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 bet 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 B_{12}, 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_{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_{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_{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_{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 7days 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_{12} supplemented. As we did not observe any significant effect on only vitamin B_{12} supplementation to nicotine treated animals in the previous study (unpublished data), therefore, nicotine + vitamin B_{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_{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 ($1.56X10^5$ cm^2 /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 H_2O_2 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 micro-pipette. 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 B_{12} 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 B_{12} 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 B_{12} 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 B_{12} showed significant ability to check the undesirable effects of nicotine on serum testosterone (folic acid alone: $P<0.05$, folic acid + vitamin B_{12}: $P<0.05$), LH (folic acid alone: $P< 0.05$, folic acid + vitamin B_{12}: $P<0.05$) and FSH level (folic acid alone: $P<0.05$, folic acid + vitamin B_{12}: $P<0.05$). 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 B_{12} (3β-HSD: $P<0.001$, 17β-HSD: $P< 0.01$).

Table 1: Protective effect of folic acid alone or in combination with vitamin B_{12} 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.

Parameters	Control (Con)	Nicotine (Nic)	Nicotine+ Folic acid (Nic+FA)	Nicotine+Folic acid+Vit B_{12} (Nic+FA+Vit B_{12})	Significance Level			
					Kruskal Wallis Non-parametric ANOVA	Mann-Whitney Test		
						Con vs Nic	Nic vs Nic+FA	Nic vs Nic+FA +Vit B_{12}
Relative testicular weights (mg/g body	9.75±0.72	7.50±0.61	8.72±1.23	9.36±1.11	P<0.05	P<0.05	NS	P<0.05

weight)								
Sperm count (10^6/ml)	41.58±2.22	19.64±1.19	24.59±2.01	31.79±2.78	P<0.001	P<0.01	P<0.01	P<0.01
Sperm motility (%)	71.00±2.37	35.42±2.58	54.00±1.94	65.40±2.01	P<0.001	P<0.01	P<0.01	P<0.01
Diameter of seminiferous tubules (µm)	236.37±5.97	176.02±4.92	193.88±2.51	202.20±3.22	P<0.001	P<0.01	P<0.05	P<0.01
Germinal cell layer thickness (µm)	87.74±1.66	60.90±1.78	62.81±2.44	70.17±2.38	P<0.01	P<0.01	NS	P<0.01
Johnsen's tubular biopsy score	9.8±0.20	7.8±0.37	8.0±0.45	8.8±0.37	P<0.01	P<0.01	NS	P<0.01

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

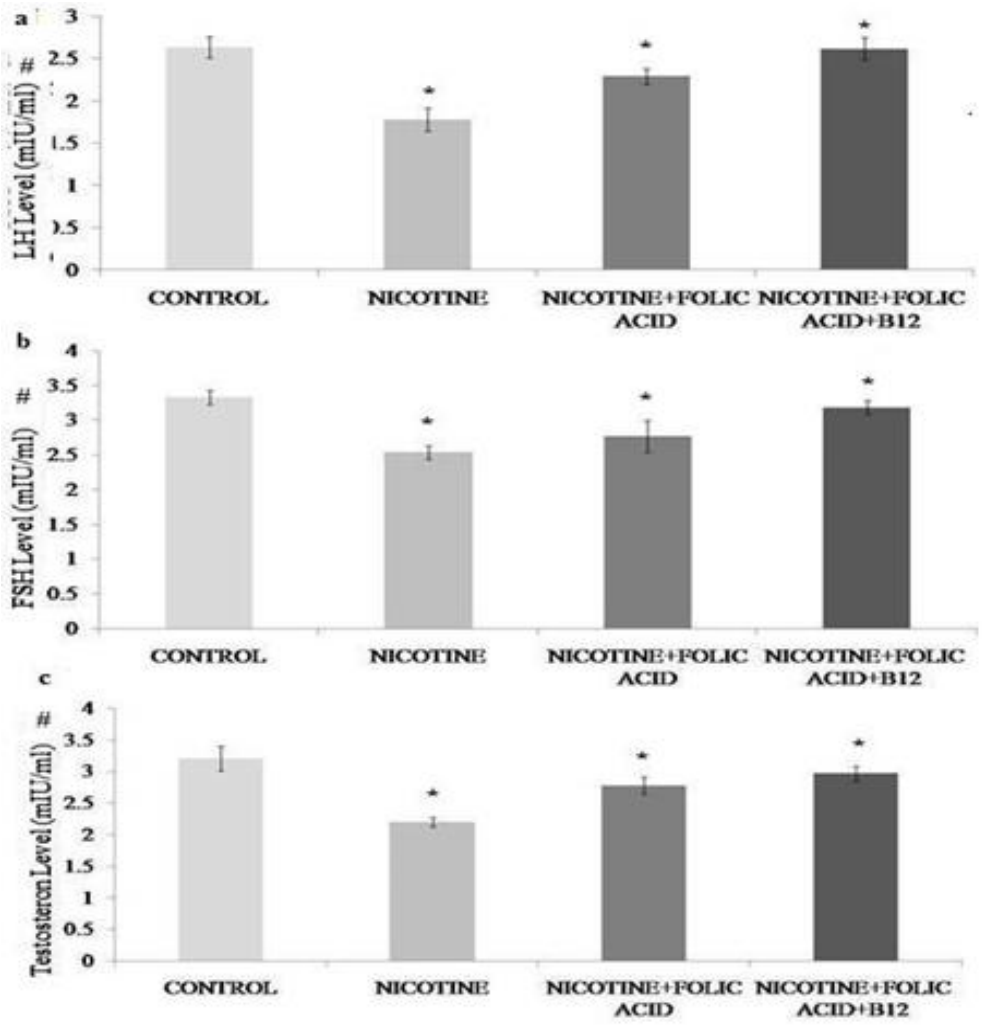

Fig.1. Effect of Folic acid (36 µg/kg body weight /day for 21 days oral administration) and folic acid + Vitamin B_{12} (0.63 µg /kg body weight/ day for 21 days oral administration) on nicotine (3 mg/kg body weight/day for 21days I.P injection) induced changes

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_{12} ($p<0.05$*).

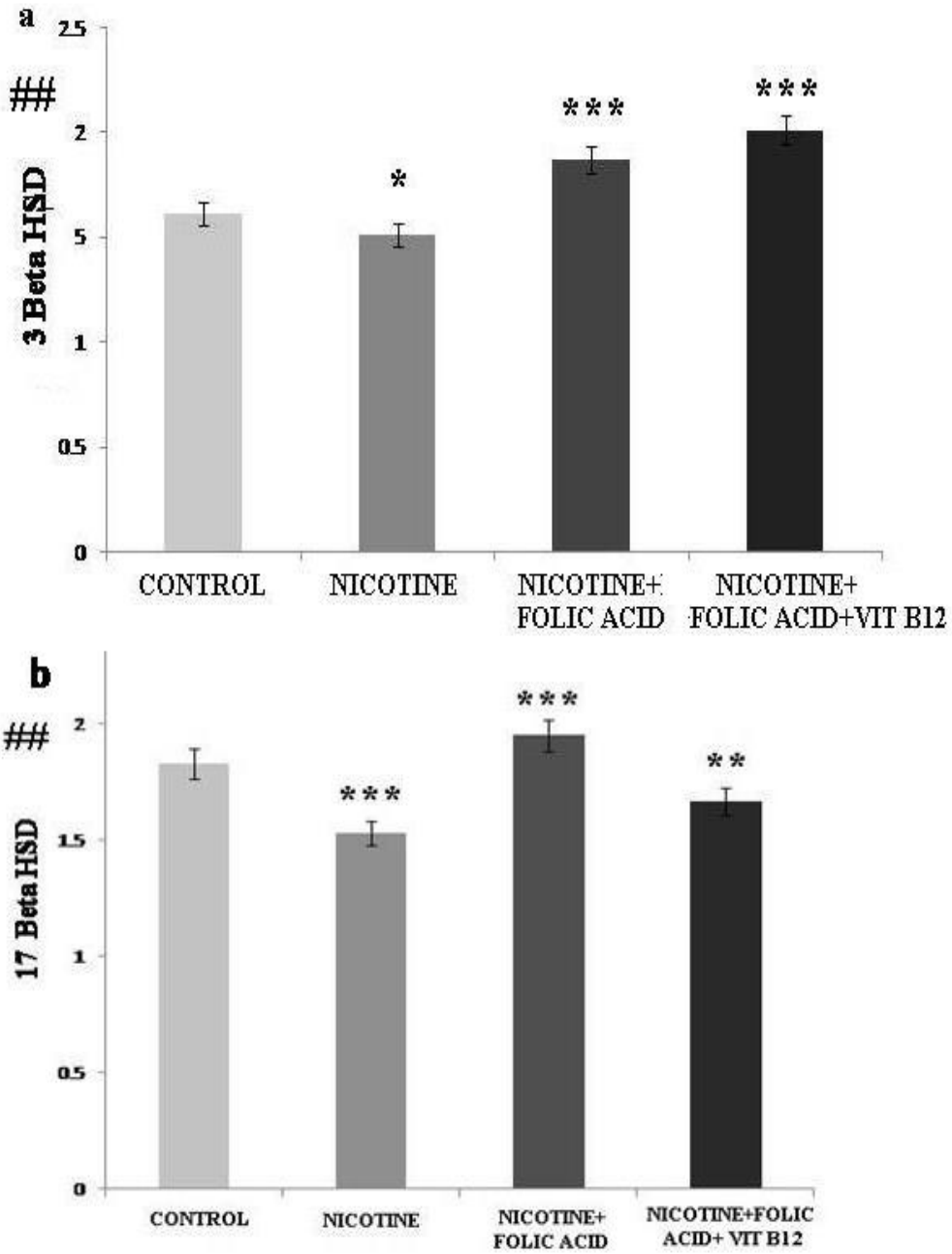

Fig.2. Effect of Folic acid (36 µg/kg body weight /day for 21 days oral administration) and Folic acid + Vitamin B_{12} (0.63 µg /kg body weight/ day for 21 days oral administration) on nicotine (3 mg/kg body weight/day for 21days I.P injection) induced changes in the activity of [a] 3β-HSD [b] 17β-HSD 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_{12} ($p<0.001$***, $p<0.01$**).

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).

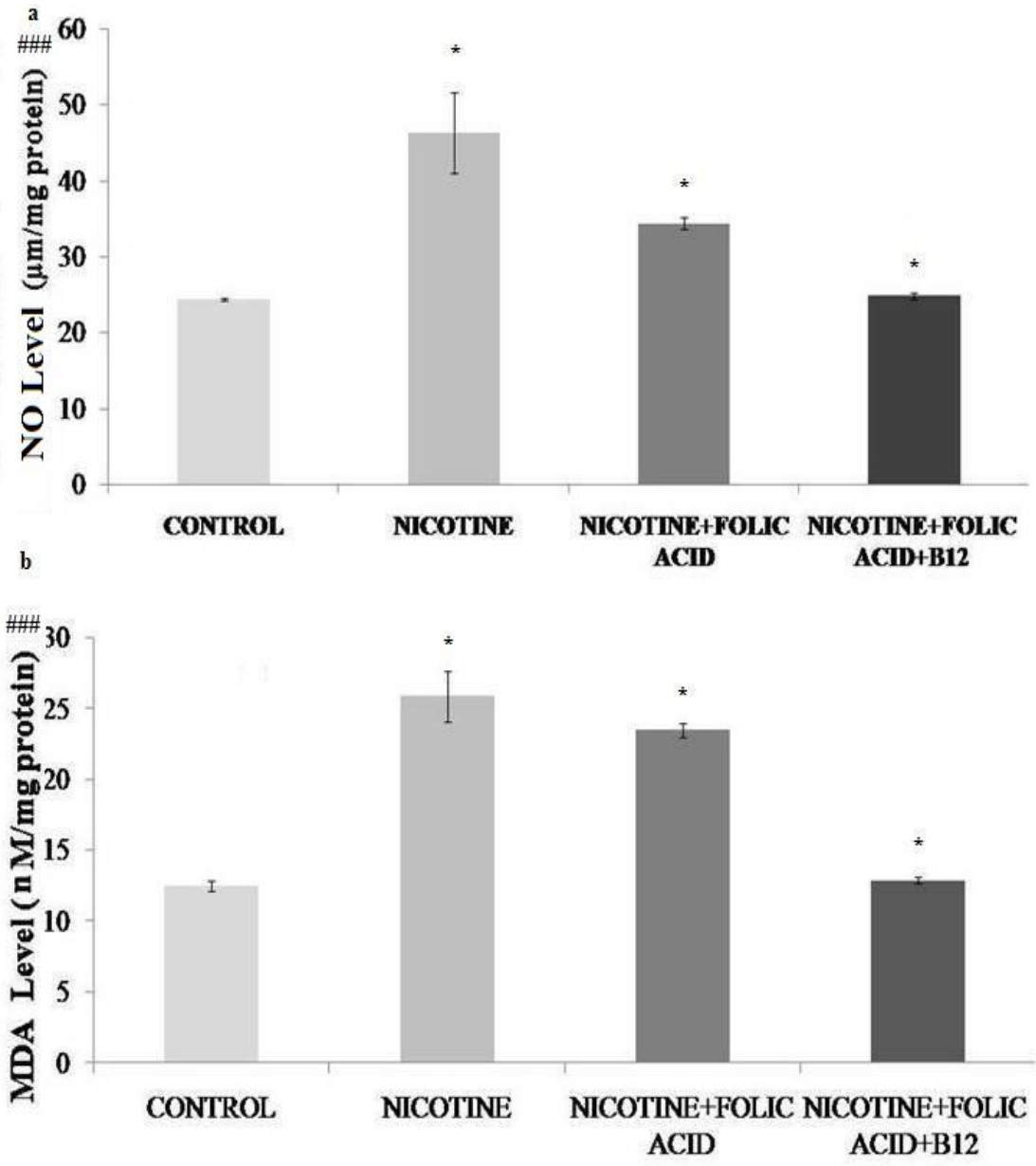

Fig.3. Effect of Folic acid (36µg/kg body weight/day for 21 days) and Folic acid+ Vitamin B_{12} (0.63µg/kg body weight/day for 21 days) on Nicotine (3 mg/kg body weight/day for 21 days) induced changes in [a] NO generation and [b] MDA production. Data expressed as Mean ± SE. Significance level based 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*).

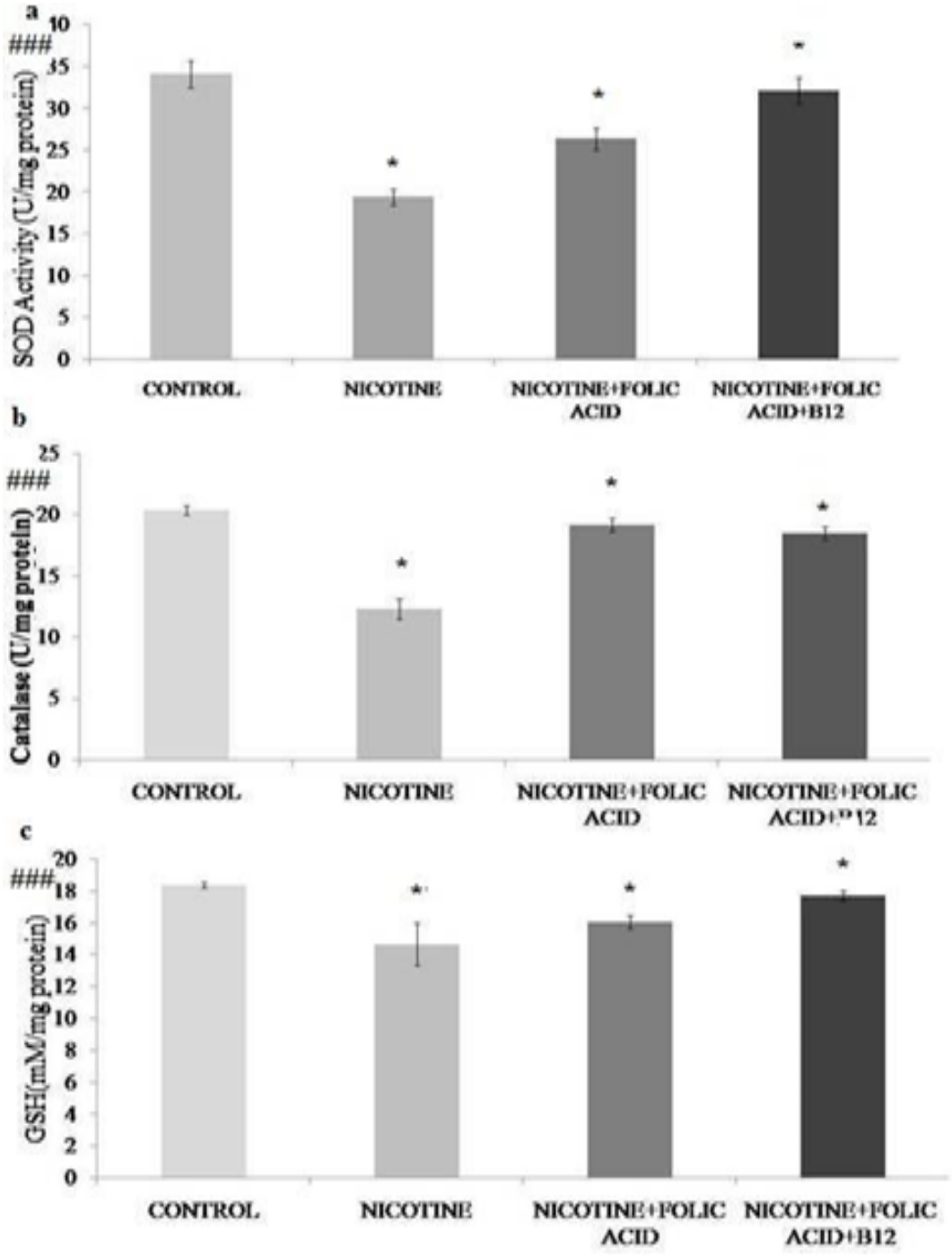

Fig. 4. Effect of Folic acid (36µg/kg body weight/day for 21 days) and Folic acid+ Vitamin B_{12} (0.63µg/kg body weight/day for 21 days) on Nicotine (3 mg/kg body weight/day for 21 days) induced changes in [a] SOD activity, [b] catalase activity and [c] GSH level. Data expressed as Mean ± SE. Significance 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).

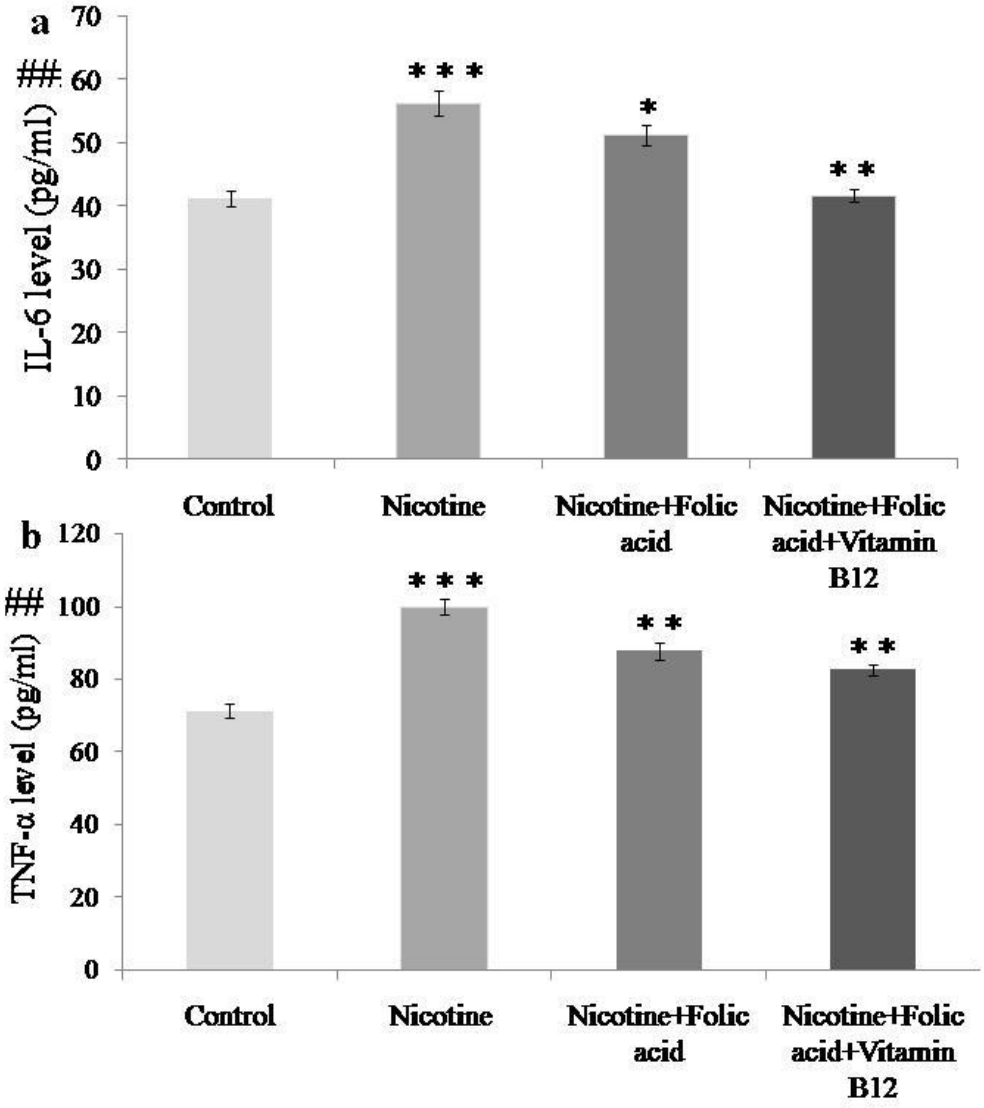

Fig.5. Effect of Folic acid (36µg/kg body weight/day for 21 days) and Folic acid+ Vitamin B_{12} (0.63µg/kg body weight/day for 21 days) on Nicotine (3 mg/kg body weight/day for 21 days) induced changes in [a] serum IL-6 level and [b] serum TNF-α level-Data expressed as Mean ± SE. Significance level based on Kruskal Wallis test (p<0.01##). Significance based on Mann-Whitney U multiple comparison test: Control vs Nicotine (p<0.001***); Nicotine vs Nicotine + Folic acid (p<0.01**, p<0.05*); Nicotine vs Folic acid+ Vitamin B_{12} (p<0.01**).

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

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_{12} 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.

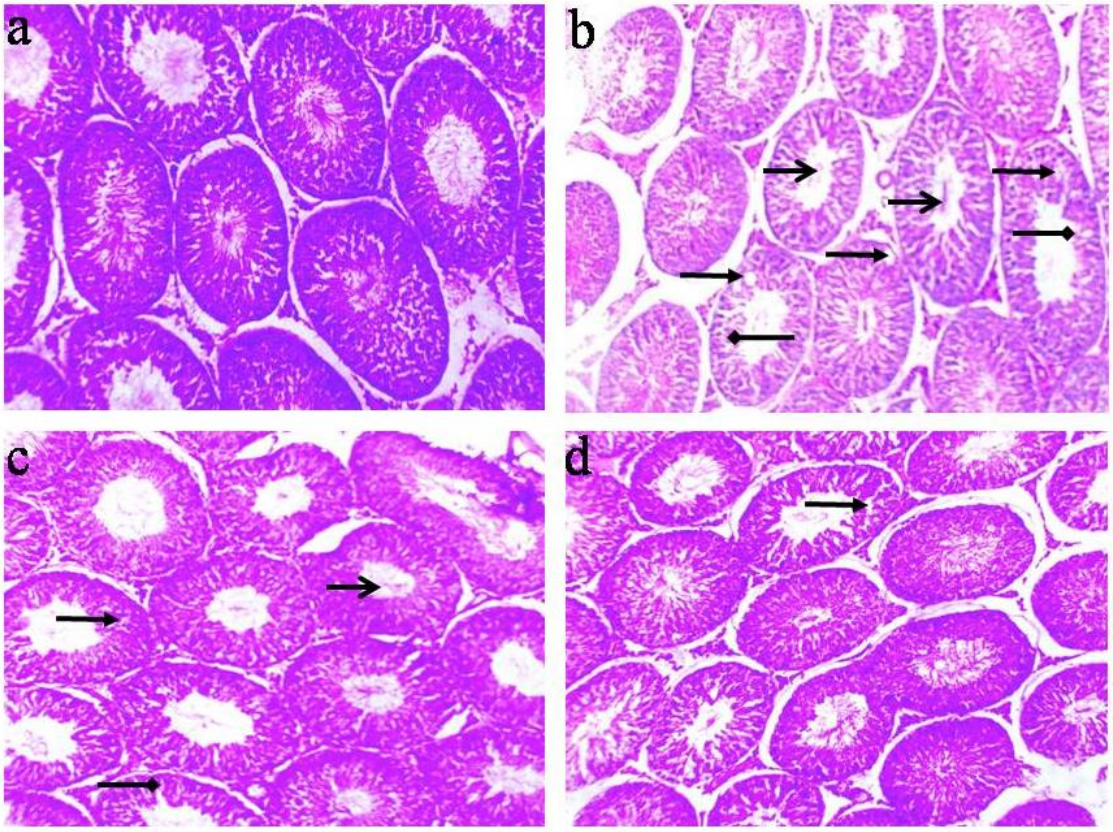

Fig.6 Representative photo micrograph of hematoxylin and eosin-stained section (400X) showing morphology of testicular tissue of [a] Control: no pathological changes were seen. Normal structural features of seminiferous tubules and interstitial tissues; [b] Nicotine (3 mg/kg body weight/d for 21 d) treated: Degenerative changes were noticed in the Sertoli cells and spermatogenic epithelium ( ). Clusters of degenerating spermatozoa and desquamated spermatogenic cells were frequently spotted deep within the lumina of the seminiferous tubules ( ) as well as decrease in germ cells ( ); [c] Nicotine +Folic acid (36 µg /kg body weight/d for 21 d), and [d] Nicotine + Folic acid+ Vitamin B_{12} (0.63 µg /kg body weight/d for 21 d) supplemented rat: prominent restoration of the nicotine mediated disruption in the testicular structure.

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_{12} against nicotine-induced male reproductive dysfunctions. The present study demonstrated that combined supplementation of folic acid and vitamin B_{12} 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 B_{12} 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 B_{12} in nicotine treated animals also exerts protection against nicotine–induced histopathological changes in rat testes. In addition, co-administration of folic acid and vitamin B_{12} 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 B_{12} 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.

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Conflict of Interest

The authors declare that they have no conflict of interest.

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