazardous dye bromothymol blue. These nanoparticles could be potentially useful in various applications in medical and environmental fields.

**Keywords:** Biosynthesis; *Penicillium* sp.; gold nanoparticles; catalytic activity; haemolysis.

INTRODUCTION

Nanotechnology is an immensely emerging concept in the field of science and technology. Nanotechnology defined as nanoparticle that acts as a whole unit in transport and other properties. The term nanotechnology was entitled by famous lecture, Richard Feyman of the American Institute of Technology. The word ‘nano’ means extremely dwarf or very small it derived from a Greek word it indicates 10⁻⁹ or billionth of a meter (1, 2). Physical, chemical and biological methods are traditionally used for the synthesis of metal nanoparticles. In the chemical methods, chemical reagents caused environmental hazards and physical methods main disadvantages are cost effective (3). However, the biological method is a good alternative for the synthesis of different metal nanoparticles with less cost and eco-friendly routes (4). Microorganisms such as bacteria, fungi, yeasts and algae are used to synthesize the Au NPs. Many fungi are a better resource for the synthesis of Au NPs because of their several superiorities, such as efficient synthesis process, high ion concentration.
tolerance, high yields and it can synthesize both intra and extracellularly (5). Previous studies have shown that some fungi like *Fusarium acuminatum* (6), *Penicillium citrinum* (7), *Aspergillus fumigatus* and *A. flavus* (8) and *Alternaria alternata* (9) could synthesis nanoparticles. Au, Te, Pt, Ag, Zn, Cd and many metal nanoparticles Au have many biological properties. Au NPs have more advantages in biomedical fields over other metallic nanoparticles due to their non-cytotoxicity and biocompatibility. This markedly leads to a wide range of applications in bio-imaging, bio-labelling, drug delivery, antimicrobial therapy, cancer treatment, biosensors and catalytic applications (10, 11).

Recent days have witnessed a shortage of safe and clean water in India and the world. The water reservoirs are facing several challenges due to the industrialization, urbanization, agriculture and mining activities (12, 13). The disposal of wastewater from dying industries has thus attracted more attention in order to a sustainable water system (14).

In the present work, reported the synthesis of Au NPs by supernatant of *Penicillium* sp. as reducing and stabilizing agent. The active isolate was characterized based on morphological features and microscopic observations. Synthesis of nanoparticles was confirmed by UV-spectrophotometer and size distribution analysis by DLS. Further mychosynthesised Au NPs studied for their haemolytic and catalytic properties.

**MATERIALS AND METHODS**

**Fungal strain and their growth conditions**

Soil sample was collected from the agricultural field near Chikka Aluvar, Kodagu, Karnataka and it was serially diluted and known dilution was plated using Sabouraud Dextrose agar and incubated at room temperature for 5 to 7 days. After incubation, the fungal culture was inoculated in 500 ml flask containing 200 mL of sterile SD Broth with final pH 5. These inoculated flasks were incubated at 28º C in a shaker at 180 rpm for 96 to 120 hours.

**Extracellular biosynthesis of Au NPs**

After incubation, the fungal culture was filtered and centrifuged at 8,000 rpm for 15 min at 4º C in a cooling centrifuge. Immediately, obtained fungal cells free extracts were used for Au NPs synthesis. For Au NPs synthesis 2 mL of fresh supernatant (obtained by centrifugation) was added to 3 mL of 1mM gold salt (HAuCl₄) in a test tube and two controls were used, one as 1mM HAuCl₄ which lacked the fungal cells extract and other control only fungal cells extract. All the experimental samples were incubated at 37 ⁰C for 48 hours. Synthesized Au NPs were separated by centrifugation at 14,000 rpm for 15 min at 4 ºC. After centrifugation metal nanoparticle were collected from the bottom of the centrifuge tube. Collected nanoparticles were repeatedly redispersed with sterile water followed by alcohol wash to remove biological molecules. The well purified colloidal solution of Au NPs was then heat dried using an oven at 50 ⁰C for overnight. The dried sample used for further analysis.

**Characterization of Au NPs**

Bio-reduction of Au NPs primarily monitored by visual observation and qualitative analyses was done by using UV-Vis spectrophotometer ranging between 300 to 800 nm at a resolution of 1 nm. The aqueous colloidal suspension was added into a clean quartz cuvette and spectral measurements were taken immediately. The surface Plasmon resonance (SPR) peaks were assessed for confirmation of Au NPs synthesis. Dynamic Light Scattering (DLS) was used to determine the particles size distribution profile. This is the most common technique used to determine the particles size and dispersity in colloidal suspensions (15).

**Hemolysis assay**

Hemolysis study was performed according to the previously reported protocol (16). The chicken blood sample was used to carry out the
hemolytic assay of Au NPs. Chicken RBCs were separated by centrifuging at 1500 rpm for 10 min and removed supernatant. The obtained pellet was further washed for several times with sodium saline (0.85%). The pellet was made up to 20 ml volume with saline and 1 ml of the RBCs were mixed with biogenic Au NPs. 1 ml of distilled water with the same volume washed RBC with saline used as negative control. All the test and control samples were incubated for 4 h at 37 °C. After incubation of test samples, the mixtures were again centrifuged at 14,000 rpm for 10 min to remove the nanoparticles. The supernatants were measured at 540 nm in UV-visible spectrophotometer.

Catalytic study of Au NPs

Catalytic activity was done by using previously reported protocol (17). Bromothymol blue (BB) was subjected to reduction by using sodium borohydride (NaBH₄, 5.28X10⁻² M) in the presence of Au NPs. 5 ml of BB (10⁻⁵ M) was mixed with 1.5 ml of freshly prepared 10⁻² M NaBH₄ and required quantities of biosynthesized Au NPs was mixed. Then the color changes were monitored by visual observation. Control was incubated at the same experimental conditions without adding nanoparticles.

**RESULTS AND DISCUSSION**

**Table1: In vitro Haemolytic activity of gold nanoparticles and percentages of haemolysis.**

<table>
<thead>
<tr>
<th>Sample</th>
<th>Content</th>
<th>UV-visible reading at 540 nm</th>
<th>% of Haemolysis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Negative control</td>
<td>Saline + RBC</td>
<td>0.012</td>
<td>0.6%</td>
</tr>
<tr>
<td>Positive control</td>
<td>Distilledwater + RBC</td>
<td>2.107</td>
<td>100%</td>
</tr>
<tr>
<td>Test sample</td>
<td>Au NPs + RBC</td>
<td>0.071</td>
<td>3.3%</td>
</tr>
</tbody>
</table>

**Fig.1: Visual observation of Penicillium sp. filtrate exposed to 1 mM gold salt concentration. A. Control without fungal supernatant, B. With 1 mM gold salt concentration, C. Culture supernatant without gold salt.**

**Visual observations**

The aqueous 1mM HAuCl₄ was added to the cell free cultures of *Penicillium* sp. and the color of the reaction mixture changed from light yellow to purple color (Fig 1) which indicated the formation of Au NPs. The color change was monitored visually which signifies the bio-reduction of gold ions and the formation of Au NPs. No color change was observed in control tubes that were incubated at the same experimental conditions without adding fungal filtrate. Similarly, in previous report aqueous HAuCl₄ was added to the leaf and twig extract of *R. tuberosa* and *P. acidus* and reaction mixture color turned from light green (plant extract) to purple color which indicated the formation of Au NPs (18).
**UV–Visible spectroscopy**

Formation of Au NPs was detected by spectral analysis under UV-Vis spectrophotometer. The SPR peak of Au NPs usually has a range of 530–580 nm in colloidal solutions depending on the size and shape of the nanoparticles. With the increase in the nanoparticles size, the SPR peak extends towards longer wavelength. In the present study, a broad SPR peak in the range of 530–560 nm was observed (Fig 2), which is indicative of the synthesis of polydispersed nanoparticles. According to the previous study, the UV-Vis spectra were recorded after the completion of the reaction, showed a distinct absorption peak at 543 nm which was similar to the present study (19).

**Dynamic Light Scattering**

DLS analysis was used to measure the size of the particles in colloidal solutions synthesized by different protocols. The particle size distribution versus intensity of the graph is shown in Fig 3. The average particle size of synthesized Au NPs by *Penicillium* sp. was found to be 130 to 150 nm. Generally, the larger particle size observed by DLS is due to the bioorganic compounds enveloping the core of the biogenic Au NPs (20).

**Properties of Au NPs**

**Hemolytic property**

Toxicity of biogenic Au NPs towards chicken red blood cells was screened using *in-vitro* hemolytic assay. In the present study, Au NPs shows a minimum level of toxicity to erythrocytes (Fig 4). The obtained results show that 3.3% of hemolysis was observed in the tested samples (Table 1). In the present study the percentage of hemolysis is very low compared to the positive control, so synthesized gold nanoparticles are biocompatible. These biogenic Au NPs could be used for biomedical applications such as drug delivery, gene delivery, cancer therapy etc. According to
Aseichev et al., 2013 Au NPs of small size (5, 10, and 20 nm) showed slightly increased hemolysis in comparison with control during their incubation (21).

Catalytic activity

A potential advantage of synthesized Au NPs as a catalyst was determined by the reduction of hazardous environmental pollutant Bromothymol Blue. The catalytic activity of Au NPs was evaluated by using NaBH₄ as a reducing agent. After adding this agent, the color of the BB changes from yellow to Blue. When a few drops of Au NPs were added to the aqueous solution of Bromothymol blue and NaBH₄ the reduction process found to be accelerated. The color of the aqueous solution turned colorless in a fraction of seconds (Fig 5). This result indicates that Au NPs synthesized from soil fungi found to be an efficient catalyst to degrade the hazardous dyes. Recent studies have reported longer time duration around 15 min for BB degradation by Iron nanoparticles (22). In the present study degradation of BB was achieved using a few drops of biogenic Au NPs in 2-3 sec. These results suggest that Au NPs also potentially useful for removal of environmental pollutants and degradation of organic pollutants. Therefore, green synthesis methods will play a significant role in the study and enhancement of the catalytic degradation of pollutant dye effluents in the environment.

CONCLUSION

A facile one step green synthesis method was developed to synthesize Au NPs successfully using fungi Penicillium sp. cell free extract without adding any external chemical agents. The fungal cell free extract act as both reducing and stabilizing agents during the synthesis of Au
NPs. The synthesis of Au NPs was confirmed by UV-visible peak obtained at 560 nm and size distribution was analysed by DLS studies. Further biogenic Au NPs showed very less and clinically acceptable level of haemolysis, this indicates Au NPs are biocompatible. Importantly, the as prepared Au NPs showed high catalytic activity for the degradation of bromothymol blue within a fraction of seconds after adding a few drops of Au NPs colloidal solutions.

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Folic acid and vitamin B_{12} ameliorate nicotine-induced testicular toxicity in rats

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ABSTRACT

Introduction and Aim: Cigarette smoking, one of the fundamental roots of preventable morbidity, has a myriad of notorious effects. Nicotine is the most bountiful and symbolic constituent of cigarette smoke. The liaison between smoking and infertility has been investigated for decades; but it’s still dubious whether the noxious effects of cigarette smoking on testis and sperm characteristics are by virtue of nicotine. Therefore, the current study interrogated the ameliorative effects of folic acid and vitamin B_{12} on nicotine induced catastrophe in testicular tissue and sperm characters in male albino rats.

Materials and Methods: Rats were treated with nicotine (3 mg/kg body weight/day, intraperitoneal) with or without folic acid (36µg/kg body weight/day, orally) and vitamin B_{12} (0.63µg/kg body weight/ day, orally) for 21 days. Sperm qualities were analyzed for motility and morphology. Various oxidative and anti-oxidative stress parameters, pro inflammatory cytokines levels, hormonal assays were performed.

Results: Findings marked that nicotine caused degenerative changes in the testicular tissue. Supplementation with folic acid and vitamin B_{12} reversed these results along with suppressing the nicotine induced changes in TNF-α, IL-6, and markers of oxidative stress. Moreover, folic acid and vitamin B_{12} in combination also significantly blunted the altered activities of testicular key androgenic enzymes, plasma levels of testosterone, LH, and FSH following nicotine exposure.

Conclusion: In closure, testimonies manifested that folic acid and vitamin B_{12} may act as plausible strategy against oxidative stress, which is a pivotal step in nicotine-induced reproductive toxicity, and bettering functional status of testicular tissue by scavenging free radicals and hindering the generation of pro-inflammatory cytokines.

Keywords: Nicotine; oxidative stress; testicular damage; folic acid; vitamin B_{12}

INTRODUCTION

Smoking and its complications are anticipated as the most critical social and health issues around the world. Cigarette smoking is very common despite of its well-known health consequences and worldwide anti-smoking campaign. Tobacco smoke contains various compounds including nicotine, irritant substances, carcinogens, carbon monoxide and other gases, which seeks medical attention (1). Nicotine is treated as the primary chemical present in tobacco which is liable for threatening tobacco use and dependence (2). Although, smoking is not always interchangeable with nicotine administration, but the toxic effect of cigarette is often being linked with nicotine content present in the cigarette (3). The use of numerous nicotine-based pharmacotherapies including nicotine
replacement therapy (NRT) has been reported to enhance the chance of a successful quit attempt for those struggling with heavy dependence. There is an increased popularity around the world in recent years about the non-combustible smoking alternatives containing nicotine (i.e., e-cigarettes) especially among adults of reproductive age. Over one third of all men globally smoke some form of tobacco and cigarette smoking is not only a crucial cause of lung cancer but also has been linked with a myriad of adverse health outcomes, including cardiovascular disease, respiratory disease, and cancer of the esophagus, pancreas, stomach, kidney, bladder and cervix (4). The association between cigarette smoking and infertility has been studied for decades and the male reproductive system is known to be highly sensitive to many toxic chemicals and drugs which lead to detrimental effects on male reproductive capacity under certain conditions. So, in order to improve reproductive outcomes, men should be advised to abstain from smoking (4).

Nicotine along with its oxidized metabolite cotinine negatively affects spermatogenesis, epididymal sperm count, sperm motility, and the fertilizing potential of sperms (5). Nicotine and cotinine are reported to cross the blood-testis barrier and be present at significant levels in seminal plasma in humans that markedly deteriorate semen quality as revealed by reduced sperm viability and altered morphology (6). Additionally, nicotine was also reported to have effect on the spermatogenesis as well. This was further explained with a previous report that showed cigarette smoking had deleterious effects on all sperm parameters, including abnormal sperm shape and motility, lower sperm count and delayed sperm maturation which suggested an early potential of infertility (7). Therefore, men with fertility issues should be advised to abstain from smoking as early as possible to improve their reproductive potential.

Currently, it is evident that the adverse toxic effects of nicotine are, may be in part, due to the increased production of reactive oxygen species (ROS). This increased ROS further damages DNA, proteins, carbohydrates, and lipids and negatively impacts enzyme activity and cellular genetic machinery (8). Indeed, it has been established that ROS inhibits steroidogenesis by interfering with cholesterol transport to mitochondria and/or catalytic function of P450 enzyme. Moreover, ROS also inhibits steroidogenesis during cholesterol transfer by suppression of the steroidogenic acute regulatory (StAR) protein expression in the MA-10 tumor Leydig cells (9). Oxidative injury is an outcome of excessive oxidative stress, inadequate antioxidant level, or an amalgamation of both. Different cellular antioxidant systems are present to protect against free radical damage. These include various enzymatic and non-enzymatic antioxidant molecules such as GSH, glutathione peroxidase and superoxide dismutase and catalase. The enzymatic scavengers of ROS may protect the cellular system from numerous toxic effects of the free radicals induced by xenobiotics in testis. The nonenzymatic antioxidant, GSH, scavenges and quenches free radicals, gets oxidized, and inactivates the process of free radical–mediated damage. When the levels of ROS outpace the cellular antioxidant capacity, cells undergo oxidative stress leading to oxidative damage. It has also been shown that chronic nicotine exposure reduces the level of cytochrome P450 IIE1, elevates free radical formation, and decreases antioxidant systems, which results in tissue oxidative damage in rats (10).

Earlier finding has reported that various nutritional interventions can better the negative impact on spermatogenesis caused by life style risk factors like smoking (11). Folates, which belong to the vitamin B group, are associated in a extensive number of biochemical processes, especially in the metabolism of homocysteine (12). Furthermore, vitamin B₁₂, a cofactor in the folate-dependent
conversion of homocysteine to methionine, is a crucial component of one-carbon metabolism. Very few have reported plasma total homocysteine concentrations despite of the fact that increased total homocysteine is a key marker for poor folate and vitamin B\textsubscript{12} function. Additionally, only a few therapeutic trials interrogating the plausible effect of folate supplementation on male fertility have been reported. Folate metabolism is engaged in numerous physiological and pathophysiological mechanisms in the field of andrology and gynecology. There is a growing body of evidence demonstrating a relationship between folate and other B vitamin deficiencies, hyper-homocysteinemia and gonadal abnormalities, such as altered spermatogenesis and impaired ovarian reserve, as well as male and female infertility (12). On the other hand, vitamin deficiency, mainly of folic acid and vitamin B\textsubscript{12} (cobalamin), is considered a major contributor to the hyper-homocysteinemia found in smokers.

Currently, there is no established report on the mechanism behind the beneficial effects of folic acid and vitamin B\textsubscript{12} on male reproductive system. Therefore, the present study was aimed to elucidate the detrimental effects of nicotine and the potential use of folic acid alone or in combination with vitamin B\textsubscript{12} on the testicular parameters and sperm qualities of rats.

**MATERIALS AND METHODS**

**Animal model**

All animal experiments were performed in the Department of Physiology, Serampore College, West Bengal, India according to the ethical guidelines suggested by the Institutional Animal Ethics Committee (IAEC) of Serampore College. Male albino rats of Wistar strains weighing between 110-125 g were used in all the experiments. Animals were adapted in an environmentally controlled animal house (temperature 24 ± 3°C) and in a 12 h light/dark schedule for 7 days with free access to food and water ad libitum before the starting of experiment. Proper hygiene was maintained by constant cleaning and expulsion of feces and spilled feed from the cages daily.

**Experimental design**

For the experiments, rats were randomly categorized into four groups comprising of five rats in each: Group A, control; Group B, nicotine-treated; Group C, nicotine + folic acid; Group D, nicotine + folic acid + vitamin B\textsubscript{12} supplemented. As we did not observe any significant effect on only vitamin B\textsubscript{12} supplementation to nicotine treated animals in the previous study (unpublished data), therefore, nicotine + vitamin B\textsubscript{12} supplemented group was not included in this current set up. Animals of all the groups were fed with a standard diet containing 71% carbohydrate, 18% protein, 7% fat and 4% salt mixture. The dose, duration and the route of administration of nicotine used were according to earlier reports (13). Animals in Group B, C and D were administered with nicotine tartrate (dissolved in 0.9% physiological saline) at a dose of 3.0 mg/kg body weight through intra-peritonial injection daily for 21 days at 16:00 h every day to avert diurnal variation. The dilution was executed in such a manner that 1 ml of physiological saline contained the recommended dose of nicotine. The animals in the control subgroup were injected with 1 ml physiological saline intra-peritonially. In addition to nicotine treatment, animals of Group C received folic acid (36 µg/kg body weight/day for 21 days) only through oral gavage and those of Group D were orally treated with folic acid and vitamin B\textsubscript{12} (0.63 µg/kg body weight/day for 21 days) respectively (13). Furthermore, to conquer the impact of any modified food consumption, animals of Group A were pair-fed with experimental groups B, C and D.

**Serum and plasma preparation**
After the treatment period was over, rats from all the groups were anaesthetized using pentobarbitone sodium (60 mg/kg body weight intraperitonially) and sacrificed by cervical dislocation. Blood samples were drawn from heart and serum was separated and used for hormone and cytokine assay.

**Preparation of testicular tissue extract**

After blood collection testis was quickly removed. For the estimation of NO, MDA, and SOD, testicular tissue extract was prepared in ice-cold Tris-HCl buffer (pH 7.4). For CAT and GSH estimations, the tissues were homogenized in ice-cold isotonic phosphate buffer (pH 7.0 and pH 8.0, respectively).

**Determination of cytokine level**

Serum level of TNF-α and IL-6 were measured by using the ELISA kit obtained from Ray Biotech (USA). All samples were assayed in duplicate. The intra-assay variations were 6.7% for TNF-α and 5.5% in case of IL-6.

**Estimation of nitric oxide production (NO) and lipid peroxidation**

The role of nitric oxide synthase (NOS) was indirectly analyzed by measuring the amount of NO produced. Nitric oxide decomposes briskly in aerated solutions to form stable nitrite/nitrate products. In this study, nitrite accumulation was estimated by Griess reaction (14) and was used as an indicator of NO production. The amount of nitrite in the sample (micromolar unit) was calculated from a sodium nitrite standard curve. The role of lipid peroxidase was determined by estimating the level of malondialdehyde (MDA) formed which acts as an index of lipid peroxidation. Quantitative analysis of lipid peroxidation was executed following the thiobarbituric acid (TBA) test (15). The extent of MDA produced was quantitated with TBA and used as an index of lipid peroxidation. The results were declared as nanomoles of MDA per milligram of protein using molar extinction coefficient (1.56X10^5 cm²/mmol).

**Estimation of superoxide dismutase activity**

The nitro blue tetrazolium (NBT) method of Beauchamp and Fridovich (16), which is established on the inhibition of NBT reduction by SOD, was used for the determination of SOD activities. The relative absorbance was then converted into unit of SOD activity per mL or per mg protein, where one unit of SOD activity was equivalent to the quantity of SOD that caused a 50% reduction in the background rate of NBT reduction.

**Estimation of catalase activity**

Catalase activity was determined according to the method described by Beers et al. (17), by following the decomposition of H₂O₂ at 240 nm and 25°C. Alteration in the rate of absorbance were transformed into unit of catalase/mg protein using a conversion factor (3.45), which correlates to the decomposition of 3.45 micromoles of hydrogen peroxide in a reaction mixture producing a reduced absorbance from 0.45 to 0.40 unit.

**Estimation of reduced glutathione content**

GSH content was measured using 5, 5'-dithiobis-2-nitrobenzoic acid (DTNB). The absorbance of reduced chromogen was followed spectrophotometrically at 412 nm. GSH level was then determined using a standard curve and expressed as mM/mg protein (18).

**Protein determination in crude extract**

The total protein content was measured by the Lowry method using BSA as standard (19).

**Hormonal assay**

Follicle stimulating hormone (FSH) and luteinizing hormone (LH) were measured using ELISA kits from MONOBIND, USA in the plasma samples so collected. Whereas plasma testosterone level was analyzed using ELISA kit from DRG Inc, Germany.
Assay of testicular 3β HSD and 17β HSD activities

To study testicular 3β HSD and 17β HSD activities, testicular tissue was homogenized separately, maintaining chilling conditions (4°C) in 20% spectroscopic-grade glycerol containing 5 mM of potassium phosphate and 1 mM of EDTA at a tissue concentration of 10 mg/ml homogenizing mixture in a homogenizer (Remi RQ-127A, Mumbai, India). This mixture was centrifuged at 10,000 g for 30 min at 4°C in a cold centrifuge (REMI-C24BL, India). The supernatant was mixed with 1 ml of 100-µM sodium pyrophosphate buffer (pH 8.9) and 40 µl of 30 µg of dehydroepiandrosterone (DHEA), making the incubation mixture a total of 3 ml. 3β HSD activity was measured after the addition of 0.5 µM of NAD to the tissue supernatant mixture in a spectrophotometer (Shimadzu 1800, Japan) at 340 nm against a blank (without NADP). For testicular 17β HSD activity measurements, the same supernatant fluid (1 ml) of homogenizing mixture was added with 440 µM of sodium pyrophosphate buffer (pH –10.2), bovine serum albumin (25 mg of crystalline BSA), and 0.3 µM of testosterone, making the incubation mixture a total of 3 ml. The enzyme activity (17β HSD) was measured after the addition of 1.1 µM nicotinamide adenine dinucleotide phosphate (NADP) to the tissue supernatant mixture in a spectrophotometer (Shimadzu 1800, Japan) at 340 nm against a blank (without NADP). One unit of enzyme activity is equivalent to a change in absorbency of 0.001/min at 340 nm.

Sperm function analysis

After exposing the reproductive tract, the caudal epididymis was attentively isolated and was minced with scissors in 1ml of physiological saline to release the sperm. Each chamber of the haemocytometer was loaded with 10µl of diluted sperm and was allowed to stand or settle for 5 minutes. Counting was then performed under light microscope at 400X magnification. Sperm morphology was analyzed using the eosin and nigrosin stain. Briefly, 10µl of eosin and nigrosin were mixed with 40µl of sperm suspension. The sperm suspension was then incubated at 40°C for 5 min and then was re-suspended with a micropipette. About 100 sperm cells per rats were morphologically inspected under the microscope at 400X magnification. Morphological anomalies were categorized as headless sperm, banana head, bent neck and bent tail. Sperm motility was tested by placing 10µl of sperm suspension on slide for microscopic investigation at a magnification of 400X. About 100 sperm cells were evaluated and classified as either motile or immotile and was declared as percentage.

Histopathological staining and analysis

The right testis and epididymis tissues were selectively taken from all groups of animals and were Bouin’s fixed. Paraffin blocks were then prepared, and 4 to 5 µm-thin sections were cut with a rotary microtome. Tissues were then be stained with haematoxylin and Eosin (H&E) and light microscopy (Carl Zeiss, Germany) was used for the histopathological evaluations. Testis sections from each study group were assessed for structural modifications. Johnsen’s tubular biopsy score (JTBS) was used for the semiquantitative analysis of spermatogenesis in 20 seminiferous tubules from each testicular section (20). From each group, testicular tubule sections were categorized by degrees, ranging from 1 to 10. In this classification, 10, signifies complete spermatogenesis and regular structure; 9 indicates many spermatozoa present and disorganized tubules; 8 expresses only a few spermatozoa present; 7 represents no spermatozoa but many spermatids present; 6 refers to no spermatozoa, only a few spermatids present; 5 marks no spermatozoa or spermatids but many spermatocytes present; 4 suggests few spermatocytes present; 3 means only spermatogonia present; 2 points out no germ
cells, only sertoli cells present; and 1 denotes complete absence of germ cells and spermatogenesis. JTBS was estimated by dividing the sum of all scores by the total number of seminiferous tubules examined.

Statistics
Data were expressed as mean ± SE. Kruskal–Wallis nonparametric ANOVA test was performed to find whether or not scores of different groups differ significantly. To test intergroup significant difference, the Mann–Whitney U multiple comparison test was performed. Differences were considered significant if p<0.05.

RESULTS
Results of the present study documented the protective effect of combined supplementation of folic acid and vitamin B₁₂ against nicotine mediated male reproductive functions in rat. Relative testicular weight was found to be decreased in nicotine treated rats compared to control (P<0.05). However, supplementation of folic acid in combination with vitamin B₁₂ successfully blunted the nicotine mediated alteration in testicular relative weight (P<0.05). Results of the sperm characteristics showed that treatment of rat with nicotine worsens sperm motility and sperm count. Significant decline in both cauda epididymal sperm count (P<0.01) and sperm motility (P<0.01) were observed in nicotine treated rats as compared to control while supplementation of both folic acid and vitamin B₁₂ appeared to minimize these detrimental effects of nicotine on male fertility (Table 1). Further, serum testosterone, LH and FSH level were found to be significantly lower (Testosterone: P<0.05, LH: P<0.05, FSH: P< 0.05) in nicotine treated rats than in the control rats (Fig.1). Folic acid alone or in combination with vitamin B₁₂ showed significant ability to check the undesirable effects of nicotine on serum testosterone (folic acid alone: P<0.05, folic acid + vitamin B₁₂: P<0.05), LH (folic acid alone: P<0.05, folic acid + vitamin B₁₂: P<0.05) and FSH level (folic acid alone: P<0.05, folic acid + vitamin B₁₂: P<0.05). In continuation with these findings, activities of both 3-β hydroxysteroid dehydrogenase (3β HSD) and 17-β hydroxysteroid dehydrogenase (17-β HSD) were found to be lower in nicotine treated rats (Fig.2). But, nicotine mediated alteration of both 3β HSD and 17α HSD activities were found to significantly abrogated by supplementation of either folic acid alone (3β-HSD: P<0.001, 17β-HSD: P< 0.001) or combined supplementation of folic acid and vitamin B₁₂ (3β-HSD: P<0.001, 17β-HSD: P< 0.01).

Table 1: Protective effect of folic acid alone or in combination with vitamin B₁₂ against nicotine induced alteration in sperm motility, sperm viability, diameter of seminiferous tubules, germinal cell layer thickness and Johnsen’s tubular biopsy score in rat.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Control (Con)</th>
<th>Nicotine (Nic)</th>
<th>Nicotine+Folic acid (Nic+FA)</th>
<th>Nicotine+Folic acid+Vit B₁₂ (Nic+FA+Vit B₁₂)</th>
<th>Significance Level</th>
</tr>
</thead>
<tbody>
<tr>
<td>Relative testicular weights (mg/g body)</td>
<td>9.75±0.72</td>
<td>7.50±0.61</td>
<td>8.72±1.23</td>
<td>9.36±1.11</td>
<td>P&lt;0.05</td>
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</tbody>
</table>
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<table>
<thead>
<tr>
<th></th>
<th>Mean ± SE</th>
<th>19.64±1.19</th>
<th>24.59±2.01</th>
<th>31.79±2.78</th>
<th>P&lt;0.001</th>
<th>P&lt;0.01</th>
<th>P&lt;0.01</th>
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<tr>
<td>Sperm count (10$^6$/ml)</td>
<td>41.58±2.22</td>
<td>19.64±1.19</td>
<td>24.59±2.01</td>
<td>31.79±2.78</td>
<td>P&lt;0.001</td>
<td>P&lt;0.01</td>
<td>P&lt;0.01</td>
</tr>
<tr>
<td>Sperm motility (%)</td>
<td>71.00±2.37</td>
<td>35.42±2.58</td>
<td>54.00±1.94</td>
<td>65.40±2.01</td>
<td>P&lt;0.001</td>
<td>P&lt;0.01</td>
<td>P&lt;0.01</td>
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<tr>
<td>Diameter of seminiferous tubules (μm)</td>
<td>236.37±5.97</td>
<td>176.02±4.92</td>
<td>193.88±2.51</td>
<td>202.20±3.22</td>
<td>P&lt;0.001</td>
<td>P&lt;0.01</td>
<td>P&lt;0.01</td>
</tr>
<tr>
<td>Germinal cell layer thickness (μm)</td>
<td>87.74±1.66</td>
<td>60.90±1.78</td>
<td>62.81±2.44</td>
<td>70.17±2.38</td>
<td>P&lt;0.01</td>
<td>NS</td>
<td>P&lt;0.01</td>
</tr>
<tr>
<td>Johnsen’s tubular biopsy score</td>
<td>9.8±0.20</td>
<td>7.8±0.37</td>
<td>8.0±0.45</td>
<td>8.8±0.37</td>
<td>P&lt;0.01</td>
<td>NS</td>
<td>P&lt;0.01</td>
</tr>
</tbody>
</table>

Data were expressed as Mean ± SE. NS: Not significant

Fig.1. Effect of Folic acid (36 μg/kg body weight/day for 21 days oral administration) and folic acid + Vitamin B$_{12}$ (0.63 μg/kg body weight/day for 21 days oral administration) on nicotine (3 mg/kg body weight/day for 21days I.P injection) induced changes.
Ray et al: Folic acid and Vitamin B<sub>12</sub>……... in rats

in the [a] plasma LH level, [b] plasma FSH level, [c] plasma testosterone level. (Error bar represents Mean ± SE (n=5).
Significance based on Kruskal-Wallis nonparametric ANOVA (p<0.05#). Significance based on Mann-Whitney U multiple comparison test: Control vs. Nicotine (p<0.05*); Nicotine vs. Nicotine + Folic acid (p<0.05*); Nicotine vs. Nicotine + folic acid + Vitamin B<sub>12</sub> (p<0.05*).

Fig.2. Effect of Folic acid (36 µg/kg body weight/day for 21 days oral administration) and Folic acid + Vitamin B<sub>12</sub> (0.63 µg/kg body weight/day for 21 days oral administration) on nicotine (3 mg/kg body weight/day for 21 days I.P injection) induced changes in the activity of [a] 3β-HSD [b] 17β-HSD in the testis. Error bar represents Mean ± SE (n=5). Significance based on Kruskal-Wallis nonparametric ANOVA (p<0.01##). Significance based on Mann-Whitney U multiple comparison tests: Control vs. Nicotine (p<0.05*, p<0.001***); Nicotine vs. Nicotine + Folic acid (p<0.001***); Nicotine vs. Nicotine + Folic acid + Vitamin B<sub>12</sub> (p<0.001***, p<0.01**).
Compared to control rats, testicular MDA level (P<0.05), the product of lipid peroxidation of the polyunsaturated fatty acid present in cell membrane, was found to be significantly higher in nicotine treated rats concomitantly with significant increase in the generation of NO (P<0.05). Both the MDA level and NO generation in nicotine treated rats were found to be reversed significantly towards control rats by supplementation of folic acid alone (MDA: P<0.05, NO: P<0.05) or in combination vitamin B_{12} (MDA: P<0.05, NO: P< 0.05) (Fig.3).

**Fig.3.** Effect of Folic acid (36μg/kg body weight/day for 21 days) and Folic acid+ Vitamin B_{12} (0.63μg/kg body weight/day for 21 days) on Nicotine (3 mg/kg body weight/day for 21 days) induced changes in [a] NO generation and [b] MDA production. Data expressed as Mean ± SE. Significance level based on Kruskal Wallis test [p<0.001##]. Significance based on Mann-Whitney U multiple comparison test: Control vs Nicotine (p<0.05*); Nicotine vs Nicotine + Folic acid (p<0.05*); Nicotine vs Nicotine+ Folic acid+ Vitamin B_{12} (p<0.05*).
In contrary, nicotine treated rats showed a drastic inhibitory response on the testicular antioxidant status as evidenced by the decreased activities of SOD (P<0.05) and catalase (P<0.05) simultaneous with reduced level of GSH (P<0.05). SOD, catalase and GSH constitute the prime antioxidant machinery in testicular tissue which protects sperm cells by scavenging ROS. Thus, reduced activities of SOD and catalase and level of GSH in testicular tissues of rat leads to facilitation of
oxidative stress. Supplementation of folic acid alone (GSH: P<0.05, SOD: P<0.05, catalase: P<0.05) or in combination with vitamin B_{12} significantly restored these testicular antioxidant and anti-oxidative enzymes activities (GSH: P<0.05, SOD: P<0.05, catalase: P<0.05) (Fig.4).

Furthermore, nicotine treatment expanded the synthesis of proinflammatory cytokines that could be involved in testicular injury. Both, IL-6 and TNF-α level were found to be increased significantly in nicotine treated rats, when compared with control (IL-6: P<0.001, TNF-α: P<0.001). These increased IL-6 and TNF-α level were anticipated in the nicotine treated rats by the supplementation of folic acid alone (IL-6: P<0.05, TNF-α: P<0.01) or combined supplementation of folic acid and vitamin B_{12} (IL-6: P<0.01, TNF-α: P<0.01) (Fig.5).

Light microscopy examination of the testes of control rats showed normal structural features of seminiferous tubules and interstitial tissue. The Sertoli cells, and the spermatogenic cells (namely, the spermatogonia, primary and secondary spermatocytes; spermatids and spermatozoa) were
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found to be resting on the thin basal lamina. After three weeks of treatment with nicotine, rats revealed cellular changes in the seminiferous tubules. Degenerative changes were noticed in the Sertoli cells and spermatogenic epithelium (Fig.6). Clusters of degenerating spermatozoa and desquamated spermatogenic cells were frequently spotted deep within the lumina of the seminiferous tubules. Supplementation of folic acid alone or combined with vitamin B<sub>12</sub> were able to blunt the nicotine mediated alteration in seminiferous tubules and interstitial tissues. Reduction in size and numbers of seminiferous tubules as well as decrease in germ cells, irregular seminiferous tubules, vacuolation and degeneration of spermatogonia and spermatocytes are the most prominent features observed in nicotine treated testicular tissue architecture. In addition, reduction in diameter of seminiferous tubules (DST) and germinal cell layer thickness (GCLT) along with decreased Johnsen’s testicular score (0-10) were noted in testes of nicotine treated rats.

**DISCUSSION**

Cigarette smoking is very common in spite of worldwide anti-smoking campaigns. Adult males in their reproductive period (between 20 and 39 years) have the highest prevalence of smoking. Therefore, the major concern of the present investigation is to assess the possible protective capability of combined supplementation of folic acid and vitamin B<sub>12</sub> against nicotine-induced male reproductive dysfunctions. The present study demonstrated that combined supplementation of folic acid and vitamin B<sub>12</sub> revoke the nicotine...
induced oxidative stress, hormonal imbalance, proinflammatory cytokine production and abnormal histology in testicular tissue of male rat simultaneously with restoration of sperm motility and sperm function.

In the present study, rats treated with nicotine for a period of 21 days showed a significant decrease in sperm count and motility. These findings further validated the earlier finding that sperm count and motility of human males were adversely affected by smoking behaviour (21). In continuation with these findings, sperm viability was also diminished in rats treated with nicotine in the present study. In addition to others, we also hypothesize that nicotine affects normal morphology of sperm and its motility. Folic acid alone or in combination with vitamin B12 reversed the nicotine-induced impairment in sperm count, viability and motility in the present study.

Results of testis histological features in nicotine treated rats gave indications to the spermatogenesis process. Nicotine treated rats showed damage to the testicular tissue architecture as evidenced by altered histological features like reduced and irregular seminiferous tubules, vacuolated and degenerated spermatogonia and spermatocyte, less germ cells, degenerated Sertoli cells as well as reduced DST, GCLT and Johnsen’s testicular score, in the present investigation. All these features in testicular tissue of nicotine treated rats clearly in favour and validate the earlier findings (5). Supplementation of folic acid in combination with vitamin B12 in nicotine treated animals also exerts protection against nicotine-induced histopathological changes in rat testes. In addition, co-administration of folic acid and vitamin B12 with nicotine on rats for a period of 21 days showed an evident improvement in the histological architecture of testes in the present study.

The data generated from this study clearly shows a significant decrease in testosterone, LH and FSH level in nicotine treated animals. The decrease in gonadotrophins may adequately explain the earlier finding (22) that cigarette smoking or nicotine treatment results in testicular degeneration, deficiency of male sex hormone and reduction in sperm count. Thus the low sperm count would probably be a result of decrease or absent androgens and LH to adequately steer the process of spermatogenesis. It was earlier reported that smoking causes decreased LH secretion without affecting the testosterone level in the male smokers (23). But, we did not dissected out whether the decreased testosterone level in the present study is the consequence of decreased LH level or caused by some other reasons. But, histological features of testicular tissue of nicotine treated rats lead us to speculate that decrease in serum testosterone level of rats treated with nicotine must have been caused by the disruption of testicular cytoarchitecture by nicotine. Consequently this might have adversely affected Leydig cell number and functioning leading to decrease serum testosterone level since Leydig cells secrete testosterone. Further, supplementation of folic acid with or without vitamin B12 anticipated the nicotine mediated changes in the LH, FSH and testosterone level.

Generally, nicotine treatment was shown earlier to increase testicular lipid peroxidation products (24) and lipid peroxidation is considered as the main mechanism by which ROS induced impairment of testicular function. In addition, nicotine tended to exert oxidative stress in germ cells by disrupting the components of the free radical defence system. In the present study, testicular tissue of nicotine treated rats contained significantly higher level of NO and MDA with simultaneous lower level of GSH and lower activities of SOD and catalase. Thus, nicotine increased testicular ROS generation, which may acts to suppress the effective concentration of antioxidants and thereby increasing the harmful effects of ROS in reproductive tissue. The free radicals produced would lead to cellular injury. Release of unsaturated fatty acid from membrane
phospholipids marked the altered cell membrane structure and fluidity, which is thought to be the consequence of disintegration of membrane phospholipids and lipid peroxidation. Nicotine mediated hindrance of sperm membrane phospholipids content in this study provides evidence that nicotine impairs sperm membrane phospholipids to cause defective sperm function. Supplementation of folic acid with or without vitamin B_{12} reversed the nicotine mediated effects on oxidative stress parameters and sperm membrane phospholipids. Taken together, we can hypothesize that decreases in sperm quality and testis histological features observed in nicotine-treated rats may be due to an increased oxidative degradation of phospholipids. In addition, an increase in reactive oxygen species (ROS) level caused by nicotine would also lead to morphological defects on sperm, since mammalian sperms are rich in polyunsaturated fatty acids and are susceptible to attack by ROS. With regard to the fact that nicotine is one of the producers of reactive oxygen species, it seems that clearing oxidative agents by chemicals like folic acid can help cure and prevent the incidence of the diseases associated with sperm cells.

Further, nicotine is reported to enhance the cytokines production, particularly TNF-α and IL-6 in liver, lung and pancreas (13). In the present study, serum TNF-α and IL-6 level were elevated in response to nicotine treatment. We speculate that this increase in pro-inflammatory cytokines results in the testicular tissue destruction and inhibition of steroidogenesis as tumor necrosis factor alpha (TNF-α) can inhibit steroidogenesis in Leydig cells at the transcriptional level of steroidogenic enzymes. Nicotine-induced production of pro-inflammatory cytokines were restricted by the supplementation of folic acid combined with vitamin B_{12}.

Folic acid, in physiological concentration, can protect bio-constituents from free radical damage at least by competition. Thus, folic acid anticipated oxidative stress in cellular as well as tissue system. Further, deficiency of folic acid and vitamin B_{12} (cobalamin) in smoker is considered a major contributor to the hyperhomocysteinemia (12). Homocysteine (Hcy) is reported to hinder the activities of antioxidant enzymes – glutathione peroxidase (GPx) and superoxide dismutase (SOD) (25). Additionally, Hcy because of its susceptibility to auto-oxidation, it increases oxidative stress with secondary ROS generation. Evidence has continued to mount showing that the intakes and serum concentrations of certain vitamins, e.g. vitamins B_{12}, B_{6}, folate and vitamin C, are required to avoid clinical deficiencies, in maintaining the health status. In addition, the risk of toxicity from folic acid and vitamin B_{12} is low, because water-soluble vitamins are regularly removed from the body through urine. Furthermore, there is a growing body of evidence demonstrating a relationship between folate and other B vitamin deficiencies, hyper homocysteinemia and gonadal abnormalities, such as altered spermatogenesis as well as male infertility. Folic acid, which is required to metabolize Hcy to methionine, lowers plasma Hcy levels in smokers. Thus, adding vitamin B_{12} reduces plasma Hcy level further. So, folic acid and vitamin B_{12} supplementation in this study possibly reduces nicotine – induced oxidative stress by lowering homocysteine level. In addition, folic acid itself acts as potent antioxidants which might be responsible further for the protection imparted by these two nutritional supplements.

In conclusion, the results of this study suggested that nicotine caused testicular damage in rats and impairs steroidogenesis by promoting the generation of oxidative stress parameters and pro-inflammatory cytokines. However, folic acid in combination with vitamin B_{12} helps to combat the nicotine mediated testicular damage in this current set up. Finally, this study recommends further studies on pre- and post-treatment of folic acid and vitamin B_{12} in nicotine treated rats in order to
extrapolate the results of the present study to human chronic smokers.

Acknowledgement

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Conflict of Interest

The authors declare that they have no conflict of interest.

REFERENCES


Spotting the spotted fever

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ABSTRACT

With a surge in rickettsial infections in our country, there is an apparent necessity to upgrade the laboratory expertise to detect the same. We were able to arrive at a diagnosis of rickettsial Spotted fever in a child who presented with fever and rash with the support of appropriate investigations.

Keywords: Indian tick typhus; immunofluorescence assay; Rickettsia conorii; Weil-Felix reactions; enzyme linked immunosorbent assay.

INTRODUCTION

Indian Tick typhus is a zoonotic and self-limiting disease caused by an obligate intracellular organism Rickettsia conorii and commonly transmitted through the bite of the brown dog tick Rhipicephalus sanguineus. This disease has been distributed in India and several countries throughout the world (1-12). Common clinical symptoms of this rickettsial infection are fever with or without headache, myalgia, and a maculopapular rash which will appear 5 days after onset of fever. Center for Disease Prevention and Control (CDC) has also included a painless eschar as one of the symptoms of Spotted fever and it is seen in 70% of the febrile patients (1). Complications are severe thrombocytopenia, purpura, neurologic deficits, respiratory and/or renal failure and death (1-3). It is a vasculotropic rickettsiosis which affects endothelial cells and the agent is disseminated through different parts of the body. Case reports of spotted fever starts appearing in Indian literature in the recent past with involvement of heart, retina, meninges and brain (9-12). Isolation of spotted fever group rickettsia can be made only in specialized laboratories with Bio-safety Level III containment facilities. Laboratory diagnosis of Rocky Mountain Spotted Fever (RMSF) and related spotted fever group is by a ‘gold standard’ serological testing Immunofluorescence Assay (IFA) (1, 6). We are reporting a child with rickettsial spotted fever (Indian tick typhus) who presented as fever without focus and then happened to develop a rash which was a leading clue to our timely and accurate diagnosis.

CASE REPORT

A four-year-old boy residing in a rural area was brought with fever of seven days’ duration which was associated with myalgia. He was admitted as a case of fever without focus initially, but on the next day he developed a popular rash which first appeared over the forearms, then to legs, face and trunk. The rash was skin colored with no erythema, non-itchy and eschar was absent (Fig.1).

Figure 1: Popular Rash with no erythema
The palms and soles were not involved and there were no genital or anal or oral lesions. No abnormalities were found during systemic examination. There was no muscle tenderness. With the above presentation, the possibilities were viral exanthema, enteric fever, rickettsial fever and incomplete Kawasaki’s disease. The child was investigated with complete blood counts which was unyielding. ESR and CRP were mildly elevated. Liver function tests and the level of serum sodium were also normal. Widal and Rapid Immuno chromatographic test (ICT) for IgM against Scrub typhus (Scrub Typhus Detect IgM Rapid Test, InBios International, Seattle, U.S.A.) were also negative. However, there was a strong suspicion of spotted fever (SF), given the setting of unsettling prolonged fever with rash and no other clinical or laboratory clues towards alternate diagnosis. Hence, Indirect Immunofluorescence Assay (IFA) testing for IgG was done for R. conorii, which is known to cause RMSF in our country (4, 5). Rickettsia conorii IgG titre in IFA (Fuller laboratories, California, USA), was 1:256 which clinched the diagnosis of rickettsial Spotted fever. In addition to IFA, Weil Felix test was done which gave the following.

RESULTS

OXK titre: 320; OX 19 titre 1: 640 and OX2 titre 1:1280. This supported the diagnosis of spotted fever. Although OXK titre was 1:320, Scrub typhus IgM ELISA was negative (Scrub Typhus IgM ELISA InBios International, Seattle, USA). Co-infection with scrub typhus was thus ruled out. Fortunately, this child had a milder form of disease and he was started on oral doxycycline at 4.4mg/kg/day in two divided doses and there was a dramatic decline in fever within a day. He was discharged after he was afebrile for 48 hours and advised with a total duration of seven days of doxycycline therapy.

DISCUSSION

Rickettsial infections are common in recent times, which are under-diagnosed because of lack of appropriate laboratory testing facilities. Variation in the clinical presentation is attributable to the genetic diversity and geographic prevalence of the rickettsial subspecies. The gold standard for the confirmation of spotted fever is the estimation of R. conorii titre by Indirect Immunofluorescence (IFA) assay, by demonstrating seroconversion or a fourfold rise in antibody titre between acute and convalescent sera or a molecular testing for rickettsial DNA by PCR, as recommended by CDC (1). Molecular testing is more useful within the first week of illness but is not readily available in all centers. In our case the IgG-IFA assay was done on ninth day of fever and came out as 1:256 which is significant. The acute phase sample was not tested as the child had the rash on the eighth day of the illness only, when RMSF was suspected. IgM for R. conorii was not done due to its known high false positivity as reported by different rickettsiologists (1, 2, 6, and 8).

Doxycycline is the first drug of choice in children of all ages, given at a dose of 4.4mg/kg/day in two divided doses for a total duration of seven days or until 3 days of subsidence of fever (1-3) but in complicated cases the duration is 10 days. Alternatives would be macrolides (oral Clarithromycin or Oral/IV azithromycin) and chloramphenicol. Azithromycin is given at a dose of 10mg/kg/day once daily for 5 days.

CONCLUSION

The purpose of this case report is to reiterate that rickettsial infections are on a surge in India and appropriate laboratory testing is the need of the hour, as many cases go undiagnosed or misdiagnosed, thus delaying the early initiation of treatment thereby worsening the prognosis. With timely diagnosis, we could initiate prompt treatment which is easily available and affordable, which in turn reduces the morbidity and mortality.

CONFLICT OF INTEREST: None

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Multiple variants of oral candidiasis as a predictor for HIV/AIDS diagnosis-A case report

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ABSTRACT

Oral candidiasis is the most common opportunistic infection in human immunodeficiency virus infection. History, clinical examination, investigation and diagnosis of oral candidiasis is important for early diagnosis and treatment of HIV infection. Pseudomembranous candidiasis and erythematous candidiasis are associated with immune compromised state and so it is of prognostic significance like reduction in the viral load and CD4 positive T lymphocytes. In this case report, we present a case of a 34-year-old physically challenged asymptomatic male who came for replacement of mandibular anterior tooth who was diagnosed with all four common variants of oral candidiasis which predicted HIV infection. Quality of life of the patient was improved after the diagnosis and treatment.

Keywords: Acquired immunodeficiency syndrome; human immunodeficiency virus; anti-retroviral therapy; oral candidiasis; opportunistic infection.

INTRODUCTION

Candida albicans is a fungal organism present as a commensal in the oral mucosa. It is a dimorphic fungus which grows both as yeast form and hyphal form (1). When the host immune system is defective, there is transformation of commensal state to the infective state (2, 3). During this process, there is a morphological alteration from yeast form to hyphal form, latter being more adherent and competes with other microflora for host binding site which leads to increased virulence of Candida species (4). Oral candidiasis is one of the HIV-defining lesions (5). Variety of oral mucosal changes like pseudomembranous candidiasis, erythematous candidiasis, angular cheilitis, hyperplastic candidiasis or candidial leukoplakia, linear gingival erythema, median rhomboid glossitis can occur (6). Treatment of oral candidiasis is by topical and systemic azoleanti fungal drugs (7, 8). The problem with oral candidiasis in HIV patients is the relapse of infection (9). In this case report, we present a case of oral candidiasis with multiple clinical variants which is rare and led to diagnosing HIV, its management and follow-up of the patient.

Case report

A 34 years’ old physically challenged male patient was reported to the Department of Oral Medicine, Radiology and Special care dentistry for replacement of missing lower front tooth. History revealed that he had trauma 2 years back after which the lower front tooth was mobile and he visited a dentist and extraction of it was done. Past medical history revealed that during his childhood he had poliomyelitis and lower half of the body was paralyzed. Personal history revealed that he was a tobacco smoker, smokes about 2 cigarettes per day for 9 years. Six months back he quit his habit. Patient was married and his wife died due to tuberculosis 6 months back and does not have any...
kids. He did not give any history of having unprotected sex. General examination revealed muscle atrophy and weakness in lower limbs. Intraoral examination reveals ill-defined erythema on the right buccal mucosa and atrophy of filiform papilla on the dorsal surface of the tongue with wrinkling and fissuring in the left angle of the mouth (Fig.1,2) multiple raised irregularly shaped whitish plaque seen on the palatal mucosa (Fig.3). Left buccal mucosa revealed a non-scrappable white patch about 1x2 cm in size which was scrapped off leaving an erythematosus base (Fig.4). The above clinical features led to a provisional diagnosis of chronic hyperplastic in palate, atrophic candidiasis in right buccal mucosa and Pseudomembranous candidiasis in the left buccal mucosa.

Suspecting HIV, Tri-Dot rapid fourth generation ELISA test which turned out to be positive (Fig.5) with routine hematological examination which showed raised ESR level of 57 mm/hour. Smear from all surface were taken which revealed candidial hyphae confirming the diagnosis (Fig.6). Thus, based on the clinical features and investigations final diagnosis of HIV induced oral candidiasis was made.

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Broad spectrum antifungal like topical clotrimazole 1% mouth paint was prescribed daily for 2 weeks and he was informed about the condition and was referred to government hospital for initiation of HAART (Highly Active Anti-Retroviral Therapy). After 2 weeks patient reported with the western blot test report which was positive and CD4 count of 248 cells per cubic millimeter. Table 1 shows the timeline management, follow-up and CD4 cell count. With proper protocol oral prophylaxis and replacement of lower anterior teeth was done and oral hygiene practices were taught. Fig. 7 shows the clinical features after 3 months of follow-up.

Table 1: Timeline management, follow-up and CD4 count.

<table>
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<th>Timeline</th>
<th>Management</th>
<th>Clinical features</th>
<th>Cd4 cell count</th>
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<tr>
<td>At diagnosis</td>
<td>Topical clotrimazole 1% thrice daily over the affected tissues.</td>
<td>Asymptomatic HC in palate, EC in right buccal mucosa and tongue, PC in left buccal mucosa, AC in left oral commisure</td>
<td>Not assessed</td>
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<td>2 weeks follow up</td>
<td>Topical clotrimazole 1% thrice daily over the affected tissues. HAART</td>
<td>Same features no change in clinical features.</td>
<td>248 cells per cubic millimeter.</td>
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<td>1 month follow up</td>
<td>Topical clotrimazole 1% thrice daily over the affected tissues. HAART</td>
<td>Reduction in the size of the lesion, completely healed AC.</td>
<td>323 cells per cubic millimeter.</td>
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<td>3 months follow up</td>
<td>Topical clotrimazole 1% thrice daily over the affected tissues. HAART</td>
<td>Relapse of all the lesions, AC in right oral commisure.</td>
<td>352 cells per cubic millimeter.</td>
</tr>
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**PC- Pseudomembranous Candidiasis, AC- Angular cheilitis, EC- Erythematous Candidiasis, HC- Hyperplastic Candidiasis, HAART- Highly Active Antiretroviral Therapy.**

**DISCUSSION**

According to WHO July 2017 report, there are approximately 36.7 million people living with HIV globally (10). A study conducted with 151 HIV/AIDS patients revealed that the probability of immune failure in the presence of oral candidiasis was 91% men, 94% in females and 96% in intravenous drug users (11). Human immunodeficiency virus infection affects the host immune system by destruction of CD4 positive T-Lymphocyte (12). When the T-Helper cells are low they fail to protect the individual and place the host at increased susceptibility to opportunistic infections. According to September 1992 consensus on classification of oral lesions associated with HIV infection oral candidiasis comes under group 1 where in lesions are strongly associated with HIV
infection (13). Four distinct clinical variety of oral candidiasis namely pseudomembranous candidiasis, erythematous candidiasis, hyperplastic candidiasis and angular cheilitis which rarely can coexist in a single patient (14). The term multifocal candidiasis is applied for either for the presentation of a single variant with multiple sites or different variants at two or more sites (15). A case report on oral mycosis in HIV infection by Samaranayake (1993) had similar clinical presentation to our case with all four variants in a single patient, such findings gives a clue to the clinician in diagnosing HIV infection (16). Highly active Anti-Retroviral Therapy first introduced in 1996 have changed the course of HIV infection and have reduced the incidence of HIV – induced oral lesions (17). WHO 2017 report shows only half of the HIV infected individuals are on therapy: 54% of adults and 43% children (10). A Greek study on the effect of HAART on the prevalence of oral candidiasis in HIV infected patients has shown that introduction of HAART was associated with a significant decrease in the prevalence of oral candidiasis with improved CD4 count (18, 19). Many HIV induced oral lesions reduce after HAART but oral candidiasis persists after the initiation of HAART and have higher tendency to recur or develop de novo which can be attributed to failure of HAART and multi-drug resistance (20). The increased susceptibility to oral candidiasis in HIV infection is due to two basic factors, one due to weakened systemic immune system and the other is due to increased virulence of candida species due to genetic switching, increased antifungal drug resistance, increased SAP production and increased candida adherence. Hence oral candidiasis can be used as a clinical marker for diagnosing HIV infection.

CONCLUSION

Oral candidiasis can be used as a predictive clinical marker for diagnosing HIV infection as it occurs at early stage of HIV infection, AIDS and during and after HAART. Dentists should be aware of HIV induced oral manifestations, different variants of oral candidiasis which will aid in immediate management thereby increasing the quality of life of the patient.

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Trace element status in a rare case of hemoglobin D Punjab trait

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ABSTRACT

Hemoglobin D Punjab is a hemoglobin variant that occurs mainly in northwest India with prevalence rate of 1%. Homozygous form is very rare and clinically more serious than heterozygous HbD. Here we present a case of HbD trait with mild anemia and slightly altered red cell indices in a 31 year old female from South India. Decreased plasma trace elements (iron, copper, zinc) and altered metalloproteins are the novel findings, emphasizing the need for periodic assessment and prompt supplementation of micronutrients to prevent red cell damage and related complications in hemoglobinopathies.

Keywords: Hemoglobin D Punjab; hemoglobin variants; trace elements.

INTRODUCTION

Hemoglobin D Punjab is a rare hemoglobinopathy with β 121(GAA →CAA) mutation leading to substitution of glutamic acid by glutamine (1). It is asymptomatic with no clinical and hematological alterations in heterozygous state, but a subset of patients may manifest with chronic mild hemolytic anemia. Patients will have HbD, small percentage of HbA depending on the type (β0, β+) mutations, normal or slightly increased HbA2 and HbF. Homozygous HbD disease is a more serious condition with splenomegaly and moderate hemolytic anemia(2). In addition to iron, trace elements copper and zinc also play a crucial role in heme biosynthesis. Further, being a part of erythrocyte membrane SOD, they may increase the RBC life span (3). We hypothesize that trace element deficiency may add to the clinical complications in hemoglobinopathies. Hence, plasma trace elements and metalloproteins were analyzed in Hb D patient.

Case: A 31 year old female, mother of six months old child complains of generalized weakness, fatigue and inability to do her routine work from previous few months. General practitioner found her to be pale with low hemoglobin, suspected iron deficiency anemia and prescribed iron supplements. When her condition did not show any improvement even after treatment for 6 months, she was referred to a hematologist. On clinical examination, the patient had no splenomegaly, blood smear showed anisopoikilocytosis with microcytic hypochromic red cells. Red cell indices revealed the following: Hemoglobin 11.5g%, RBC count 3.9 x 106/µL, PCV 34.9%, reticulocytes 0.8%, MCV 88.2fL, MCHC 32.9 g%, MCH 29 pg, RDW 15.1%, WBC count 6600 cells/cumm, platelet count 345000 cells/cumm. Further LDH levels were normal (192U/L) and sickling test was negative.

Even with oral iron supplements her hemoglobin and ferritin levels were lower, hence hemoglobin variant analysis was recommended, to rule out any hemoglobinopathies. Hb analysis by HPLC quantified HbA at 53.9%, HbA2 3.5%, Hb F< 0.8%. The chromatogram showed an additional peak with retention time of 3.93mins corresponding to Hb D Punjab with 40.7% of the
confirmed a rare case of Hb D Punjab trait. The chromatogram confirmed a rare case of Hb D Punjab trait. 

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</table>

<table>
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<tr>
<th>Concentration</th>
<th>%</th>
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</thead>
<tbody>
<tr>
<td>F</td>
<td>&lt; 0.8*</td>
</tr>
<tr>
<td>A1C</td>
<td>5.2</td>
</tr>
<tr>
<td>A2</td>
<td>3.5</td>
</tr>
</tbody>
</table>

**Fig. 1: Chromatographic profile of HbD Punjab trait**

Since plasma iron and ferritin were towards lower side of the normal, we estimated other trace elements copper and zinc that had a role in heme metabolism and related metallo-proteins (Table 1). Both copper and zinc levels were lower in the patient studied and SOD was higher than the normal range.

**Table 1: Plasma trace elements and metalloproteins in HbD Punjab**

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Normal values</th>
<th>HbD Punjab</th>
</tr>
</thead>
<tbody>
<tr>
<td>Iron (µg/dL)</td>
<td>37 -145</td>
<td>60.63</td>
</tr>
<tr>
<td>Copper (µg/dL)</td>
<td>83 - 109</td>
<td>83.2</td>
</tr>
<tr>
<td>Zinc (ppm)</td>
<td>0.78 – 0.88</td>
<td>0.5</td>
</tr>
<tr>
<td>Ferritin (ng/mL)</td>
<td>13 - 150</td>
<td>19.54</td>
</tr>
<tr>
<td>Ceruloplasmin (mg/dL)</td>
<td>20 - 40</td>
<td>28.4</td>
</tr>
<tr>
<td>SOD (U/mL)</td>
<td>2 – 3.4</td>
<td>3.6</td>
</tr>
</tbody>
</table>

**DISCUSSION**

Mutations in genes coding for hemoglobin chains is seen in about 7% of worldwide population, that can either decrease the rate of globin chain synthesis as in case of thalassemia’s or modify amino acid make up generating hemoglobin variants. Hb S, C, D are some of the variants produced as a consequence of point mutation (1). Different to other hemoglobinopathies, Hb D is still poorly studied though it is prevalent in Punjab and north western India with an estimated frequency of 1%. Hb D occurs in 4 forms: HbD trait, HbD thalassemia, HbSD disease and HbD disease. HbD disease usually presents as mild to moderate hemolytic anemia with moderate splenomegaly. Adachi et al., (4) published a study on HbSD with severe clinical symptoms and opined that HbD favors polymerization of HbS, which was later confirmed by Patel et al., (5). HbD trait patients are mostly asymptomatic with no changes in the blood indices but the case studied by us had mild anemia and minor hematological...
variations showing anisopoikilocytosis. In one of the earlier studies done on 30 HbD trait patients, 24 patients were asymptomatic, and 6 patients showed clinical symptoms and hematological alterations similar to the findings in our patient(6). Trace elements viz., copper and zinc play a major role in erythrocyte function by being an integral part of metalloproteins like SOD and ceruloplasmin. A chronic imbalance in oxidants within RBC makes subjects with haemoglobinopathies more prone to oxidative damage, accelerating cell turnover (7). Erythrocytes act as a sink of superoxide ions and a rise in plasma SOD indicates oxidant surge in the patient studied. Ceruloplasmin oxidizes iron and incorporates it to Apo transferrin and transports it to bone marrow for erythropoiesis. Several researchers have also reported zinc and copper deficiency in patients with hemoglobinopathies (8). Hence, trace element deficiency might have added to the morbidity in Hb D Trait studied.

The study highlights the need for periodic assessment and prompt administration of micronutrients to reduce the extent of oxidative damage to erythrocytes and related complications in hemoglobinopathies.

REFERENCES


Efficacy of *Vitex agnus* in lowering prolactin level in mammary tumor induced SD rats

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ABSTRACT

Introduction and Aim: Breast cancer accounts for about 30% of all cancers in women. The present study aims to see the role of prolactin and *Vitex agnus* fruit extract in breast cancer progression in mammary tumor induced Sprague Dawley (SD) rats.

Materials and Methods: Thirty-day old inbred SD female rats of body weight 70-80 grams were taken for this study. The rats were induced with N- Methyl-Nitroso-Urea for mammary tumor development. After the development of palpable and visible tumor the rats were treated with anti-prolactin drug (Cabergoline) and a prolactin lowering herb *Vitex agnus* Castus (VAC) for two months. After the treatment the rats were sacrificed for antioxidants estimation and histopathological section examination.

Results: The rats treated with anti-prolactin drug showed benign tumors with hyperplasia and lactational change proving the presence of prolactin in the tumor tissue, whereas the plant extract showed mammary tumor regression by the presence of foamy macrophages in the histopathological sections. Results also showed treatment with cabergoline increased the GSH level and decreased the MDA level compared to tumor induced group.

Conclusion: Prolactin may have a potential role in progression of breast cancer and *Vitex agnus* extract showed a prolactin lowering effect and facilitated in regression of the tumor.

Keywords: Breast cancer; prolactin; cabergoline; *Vitex agnus*; histopathology; GSH; MDA.

INTRODUCTION

Breast cancer is one of the leading health problems throughout the world. One in ten of all new cancers diagnosed worldwide each year is a cancer of the female breast. It is also the principal cause of death from cancer among women globally. Breast cancer accounts for about 30% of all cancers in women and is one of the leading causes of cancer-related mortality in females (1, 2). Metastatic breast cancer develops in 30-40% of patients with breast carcinoma and is essentially incurable with standard therapy (3).
Recent studies done on breast cancer shows, the emerging factor that has gained maximum focus in the association of prolactin with breast cancer. Several studies done on breast cancer from past few decades have led to the possibility that prolactin plays an important role in human breast cancer. Prolactin, a pituitary hormone, is mainly involved in the production of milk protein and plays a very important role in the mammary gland development (4). The literature reveals that prolactin belongs to the same gene family as Growth Hormone and placental lactogenes (PLs). The PRL receptor (5); (PRLR) and the GH receptor (GHR; 6, 7) are members of the cytokine receptor superfamily. Ligand binding induces homo-dimerization of two receptor molecules followed by activation of tyrosine kinases in the Janus family, phosphorylating members of the STAT family and proteins involved in the MAP kinase signaling pathway. Previous studies done on mammary tissue show the presence of receptors for both GH and PRL. It is evident from the studies that breast cancer and mammary tumor cell lines express the PRLR (8). A study showed that if the prolactin receptor was blocked it resulted in the inhibition of growth in cultured tumor cells (9). From the literature it is also seen that GHR is also expressed in mammary tumors and tumor cell lines (10). For a long time, plants have played an important role in human life, and the use of plants as treatments is still very important (11). From the study it has been estimated that more than 80% of the world’s population utilizes plants as their primary source of medicinal agents (12), due to many reasons but primarily due to the high cost of Western pharmaceuticals and also because the traditional medicines are generally more acceptable from a cultural and spiritual perspective. Studies are showing that even in the Western world, use of the herbal medicines is steadily growing, with approximately 40% of the population reporting use of herbs to treat medicinal illness (13).

**Vitex agnus** Castus is an anti-prolactin herb which works by acting on the pituitary gland and the hypothalamus. These two structures are responsible for the release of hormones or for triggering hormone responses throughout the reproductive system (14). Many of the in-vitro studies done on **Vitex agnus** describe the dopaminergic effects of Vitex via a dose-dependent binding of dopamine-2 receptors, yielding a potent inhibition of prolactin in cultured pituitary cells (15).

Therefore, the present study aimed to evaluate the role of prolactin and assess the anti-prolactin effect of **Vitex agnus** Castus seed extract in breast cancer etiology, progression and treatment.

**MATERIALS AND METHODS**

**Animals**

After obtaining the permission from the institutional animal ethical committee dated 28/02/14, healthy, 30 days old inbred Sprague Dawley female rats of body weight 70-80 grams were taken for this study. Animals were maintained according to prescribed guidelines of a committee for the purpose of control and supervision of experts on animals (CPCSEA), Govt. of India, for the use of laboratory animals. All animals were maintained under normal day-night environment in temperature controlled institutional animal house. Animals were housed in polypropylene cages; paddy husk was used for bedding. Animals were given water *ad libitum* and standard rat feed pellet was used for feeding.

**Induction of tumor**

**Preparation of the carcinogen**

Mammary tumor was induced by injecting N-Methyl-N-Nitrosourea dissolved in normal saline with pH- 4 maintained by adding 3% glacial acetic acid. The SD rats were given a single intraperitoneal dose of 50 mg/kgbw of MNU and kept for observation for the mammary tumor development.

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Collection of plant material

The plant *Vitex agnus* Castus also known as *Vitex negundo* or *Vitex nirgundi* was collected from Vijaynagar; Mysore, Karnataka and the fruits and leaves of the plant were air dried and powered for the extraction.

Preparation of *Vitex agnus* extract

Hydro-alcoholic extract of the plant seeds was prepared using Soxhlet apparatus where 100gm of dry seed powder was suspended in 100 ml of 50% methanol (1:1 ratio) and refluxed at 50°C for 72 hours. The crude extract was concentrated by rotary flash vacuum evaporator and the concentrate was collected in an air tight container which was stored at 4°C for subsequent use. Stock and working standard solutions of the concentrated crude extract were prepared for the study.

The dosage was selected on the basis of previous literature as 200mg/kg bw administered orally once daily for 2 months (16). Cabergoline (anti prolactin drug) was given at a dose of .05mg/kg bw once daily through oral route for 2 months.

Histopathology

Histopathological section of the mammary tumor of the SD-rats treated with / without plant extract was done on the sixteenth day after finishing the treatment plan. The rats were anesthetized and the tumor was excised from the mammary region for the same. The tumor tissue was kept at 10% buffered formalin and then sectioned for Hematoxylin and Eosin staining.

Anti-oxidant estimation

**Reduced glutathione (GSH; 17)**

The samples (0.5ml of serum) were treated with 1.5mL of precipitating solution and kept for 10 minutes for the precipitation to complete and then filtered through a Whatmann No.1 filter paper. 500µL of the filtrate is taken and to this 2mL of phosphate solution and 250µL of DTNB solution is added. Simultaneously a blank is maintained containing 200µL of distilled water, 300µL of precipitating solution, 2mL of phosphate solution and 250µL of DTNB. The intensity of the yellow color formed is spectrophotometrically read immediately (within ten minutes) at 412nm against the blank.

**Lipid peroxidation (LPO; 18)**

100µL serum is diluted to 500µL with distilled water. To the diluted sample 1mL of TCA-TBA-HCl reagent is added and vortex is done for 2 minutes. The samples are kept in boiling water bath for 15 minutes. The reaction mixture is cooled and centrifuged. The supernatant is taken and the optical density of the pink color formed is read at 535nm.

**Estimation of prolactin**

Quantitative estimation of serum prolactin levels was done by using a commercially available in vitro Enzyme Linked Immuno-Sorbent Assay (ELISA) kit procured from Ray Biotech.

**Principle:** The assay is based on biotinylated double antibody sandwich technology which employs a prolactin specific antibody.

Statistical analysis

The statistical analysis was done by ANOVA followed by Tukey’s post hoc test.

RESULTS

**Antioxidant estimation**

The antioxidant status (Table 1) was found to be significantly lowered in tumor induced group and significantly increased in *Vitex* seed extract treated group. The GSH levels were significantly reduced in the tumor induced and cabergoline treated groups. Themaldialdehyde (MDA) levels were found to be increased in tumor induced groups as compared to control and treated group showing increased lipid peroxidation in tumor tissue. Cabergoline treated group also showed a decrease in MDA level and an increase in antioxidant level compared to tumor induced group. However, plant
Kour et al: Efficacy induced SD rats

The extract group showed better response than Cabergoline treated group.

Table 1: Antioxidant parameters of the experimental groups

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Control</th>
<th>Tumor induced</th>
<th>Cabergoline treated</th>
<th>Vitex treated</th>
<th>p-value</th>
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<tbody>
<tr>
<td>GSH (µmol/ml)</td>
<td>7.33± 0.09</td>
<td>3.15±0.07</td>
<td>5.27±0.08</td>
<td>7.94±0.06</td>
<td>&lt; 0.001*&lt;sup&gt;S&lt;/sup&gt;</td>
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<tr>
<td>MDA (µmol/ml)</td>
<td>0.91±0.11</td>
<td>1.55±0.02</td>
<td>0.95±0.02</td>
<td>0.72±0.03</td>
<td>&lt; 0.05*&lt;sup&gt;#&lt;sup&gt;S&lt;/sup&gt;</td>
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</table>

Results are expressed as Mean ± SD
Symbols *, #, $ indicate comparison between Tumor induced vs Control, Tumor induced vs Cabergoline and Tumor induced vs Vitex treatment groups respectively.

ELISA of prolactin levels

The prolactin levels of the groups were estimated in the groups in order to re-evaluate the role of prolactin in the mammary tumor progression and if the reduction of prolactin levels reduces the tumor progression. A significant increase in prolactin levels were observed in the tumor induced and cabergoline treated groups. A significant decrease in prolactin levels was observed in the Vitex treated group when compared to the tumor induced and cabergoline treated group.

Table 2: Levels of prolactin in experimental groups

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Control</th>
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<th>Cabergoline treated</th>
<th>Vitex treated</th>
<th>p-value</th>
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</thead>
<tbody>
<tr>
<td>Prolactin (ng/ml)</td>
<td>0.55±0.19</td>
<td>1.17±0.40&lt;sup&gt;#&lt;/sup&gt;</td>
<td>1.01±0.42&lt;sup&gt;*&lt;/sup&gt;</td>
<td>0.89±0.32&lt;sup&gt;##&lt;/sup&gt;</td>
<td>P&lt;0.05</td>
</tr>
</tbody>
</table>

Results are expressed as mean ± SD
Symbols *, #, $ indicate comparison between Tumor induced vs Control, Tumor induced vs cabergoline and Tumor induced vs Vitex treatment groups respectively.

Histopathology

The histopathological section of normal control rats showed the mammary ducts, breast parenchyma and sebaceous ducts (Fig.1). The tumor induced SD rats showed infiltrating ductal carcinoma (IDC) with sheets of malignant ductal cells and stromal infiltration, tubular adenosis and malignant ductal cells infiltrating the squamous epithelial layer of the skin (Fig.2). The histopathological section of Cabergoline (anti prolactin drug) treated group showed intraductal papilloma, lactational change, and adenomyoepithelial hyperplasia (AMH; Fig.3). The histopathological section of *Vitex agnus* seed extract treated group showed atrophic ducts and absence of malignant tumor cells and fibroblastic proliferation (Fig. 4). The findings are tabulated in Table 3.
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Fig.1: Shows mammary gland, sweat glands and the breast tissue of rats. Fig.2: shows ductal carcinoma, necrosis & stromal infiltration, tubular adenosis & cribriform Ductal Carcinoma in Situ (DCIS) with single intraperitoneal dose of MNU at 50 mg/kg. Fig.3: Intraductal papilloma, benign and unremarkable breast tissue, lactational change Adenomyoepithelial hyperplasia (AMH) Fig.4: Shows atrophic ducts and absence of malignant tumor cells and fibroblastic proliferation in the stroma. Presence of foamy macrophages or histiocytes was observed which indicates the tumor regression.

**Table 3: Histopathological findings of the different experimental groups**

<table>
<thead>
<tr>
<th>Groups</th>
<th>FA</th>
<th>ADH</th>
<th>DPA, IDP</th>
<th>AME</th>
<th>PDC</th>
<th>DCIS</th>
<th>Macrophages histiocytes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Tumor MNU induced</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>++</td>
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</tr>
<tr>
<td>cabergoline</td>
<td>++</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Vitex treated</td>
<td>+</td>
<td>-</td>
<td>++</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
</tbody>
</table>


**DISCUSSION**

Breast cancer is a major public health problem throughout the world. Metastatic breast cancer develops in 30–40% of patients with breast carcinoma and is essentially incurable with standard therapy (1). The cause for breast cancer still remains under search but the diagnostic test to evaluate the early detection of breast cancer has always been under the prime focus. Hormones have played a major role in the development of breast cancer (19). Post-menopausal women have always been under the risk of developing cancer due to the fluctuations in the estrogen levels. In the recent research on the diagnosis of breast cancer has led to the information that prolactin is secreted in the breast tissue and increase in the levels of prolactin from the mammary tissue can be the major reason for the development of the breast cancer. Studies carried out on mammary cancer cell lines have given a brighter insight about the role of prolactin but there is a lack of sufficient literature about In-vivo studies to find out the role of prolactin in mammary cancer development (20). The present study is an In-vivo study to find out the role of prolactin in progression of breast cancer.

The antioxidant estimation showed a decrease in GSH levels and higher MDA levels in tumor induced group. This is suggestive of the oxidative stress present in the tumor tissue. Plants have always been a great source of antioxidants providing a properly oxidized state of the tissue. *Vitex agnus* treatment showed significant improvement in both MDA and GSH suggestive of its antioxidant potential (21, 22). Treatment with cabergoline showed improvement in lipid peroxidation as indicated by the MDA levels compared to other groups. Therefore, the antioxidant estimation of the groups indicated the oxidative stress in the tumor induced group and improvement in the groups where the tumor progression was reduced due to the decrease in prolactin levels. The prolactin levels estimated through rat ELISA kits showed increased levels of prolactin the tumor induced and cabergoline treated groups. This can be attributed to the production of local prolactin in the vicinity of mammary tissue. The higher levels of prolactin in the cabergoline treated animals confirms the local release of prolactin. The presence of prolactin in spite of pituitary inhibition confirms the local release of prolactin. The Vitex agnus treated group showed significant decrease in the prolactin levels indicating the prolactin lowering activity of this herb extract.

The histopathological section of MNU induced rats showed the presence of stromal infiltration and ductal carcinoma in situ (DCIS) marking the presence of malignant tumor when compared to normal rats showing normal sweat glands and
sebaceous glands. Rats treated with cabergoline at a dose of 0.5mg/kg bw for 2 months showed Intraductal papilloma, benign and unremarkable breast tissue, adenomyoepithelial hyperplasia (AMH) along with lactational change indicating the presence of prolactin in the breast tissue. In Vitex treated group the histopathological section shows the presence of atrophic ducts and absence of malignant tumor cells along with fibroblastic proliferation in the stroma. Also presence of foamy macrophages or histocytes was observed which indicated the tumor regression. Therefore, from the histopathological study it was evident that prolactin was present in the mammary tissue inspite of its pituitary inhibition by cabergoline and the prolactin lowering herb administered showed the regression of mammary tumor.

CONCLUSION

The presence of prolactin in mammary tumor in spite of its pituitary inhibition with cabergoline was evident and Vitex agnus treatment helped in reduction of prolactin levels and hence regression of mammary tumor which can be used in the management of breast cancer.

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