Research article

The protective role of adiponectin in restoring spermatogenesis in mice fed on methionine-choline deficient diet

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(Received: September 2022 Reviewed: November 2022 Accepted: December 2022)

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ABSTRACT

Introduction and Aim: The process of spermatogenesis requires a continuous supply of nutrients for producing normal sperms. Methionine and choline are considered important amino acids in transport of energy substrates, redox balance, and in DNA methylation while the protein adiponectin is known for its anti-inflammatory and anti-apoptotic function. In this study, we aimed to investigate the effect of methionine and choline on mice testis when fed with a methionine choline deficient diet (MCDD) as well as the protective effect of adiponectin on mice testis.

Materials and Methods: This study comprised of 25 mice, which were divided into three groups: Control (n=5); M1 (n=10 that were fed with MCDD for three weeks) and M2 (n=10 that were fed with MCDD for three weeks and in the third week were administered with daily injections of adiponectin for one week).

Results: The present study revealed MCDD caused a significant reduction in body weight and increase in the incidence of apoptosis where P value was 0.001; caspase-3 expression was higher than that of Bcl2 in group M1. Adiponectin therapy in the last week showed the potential to restore body weight, reduce apoptotic rate, and the expression of Bcl2 which was higher than that of caspase-3.

Conclusion: A diet lacking in methionine and choline not only significantly reduced body weight in mice, but also increased the apoptosis rate of spermatogenic cells. Adiponectin therapy in MCDD fed mice reduced the apoptotic rate and restored spermatogenesis.

Key words: Methionine choline deficient diet; caspase-3; Bcl2; adiponectin.

INTRODUCTION

Infertility affects more than 60 million couples worldwide, with male factors accounting for approximately 40-50% of cases. A meta-analysis study found that seminal fluid quality (sperm count and concentration) had fallen by 50-60% worldwide in the previous four decades, raising severe concerns about fertility in the near future (1). Anti-testicular antibodies (ATCA) were most prevalent contributing to infertility (2), but malnutrition, sedentary lifestyles, alcohol consumption, smoking, pollution, underlying health conditions, and stress have also been implicated as major causes of male infertility (3). It has been estimated that a healthy male produces over 200 million sperm cells daily within the testis through the spermatogenesis process that involves the continuous production of spermatozoa cells (4). The steroid testosterone, produced by the testis is essential in maintaining and regulating normal spermatogenesis (5). Studies have shown that spermatogenesis is greatly influenced by genetic, nutritional and lifestyle factors which play an essential role in male reproductive health and fertility (2). Research conducted in recent years has shown diet, balanced nutrition to have a significant impact on male fertility (6,7,8). Vitamins, minerals, fatty acids, amino acids, and antioxidants have been shown to increase sperm cell physiology and function through a variety of pathways, including ROS reduction, antioxidant defence system restoration, and inflammation inhibition (6,7,9). Although several studies have implicated diet to play a key role in the improvement of sperm parameters, this is paucity of studies considering the vast field of nutrition. The influence of methionine and choline on sperm quality and function has either not been investigated so far or is limited. Methionine is an essential amino acid that plays a crucial role in DNA methylation, cellular redox balance (10), and DNA synthesis (11), while choline is an integral part of cell membranes important in maintenance of cell structure and function. A diet deficient in choline has been thought to be associated with a broad spectrum of diseases like non-alcoholic liver disease, atherosclerosis, and neurological diseases (12). Hence, in this study we aimed at investigating the effect of methionine choline deficient diet (MCDD) on the histology of the testis. Furthermore, appropriate antioxidant intake has been proven to be quite effective in the prevention and treatment of male infertility, therefore this study also looked into the putative protective effect of the anti-inflammatory, anti-fibrotic, and antioxidant hormone adiponectin on the testis of mice fed MCDD.

DOI: https://doi.org/10.51248/v.42i6.2434

Biomedicine- Vol. 42 No. 6: 2022

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MATERIALS AND METHODS

Animals

Twenty-five albino male mice weighing around 18-30 g and 7-8 weeks old were used in this experiment. The experiments were carried out in the Department of Human Anatomy, Medical College, Mustansiriyah University, Iraq and lasted three weeks. The mice were kept in plastic cages under controlled temperature and humidity with a 12:12 hour light: dark cycle for one week to adapt to the lab environment. The mice were fed with regular mouse chow and water before diet manipulation. The experiment was performed and the guidelines of treatment of animals were followed according to Ethical committee No.27 on 23/1/2022.

Mice were fed with a methionine-choline deficient diet prepared according to the formula set by Isebella et al., (13). The ingredients and quantity used are given in Table 1. The ingredients were mixed with water to form pellets, baked at 55°C for 5 h and dried. The pellets were stored in a refrigerator (2-7 °C) until use.

Table 1: Composition of methionine choline deficient diet (MCDD)

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Quantity (g/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sucrose</td>
<td>455.3</td>
</tr>
<tr>
<td>Corn Starch</td>
<td>200</td>
</tr>
<tr>
<td>Corn oil</td>
<td>100</td>
</tr>
<tr>
<td>Non-nutritive bulk</td>
<td>30</td>
</tr>
<tr>
<td>Amino Acid Mix (No methionine)</td>
<td>174.4</td>
</tr>
<tr>
<td>Mineral mix</td>
<td>35</td>
</tr>
<tr>
<td>Dicalcium phosphate</td>
<td>3</td>
</tr>
<tr>
<td>Vitamin Mix (no choline)</td>
<td>2.3</td>
</tr>
</tbody>
</table>

Recombinant mouse adiponectin protein (ab285603) was procured from Abcam (UK). It was delivered as a fluid in a concentration of 1mg/ml. It was aliquoted into 100 μg portions and stored in the freezer at -20°C.

Experimental design

The mice were divided into three experimental groups as follows:

- Control group: Five mice were used and were nourished by regular chow for 21 days and were injected with 0.9% saline intraperitoneally.
- Group M1: Ten mice were fed MCDD for 21 days. On the 21st day they were injected with 0.9% saline intraperitoneally.
- Group M2: Ten mice fed MCDD for 21 days followed by adiponectin intraperitoneal injection given at a dose of 1.5mg/kg/day for one week as described earlier (14).

Histological study

To evaluate the effect of MCDD on testicular histology changes and spermatogenesis, mice testis seminiferous tubule tissue was fixed with Bouin solution for 1 day, then dehydrated by immersing the testes in increasing concentrations of alcohol, followed by wax impregnation and embedding to create wax blocks. Serial sections of 5μm in thickness were cut using a microtome, the sections were placed on slides, dewaxed using xylene, treated with decreasing alcohol concentrations, followed by hematoxylin and eosin staining.

Immunohistochemical study

Two serial sections of 4μm thickness were taken from each sample, placed on positive charge slides for immunohistochemical staining. Anti-Bcl2 and Anti-Caspase primary antibodies (Santa Cruz) were used. The secondary antibodies HRP/DAB (ab64261, Abcam, UK) used was mouse specific. The primary antibodies were diluted for 1:200 by using a reducing dilution buffer (Abcam, ab64211) and kept at room temperature for 30 minutes. Detection was by using labeled streptavidin biotin from secondary detection kit, then DAB followed by chromogen staining. The sections were counterstained by haematoxylin and mounted by DPX (15). The examinations were done blindly, Anti-Bcl2 and Anti-Caspase staining showed brown stains in the nuclei and cytoplasm. The tissues were examined at low power (10X) as well as high power (40X) for staining of cells and scored semi-quantitatively by counting the percentage of positive staining cells (nuclei or cytoplasm) over the total number of the examined cells. The positive cells were evaluated for their intensity of immune reactivity in a scale from 0 to 3+. The indices were obtained by multiplying the percentage of positive cells by the intensity of immune reactivity (16). The stain intensity score was given as 0 (no staining), 1+ (weak staining), 2+ (moderate staining), 3+ (strong staining). Staining percentage was 0 for no staining, 1+ <10%, 2+ 10-50%, 3+ >50%. The final score was obtained by multiplying the intensity by the percentage of staining. The results of microscopic examination were further confirmed using the Immunohistochemical Profiler Plugin and Macro in image J computerized software.

Statistical analysis

Data obtained was analyzed using the SPSS-26 (2019, IBM Corp.®) statistical package and Microsoft Excel 2019 (Microsoft ©). Data are presented as mean ±SD and tested by student t-test and ANOVA test. The statistical significance of the results was tested at a P-value ≤0.05 with 95% confidence interval.

RESULTS

The present study showed that mice given MCDD (group M1) had a significant progressive weight loss from the end of the first week to the end of the third week. The weight reduction was about 37.58% of body...
weight. While in the second group which received adiponectin after feeding with MCDD (group M2) the weight reduction was about 27.57%, as in Table 2.

**Table 2:** Demonstrating the mean of body weight (gm) of the three groups

<table>
<thead>
<tr>
<th>Groups</th>
<th>Means of body weight (gm)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Week 1</td>
<td>Week 2</td>
</tr>
<tr>
<td>M1</td>
<td>27.357±1.53</td>
<td>23.243±1.52</td>
</tr>
<tr>
<td>M2</td>
<td>28.9±0.8</td>
<td>27.2±0.7</td>
</tr>
<tr>
<td>Control</td>
<td>29.2±0.5</td>
<td>30.8±0.8</td>
</tr>
</tbody>
</table>

*Significant difference in t test at 0.05 level
# Significant difference in ANOVA test at 0.05 level

Histologically studies revealed normal spermatogenesis and organization of spermatogenic epithelium with appearance of spermatozoa filling the lumen of seminiferous tubules and illustrating the absence of any immature germ cells inside the lumen among mice testis of control group (Fig.1).

**Fig. 1:** Section of seminiferous tubule showing the normal spermatogenic epithelium of control group

On the other hand, the testes of mice fed with MCDD, (group M1) showed immature germ cells and apoptotic bodies sloughed into the lumen of seminiferous tubules with appearance of vacuoles inside the cytoplasm of Sertoli cells. Moreover, the spermatogenic epithelium was disrupted and disorganized with absence of sperms from the lumen (Fig. 2).

**Fig.2** The left: a section of mouse testis treated with methionine-choline deficient (M1 group) shows the reduction in the number of spermatogenic cells in the seminiferous epithilium with dark nuclei and multiple vacuoles (black arrows). The right: A section from the seminiferous tubule of diet (M1 group) shows multiple apoptotic germ cells with dark nuclei sloughed from the seminiferous epithilium (inside the black circle).

However, the testes of M2 group reveal the restoring of spermatogenesis and reappearance of normal spermatogenic epithelium with absence of all the signs of spermatogenic degenerations like Sertoli cells vacuolation and germ cell sloughing (Fig.3). The means of apoptotic indices of the groups demonstrating that the apoptosis was significant in group M1 (0.6421) in comparison to control group (0.1914), then the apoptotic index reduced to (0.4671) in group M2, (Table 3).

**Fig.3:** Histological section of testis (M2 group) showing restoration of spermatogenesis, the lumen illustrates large number of sperms

**Table 3:** Apoptotic indices of the three groups

<table>
<thead>
<tr>
<th>Groups</th>
<th>Mean± Std.Deviation</th>
<th>Std. Error Mean</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.1914±0.01512</td>
<td>0.00404</td>
<td></td>
</tr>
<tr>
<td>M1</td>
<td>0.6421±0.03093</td>
<td>0.00827</td>
<td>0.001</td>
</tr>
<tr>
<td>M2</td>
<td>0.4671±0.04177</td>
<td>0.01116</td>
<td></td>
</tr>
</tbody>
</table>

*Significant difference in t test for at 0.05 level

Results for immunohistochemical studies are presented in Fig.4 and the scoring obtained for each group is graphically represented (Fig.5). Caspase scoring was observed to significantly increase in group M1 and then decreased after treating adiponectin in group M2. On the other hand, Bcl2 scoring was significantly reduced in group M1 and returned back to increase significantly in group M2 (Fig.5).
DISCUSSION

This study evaluated the effect of methionine choline deficient diet (MCDD) on mice testes. Methionine and choline are methyl donors which maintain S-adenosylmethionine levels. Methionine and choline affect the energy metabolism and the synthesis of proteins due to limitation in the formation of VLDL proteins that are responsible for the synthesis of triglycerides from liver (17). Reduction of serum triglyceride results in reduction in fatty acid supply for the tissues like testis. Methionine and choline are crucial for phosphatidylcholine formation, which are important factors in mitochondrial function (18). This alteration results in the reduction of energy supply which explains the reduction in the weight of mice, which are fed with MCDD. Our study also shows that there are germ cell losses by sloughing and degeneration and also demonstrated that apoptotic index to be high in mice fed with MCDD. Bcl2 is a protein which has anti-apoptotic activity by regulating the apoptosis through maintaining the integrity of the mitochondrial membrane. Previous studies have shown that MCDD can cause hypo-methylation of the genome which in turn can affect lipid metabolism (19), while another study pointed out that MCDD can cause disruption in mitochondrial function (20). These might change the balance between Bcl2 and Caspase, as it appears in our study that caspase expression was higher than that of Bcl2 in the group which was fed with MCDD which probably explains the high expression of caspase and the high apoptotic index observed in this group.

The loss of spermatogenic cells might be due to deficiency in fatty acids which are the key components of the phospholipids of the plasma membrane of spermatogonia, spermatocytes, round spermatids, and also mature sperms (21). Alteration in energy homeostasis results in the reduction in the energy during spermatogenesis (22). Our study demonstrated that adiponectin treatment reduces weight loss in mice fed with a MCDD diet indicating the role of adiponectin in regulating the transport of energy substrates like glucose and lactate (23). In our experiment there was no complete restoration in body weight, which probably may be due to the short duration of adiponectin treatment given and therefore it is expected that weight restoration might need longer duration of adiponectin therapy than just 1 week as given in this study.

As demonstrated in Table 3, group M2, which received adiponectin in the third week, showed restoration of normal spermatogenesis with absence of cell sloughing and a lower apoptotic index than group M1. Previous studies demonstrated that adiponectin treatment stimulates cellular proliferation, enhancing germ cell survival and preventing cell apoptosis by its antioxidant action and through reduction in nitric oxide (24). Additionally, adiponectin can reduce the rate of apoptosis by enhancing the transfer of energy molecules (23). Our experiment illustrated that caspase expression in group M2 reduced and was lower than that of group M1. Adiponectin therapy has been shown to reduce apoptosis by reducing caspase expression (25). The effect of adiponectin and its receptors physiologically on testes is unclear. However, the presence of adiponectin receptor ADIPOR2 (functional receptor) founds to have a crucial role in testicular function in mice, as ADIPO knocking mice develop reduction in testicular weight, atrophy in the seminiferous epithelium, and azospermia, while the testosterone levels were not affected (26). Adiponectin gene deletion in mice, however, does not appear to affect male or female fertility (27), suggesting that the adiponectin-like ligands activate ADIPOR2 receptors and compensate for the absence of adiponectin. According to our findings, adiponectin has the potential to restore the normal histological appearance of seminiferous epithelium and the normal process of spermatogenesis after being affected by a methionine-choline deficient diet.

CONCLUSION

Based on our findings, MCDD considerably reduces body weight because methionine and choline are key amino acids for the transfer of energy substrates. Furthermore, because caspase expression was higher than that of Bcl2, MCDD can increase the rate of apoptosis of spermatogenic cells by activation of caspase. Furthermore, adiponectin therapy to MCDD-fed mice can lower apoptotic rate and restore spermatogenesis, although full body weight restoration may require a longer time of therapy.

CONFLICT OF INTEREST

The authors declare no conflicts of interest.

REFERENCES

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