Antioxidant Effects of Spinach on Intra-testicular Testosterone Levels in Obese Rats

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ABSTRACT

Objective:
To determine the antioxidant effects of spinach on Intra-testicular testosterone levels and histopathological changes in testicular tissue in obese Sprague Dawley rats.

Study design:
It was an experimental, randomized control trail.

Place and duration:
The research was performed in the multidisciplinary research laboratory of Islamic International Medical College, Rawalpindi in association with the Animal House at National Institute of Health (NIH), Islamabad, Pakistan from April 2016 to March 2017.

Materials and Methods:
In this study, 40 male Sprague Dawley rats were randomly allocated into Group A-control group and Experimental group. Then for the duration of six weeks, control group was fed on standard diet while Experimental group was fed on high fat diet to induce obesity. After inducing obesity, Experimental group was divided into Group B-Obesity control group and Group C-Spinach treated group. Intra-testicular testosterone levels and testicular tissue histology of Group A and Group B rats were evaluated. Afterward 5% spinach hot water extract along with high fat diet was given to Group C for four weeks and finally Intra-testicular testosterone levels and testicular tissue histology were measured in this group.

Results:
Intra-testicular testosterone levels of Group B rats was significantly decreased ($P<0.001$) as compared to Group A rats. However, Intra-testicular testosterone levelsof Group C rats was significantly increased ($P<0.001$) as compared to Group B rats after spinach administration. Testicular tissue histology of Group A ratsshowed normal shaped seminiferous tubules with eight to nine layers of spermatogenetic cells, while atrophic seminiferous tubules with five to six layers of spermatogenetic cells were observed in Group B rats. However administration of spinach in Group C rats showed seminiferous tubules with increased number of mature spermatozoa in their lumen and eight to nine layers of spermatogenetic cells.

Conclusion:
Spinach has restorative effects on male reproductive system by normalizing the intra-testicular testosterone levelsand by restoring the structure of testicular tissuesin response to obesity-induced oxidative stress.

Key word:
Antioxidant, Intra-testicular testosterone, Obesity, Spinach

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INTRODUCTION

Obesity is an intricate medical state described as the excessive fat accrual in the adipose tissues with the body mass index of more than 28kg/m^2.[1]According to the WHO, approximately 39% of adults aged 18 years and above were overweight and 13% were obese in 2016.[2] Two most fundamental reasons of obesity are intake of fatty diet and reduced physical activity.[3]

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Obesity is not only the risk factor of various diseases but also affects the functions of male reproductive system.[4,5] One of the connections between obesity and impaired reproductive function is the production of reactive oxygen species.[6] In a normal person, a fine equilibrium is maintained between the generation of reactive oxygen species and utilization of antioxidants. However, in obesity, production of reactive oxygen species is increased which are highly reactive species accountable for damage to surrounding tissues, thus cause negative impact on fertility and male reproductive function.[7]

These reactive oxygen species not only affect the process of spermatogenesis that occurs in the seminiferous tubules but also affect the secretion of testosterone hormone.[8-10] Thus, in order to avoid these consequences discovery of new, affordable, easily accessible and less perilous strategies is very imperative, where natural antioxidants found in our diet can play a vital role.[10,11]

Spinach is a globally cultivated green flourishing vegetable. It is rich in micronutrients, minerals and phytochemical such as carotenoids and phenols.[12,13] Spinach works as an antioxidant and provide a shield to numerous significant macromolecules from the injury resulting from reactive oxygen species as demonstrated in numerous studies on animal models.[14-16]

However, information regarding its therapeutic role against the obesity induced decrease in intra-testicular testosterone secretion was still lacking. Therefore, this study was designed to find out ameliorative effects of spinach on intra-testicular testosterone levelsand testicular tissues in obesity-induced oxidative stress.

MATERIAL AND METHODS:

Nutritional composition:

High fat diet: Standard diet supplemented with 20% butter.

Spinach treated diet: High fat diet augmented with 5% spinach hot water extract. For the preparation of spinach hot water extract, fresh spinach leaves were bought from local market and then identified by taxonomist of National Herbarium department in National Agriculture Research Centre (NARC) Islamabad. 5% spinach hot water extract was prepared by the technique used by *Ko et al.*, 2014.

Methodology:

After being approved by the ethical review committee of Islamic International Medical College, Riphah International University, Islamabad. This investigational study was performed in the multidisciplinary research laboratory of Islamic International Medical College, Rawalpindi in association with the Animal House at National Institute of Health (NIH), Islamabad, Pakistan from April 2016 to March 2017.

In this randomized control trial, 40 male Sprague Dawley rats (8 weeks age) weighing from 160-200g were allowed to acclimatize to NIH Animal house environment for seven days; at humidity-50-70% and at a room temperature-24 + 2 °C, maintaining a 12 hour light and dark cycle. The food-a standard diet in pellet form and water was provided ad libitum.

After acclimatization, rats were randomly distributed into Group A- Control group (n=13, Fed on standard diet) and Experimental group (n=27, Fed on High fat diet to induce obesity). At the end of 6th weeks, when rats of Experimental group had gained 20% weight above that of rats of Group A , the Experimental group was then subdivided into Group B-Obesity control group (n=13) and Group C-Spinach treated group (n=14). Then first sampling was done from Group A rats-Control group (n=13) and Group B rats-Obesity control group (n=13) to measure the intra-testicular testosterone levels (ng/ml) and to examine the histopathological changes in testicular tissues.

For measuring the intra-testicular testosterone levels (ng/ml), aspiration of intra-testicular fluid was performed on left testes of Group A and Group B rats, using a 21-gauge butterfly needle with tubing attached to a 5-ml syringe containing 2 ml normal saline. Needle was inserted into the anterior portion of the testicle and gentle pressure was applied to the testes until an adequate amount of testicular fluid was withdrawn into the tubing.Intra-Testicular fluid sample in the tubing was shifted to laboratory where it was transferred into labelled gel tubes for centrifugation. Intra-Testicular fluid sample was centrifuged at 6000 rpm for 10 minutes to remove cellular components and then used for determining the levels of intra-testicular testosterone (ng/ml) by using ELISA Kit from Elabscience Biotechnology *Co.Ltd., Japan.*

For testicular tissue histology, right testes of Group A and Group B rats were fixed in formalin saline solution for 24 hours. Ascending grades of ethanol were used to dehydrate the tissue. It was cleared in xylene and embedded in paraffin for 2 hours. Then next day testicular tissue is sectioned by using microtome (thickness of 5µm). Testicular tissue sections were deparaffinized by xylene, hydrated through an ethanol series of 100%, 90%, 80%, 70%, and 50%, and finally Staining of slides was done using haematoxylin-eosin (HE) stain for the determination of testicular pathology (size

of seminiferous tubules, lumen of seminiferous tubules and spermatogenetic cell layers) by observing four random fields under light microscope.

After first sampling, 5% spinach hot water extract was orally administered to Group C-Spinach treated group along with persistence of high fat diet for period of four weeks. Then after 4 weeks, intra-testicular testosterone levels (ng/ml) and testicular tissue histology was done on Group C-Spinach treated group rats similar to the method used for the first sample collection.

Data analysis:

Statistical analysis was performed by using Statistical package of social sciences (SPSS 21) version. Results of intra-testicular testosterone levels (ng/ml) were expressed as Mean ± SEM. Comparisons among the groups was evaluated by using the independent sample t-test. p value of <0.05 was regarded as significant.

RESULTS

In our research, a total of 40 male Sprague Dawley rats were initially divided into two groups, Group A (Control group) and Experimental Group.On comparison, weight of Experimental Group rats-fed on high fat diet (226.4 ± 5.74g) was significantly increased (p<0.001) as compared to weight of Group A rats-fed on normal standard diet (179.2 ± 3.74g).

Intra-testicular testosterone (ng/ml) levels of Group B rats were observed to be (2.48 ± 0.22ng/ml) which were significantly decreased (p<0.001) as compared to the Group A rats (5.22 ± 0.24ng/ml). After administration of spinach hot water extract, intra-testicular testosterone levels of Group C rats (4.64 ± 0.19ng/ml) were significantly raised (p<0.001) as compared to the Group B rats (2.48 ± 0.22ng/ml). Comparison of Mean ± SEM of intra-testicular testosterone (ng/ml) levels in all three groups (A, B, C) is shown in Figure 1.1.

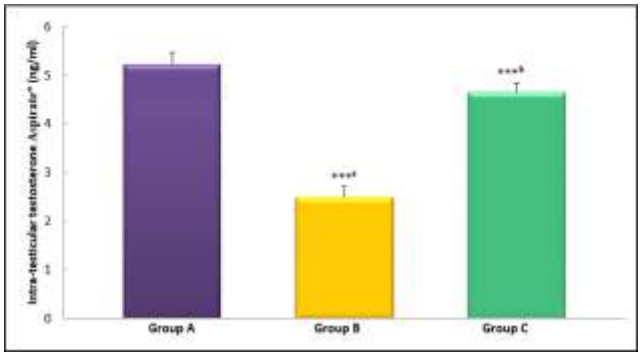

Figure 1.1: Comparison of Mean ± SEM of Intra-testicular Testosterone (ng/ml) levels of Sprague Dawley rats in the following three groups:

Group A: Control group (n=13)

Group B: Obesity control group (n=13)

Group C: Spinach treated group (n=14)

*= intra-testicular testosterone diluted in 2 ml of normal saline.

***= p< 0.001 is considered statistically significant.

***[a]= Group A vs B

***[b]= Group B vs C

Under light microscope (40 and 100 x Magnification), seminiferous tubules of testicular tissue were observed in all three groups. Group A (Control group) Sprague Dawley rats showed normal shaped seminiferous tubules with eight to nine layers of spermatogenetic cells and lumen of seminiferous tubules was filled with large number of spermatozoa. While atrophic seminiferous tubules with five to six layers of spermatogenetic cells and widening of lumen were observed in Group B (Obesity control group) Sprague Dawley rats. However administration of spinach hot water extract in Group C (Spinach treated group) Sprague Dawley rats showed normal shaped seminiferous tubules with increased number of mature spermatozoa in their lumen and eight to nine layers of spermatogenetic cells. The histological changes in testicular segment of all three groups (A, B, C) are shown in Figure 1.2, 1.3, 1.4.

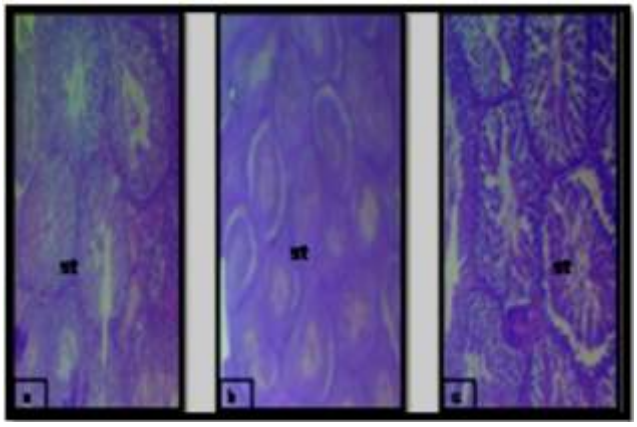

Figure 1.2: Histological changes in the testicular tissue of Group A (Control group), Group B (Obesity control group) and Group C (Spinach treated group) Sprague Dawley rats (Under 40 x Magnification).

a. Testicular segment of Group A (Control group) Sprague Dawley rat showing normal seminiferous tubules (st).

a. Testicular section of Group B (Obesity control group) Sprague Dawley rat showing atrophic seminiferous tubules (st).

b. Testicular section of Group C (Spinach treated group) Sprague Dawley rats showing normal seminiferous tubular (st).

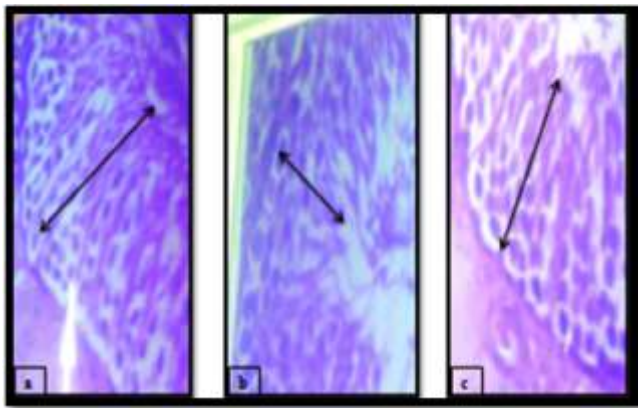

Figure 1.3: Layers of spermatogenetic cells in the testicular tissue of male Sprague Dawley rats in Group A (Control group), Group B (Obesity control group) and Group C (Spinach treated group) (100 x Magnification).

a. Testicular segment of Group A (Control group) Sprague Dawley rat showing 8-9 layers of spermatogenetic cells (double head arrow).

b. Testicular section of Group B (Obesity control group) Sprague Dawley rat showing 5-6 layers of spermatogenetic cells.

c. Testicular section of Group C (Spinach treated group) Sprague Dawley rats showing 8-9 layers of spermatogenetic cells.

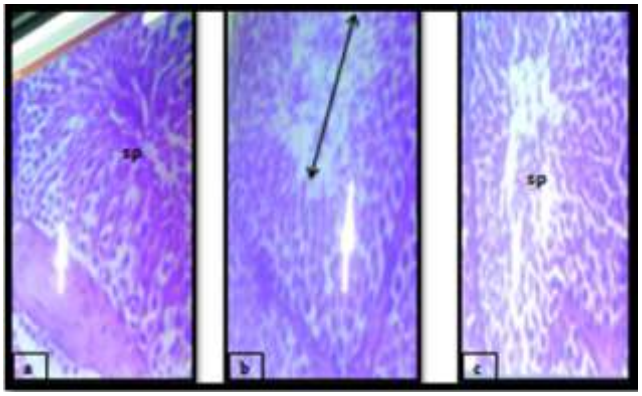

Figure 1.4: Number of spermatozoa in the lumen of seminiferous tubules in Group A (Control group), Group B (Obesity control group and Group C (Spinach treated group) Sprague Dawley rats (100 x Magnification).

a. Testicular segment of Group A (Control group) Sprague Dawley rat showing spermatozoa (sp) in the lumen of seminiferous tubules.

b. Testicular section of Group B (Obesity control group) Sprague Dawley rat showing decrease number of spermatozoa and widening of lumen of seminiferous tubules (double head arrow).

c. Testicular section of Group C (Spinach treated group) Sprague Dawley rats showing increase in number of spermatozoa in lumen of seminiferous tubules.

DISCUSSION

Obesity, one of the utmost prevalent metabolic disorders, is closely associated to many illnesses such as cardiovascular diseases, hypertension, insulin resistance and endocrine disorders.[17-19]Moreover, growing substantiation suggests that increased body fat also affect the functions of male reproductive system and cause male infertility.[20,21]

Therefore, our study explored the injurious effects of obesity on male reproductive system as well as the beneficent role of spinach (*Spinacia oleracea*) in restoring fertility. In present study, animal model of high fat diet-induced obesity was chosen, in which obesity was induced over a period of 6 weeks.

In our study, we measured intra-testicular testosterone levels as there disturbance is mainly suspected to trigger the change in process of spermatogenesis. The findings of present study showed that in obesity, there is significant decrease in levels of serum and intra-testicular testosterone. These findings are in accordance with Cui, Long, Tian & Zhu, (2017) results that showed that high fat diet-induced obesity cause significant decrease in testosterone levels.[22]This lower level of testosterone in obese rats is mainly caused by obesity-induced oxidative stress that affects the structure and function of Leydig cells. However the usage of spinach (*Spinacia oleracea*) cause significant increase in testosterone levels and these results are in accordance with *Türk et al.*, (2008) who also explored that use of natural antioxidants such as pomegranate elevates the testosterone levels by down-regulating the oxidative stress.[23]

While observing the testicular tissues after HE staining, present study findings demonstrated that obesity also leads to atrophy of the testes.In obese rats, there was also decease in spermatogenetic cell layers and number of spermatocytes in the lumen of seminiferous tubules, ultimately affecting the process of spermatogenesis. These

findings are in agreement with various reports submitted by Cui Long, Tian & Zhu, (2017); *Alhashem et al*., (2014); *Yan et al.,* (2015).[22,24-25]Our study shows that consumption of spinach (*Spinacia oleracea*) has the potential to improve the histopathological changes in testicular tissue in obese animal model by restoring the oxidative damage to testicular structure caused by over production of reactive oxygen species.

Thus in present study, we re-establish the fact that obesity provokes oxidative stress and these findings were in accordance with other studies done by *Cui et al*., 2017; Feillet-Coudray et al., 2009.[22,26] Similarly, these results are in accordance to *Mortazavi et al*., (2014) research outcomes that supported the fact that use of antioxidants has favorable effects on structure and functions of male reproductive system.[10]

Thus in current study, we re-establish the fact that obesity aggravates oxidative stress and these findings were in accordance with other studies done by *Cui et al*., 2017; *Feillet-Coudray et al*., 2009.[22, 26]However, the usage of spinach (*Spinacia oleracea*)has the ability to restores the functions of male reproductive system by normalizing the levels of intra-testicular testosterone. .

CONCLUSION

In brief, it is obvious from the outcomes of the present research that spinach (*Spinacia oleracea*) has potential to restore normal functions of reproductive system by normalizing intra-testicular testosterone levelsand by restoring the structure of testicular tissue in response to obesity-induced oxidative stress. Further, this research will help fertility specialists in counseling their patients and in adapting the appropriate treatment for infertility.

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