

Potential Protective Effect of Pomegranate (*Punica Granatum*) Juice on Monosodium Glutamate Induced Seminiferous Tubules Changes in Adult Male Albino Rats; Histological Study

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Monosodium glutamate (MSG) has been recognized as flavor enhancer that adversely affects male reproductive systems. The present study was designed to investigate the potential protective effects of pomegranate juice on MSG induced histopathological changes in the seminiferous tubules of rats. Fifty adult male albino rats were divided into five groups of ten rats each; Group I (Control group), received daily standard diet only for one month. Group II (Pomegranate group), received daily pomegranate juice only for one month. Group III (MSG group), received daily a single dose of 60 mg/kg body weight of MSG for one month. Group IV (MSG and Pomegranate group), received daily a single dose of 60 mg/kg of MSG concomitant with pomegranate juice for one month. Group V (MSG withdrawal group), received daily a single dose of 60 mg/kg body weight of MSG for one month then leaved for another one month. The testis was subjected to histological study, using light and electron microscopes, and the cauda epididymis was used for caudal sperm count. MSG induced toxicity in testicular tissues. Pomegranate juice resulted in improving the MSG induced changes, and it had the ability to increase sperms number and to reduce sperms abnormalities. Supplementation of pomegranate juice could ameliorate the MSG induced testicular toxicity. Thus, it could have a role in improving male fertility.

Keywords: Monosodium glutamate, Sperm, Testicular morphology, *Punica granatum*, pomegranate juice and seminiferous tubules.

Consumption of healthy and nutritious diet is important to enjoy a good quality of life. Food additives, natural or artificial, is a common practice in food industry worldwide to amplify the taste and flavor so making food edible, pleasurable and attractable¹. Monosodium glutamate (MSG), a glutamic acid in the form of sodium salt, is a food additive flavor that has characteristic

umami taste described by Ikeda 1908 and it is manufactured by bacterial fermentation of sugar cane, sugar beet or molasses². MSG is classified as “generally recognized as safe” without a specific acceptable daily intake (ADI) by several reputable international organizations such as the food and drug administration (FDA) in the United States, European Commission’s, food

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and agriculture organization (FAO)³. However, since the potential role of MSG in the “Chinese Restaurant Syndrome (CRS)” this recognized safety has been questioned by researchers. Moreover, ensured safety might encourage MSG overuse, intentionally or unintentionally, in human diet which needs to highlight its potential harmful effect and ensured it is used wisely⁴⁻⁶. Additionally, safety concerns, in the form of several adverse effects seen in animal studies investigating MSG effect on different organs, haven’t been seen in human clinical trials^{7,8}.

Several MSG adverse effects have been revealed in different *in vivo* animal studies and in clinical trials⁹. MSG is a source of glutamate which is an important neurotransmitter in central nervous system¹⁰ when taken in excess might result in neurological toxicity¹¹. An *in vitro* study revealed nerve cell death caused by higher MSG doses¹². Abnormal liver changes were observed in rats after higher doses of MSG for six-week¹³, also in mice following ten weeks treatments¹⁴. A two-month study on rats treated with MSG showed changes in testis seminiferous tubules and interstitial tissue¹⁵. Adult rats kidney showed some toxic effect following MSG supplementation¹⁶. Another study on rats showed that MSG result in changes in fertility parameters such as testicular tissue and sex related hormones after six weeks¹⁷. A one-month duration of low doses of MSG did not result in variations in sex hormones and antioxidant hormones in rodents¹⁸. In female rats, MSG supplementation has showed some degenerative changes affecting the oviduct¹⁹. While in male rats MSG resulted in an increased abnormal sperms and reduced its count²⁰. Another study on dwarf goats showed decrease sex hormones levels in blood following a 4-week of MSG treatment²¹.

In human, Jiangsu Nutrition Study (JIN) results showed elevated hemoglobin level in males with increased MSG in their diet²². Five days of high MSG doses intake were associated with headache, nausea and an increase in blood pressure parameters with no pain in mastication muscles²³. MSG side effects were more apparent when higher doses were administered for long periods in animal studies. In humans such side effects were less apparent due to mode of intake, and the differences in both models (human vs animal), thus, necessitating further research⁹. In the China Health

and Nutrition Survey (CHNS) a conclusion was made regarding a link between consumption of MSG containing food and the increased in weight²⁴. Orally taken MSG was associated with symptoms similar to Chinese restaurant syndrome includes, headache, muscle tightness, numbness/tingling, general weakness and flushing²⁵.

A number of products were used to moderate the oxidative effects of MSG on testis, for example, Propolis, a bee product, was investigated for its beneficial influence on male rat testis function and structure against MSG chemotoxicity by moderating the oxidative stress effect²⁶. The nutritive value of the pomegranate fruit was documented and that a 250 ml glass of pomegranate juice (PJ) provides approximately 50% of an adult’s recommended daily allowance of the vitamins A, C and E. Pomegranate contains a number of polyphenols, including anthocyanins, minor flavonoids and punicalagin, which is the most important member of the ellagitannins family. The punicalagin is the largest polyphenol among the pomegranate ellagitannins and it is responsible for most of the antioxidant activity of the PJ²⁷. The potent antioxidant effects of polyphenols have been demonstrated in clinical and experimental studies^{28,29}. Scientific research has highlighted the relationship between oxidative stress that result in reactive oxygen species (ROS) production and male infertility^{30,31}, pomegranate fruit is rich with antioxidant ingredients which could be recommended as protective constituent of a healthy diet against the adverse effect of stressful agents as MSG³²⁻³⁴.

This preclinical animal study was aimed to assess the potential protective effect of PJ against monosodium glutamate induced morphological alterations in adult male rats reproductive system using histology methods and semen analysis.

MATERIAL AND METHODS

Pomegranate Processing

Fresh pomegranates were washed, crushed, and squeezed. The juice was filtered, pasteurized, and stored at -18°C.

Chemicals

Monosodium glutamate crystalline powder (99% NT purity, Sigma chemical Company, ST Louis, MO, USA) was used to prepare a stock

solution by dissolving 60 g of MSG crystals in 100 ml of distilled water. A working dose of 60 mg/Kg (15) was administered by orogastric tube to adjust the proper dose and according to the dose schedule per group and as per their respective weight.

Study Design

This study was approved by the Scientific and Ethics Committee at Faculty of Medicine Mutah University. Fifty adult male Sprague-Dawley rats (150-200 gm) were housed in cages under standard

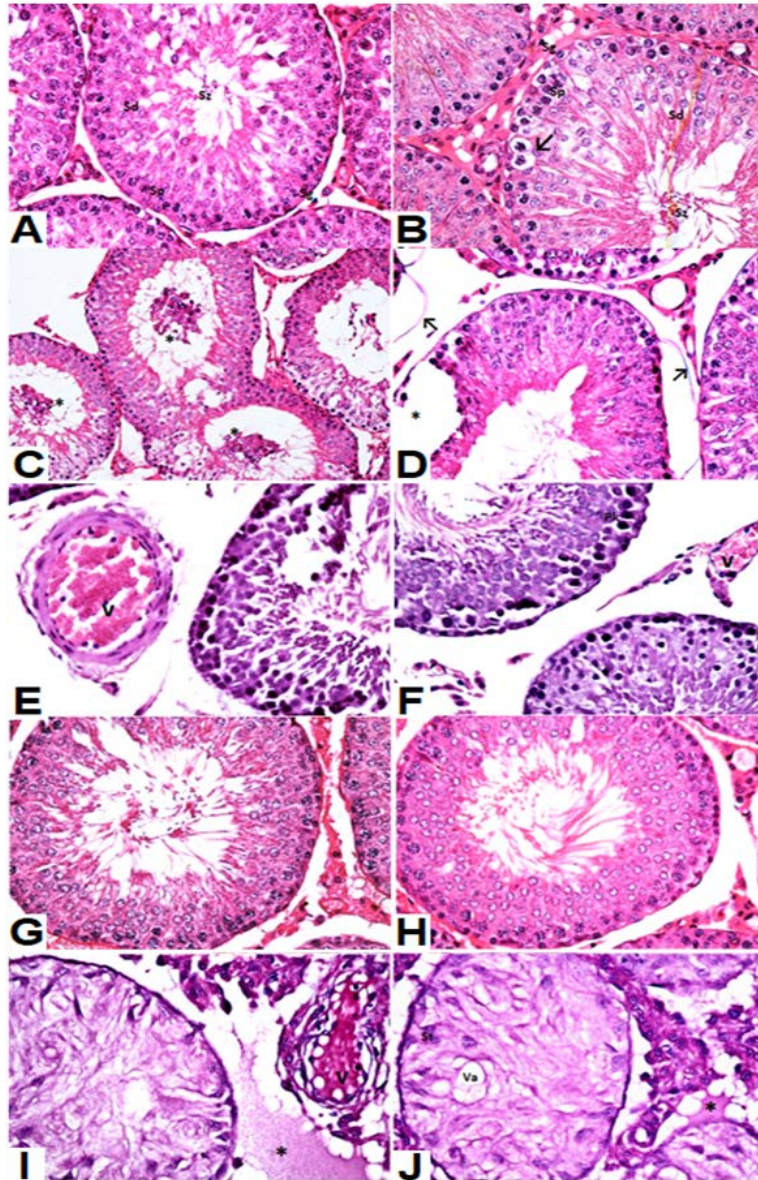


Fig. 1. Histological sections of rat testis. A: Normal rats with normal seminiferous tubules. B: Rats received PJ only show increased sperms number. C-F: MSG treated rats showing abnormal seminiferous tubules and degenerated spermatogenic cells. G-H: PJ concomitant with MSG rats showing, nearly normal seminiferous. I-J: Withdrawal MSG rats showing degeneration of spermatogenic cells. Sertoli cell (St), vacuolation (Va), hyaline material (*), blood vessels (V), splitting of the basal lamina (+), pyknotic nuclei of, loss of cells and its exfoliation in the lumen of the tubules (*), spermatogonia (Sg), primary spermatocytes (Sp) and spermatids (Sd) sperms (Sz) and mitotic figure (+). (H&E stain X400)

laboratory conditions with dark and light cycle. They were permitted free access to standard diet and water *ad libitum*. In the experiment the animals were randomly divided into 5 groups each has 10 rats: Group I (Normal control): received daily standard diet only for one-month, free access to water with no treatment throughout the study period. Group II (Positive control/Pomegranate juice): received daily pomegranate juice 2 ml / day via gastric gavage only for one month. Group III (MSG treated): received daily a single dose of 60 mg/kg body weight of MSG for one month based on the previously identified toxic dose *in vivo*. Group IV (MSG and Pomegranate treated / Protected group): received daily a single dose of 60 mg/kg of MSG concomitant with pomegranate juice 2 ml / day for one month. Group V (MSG Withdrawal

group): received daily a single dose of 60 mg/kg body weight of MSG for one month then leaved without any treatment for another one month.

All administrations were given orally by gavage. Body weight of each animal was determined at the start of the treatments and at time when sacrificed. The animals were sacrificed by decapitation after ether anesthesia. The cauda epididymis was chipped in 1 ml of normal physiological saline and the clear fluid was used for semen analysis using Neubauer's hemocytometer at a 1: 19 dilutions. Sperm count, motility, viability, and abnormality were assessed as described in WHO laboratory manual [35]. The testicles were dissected out from each rat, weighed then cut into two specimens and subjected to histological and semen analysis. Optical microscopy: specimen was

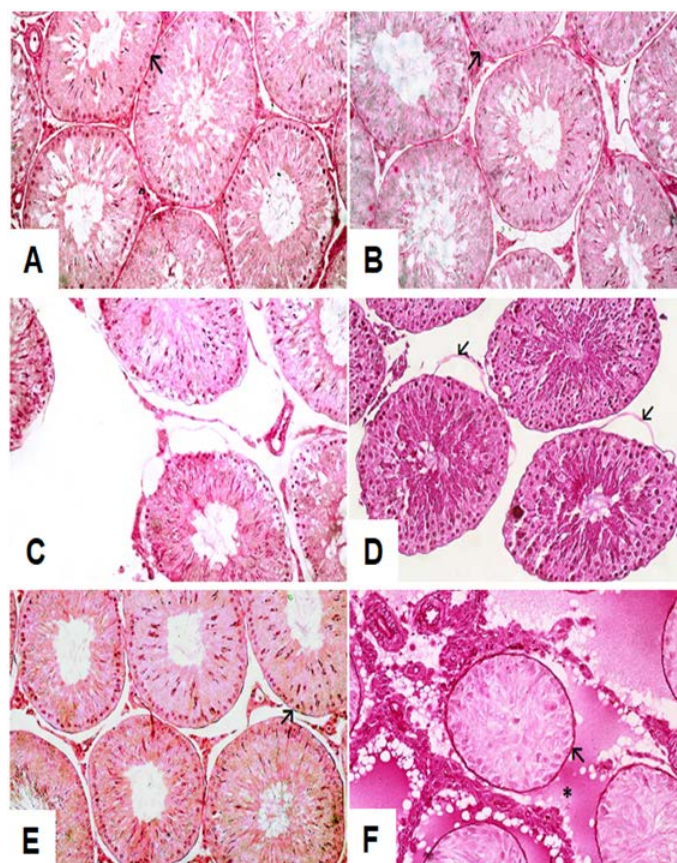


Fig. 2. Histological sections of rat testis. A: Normal rats with normal seminiferous tubules. B: Rats received PJ only showing normal thickness of the basal lamina. C-D: MSG treated rats showing, thin and splitting of the basal lamina (+) atrophied and widely separated seminiferous tubules E: PJ concomitant with MSG rats showing normal thickness of the basal lamina. F: MSG withdrawal rats showing thick and darkly stained basal lamina. Note vacuolated hyaline material between the seminiferous tubules (*). (PAS stain, X200)

fixed in Bouin's fixative solution and histologically processed to obtain 6µm thick paraffin sections. Tissue sections were dehydrated in an ascending grade of alcohol (ethanol), cleared in xylene ready for staining. Some sections were stained with Haematoxylin and Eosin (H&E) stain and Periodic acid Schiff (PAS) stain for light microscopic examination [36]. Other sections were labelled using activated Caspase 3 (apoptotic marker) for Immunohistochemical analysis. Transmission electron microscopy: specimen were immediately fixed in 3 % glutaraldehyde solution and each specimen was processed and examined using transmission electron microscope.

Statistical Analysis

Analyses were performed using IBM SPSS Statistics v.19. Biochemical parameters were compared by one way-ANOVA and multiple comparisons were done with Tukey's Post-hoc tests. $P < 0.05$ was accepted as statistically significant.

RESULTS

Optical microscopy

Haematoxylin and Eosin (H&E), and periodic acid Schiff (PAS) stained sections of testicular tissue in group I and II revealed normal architecture of seminiferous tubules lined with

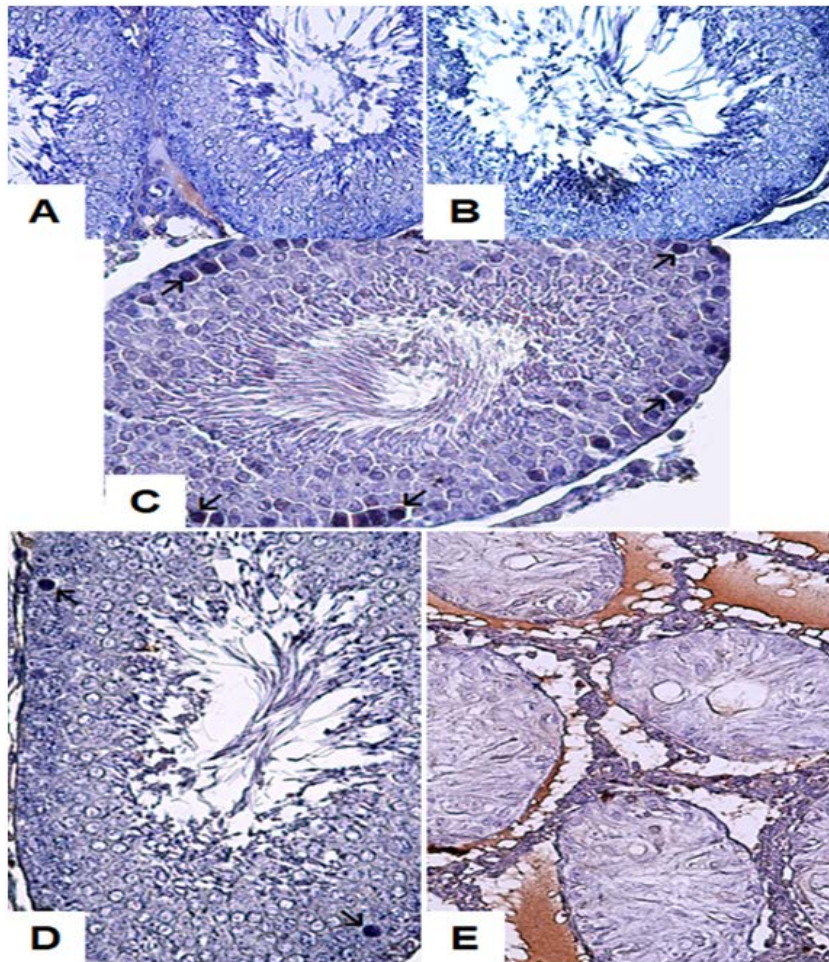


Fig. 3. Histological sections of rat testis. A: Normal rats testicular tissue. B: Rats received PJ only showing, normal immunohistochemical reaction of activated Caspase - 3 in the spermatogenic cells. C: MSG treated rats revealing, numerous dark pyknotic cells (apoptotic cells (+)). D: PJ concomitant with MSG rats showing few dark apoptotic cells (+). E: MSG withdrawal rats revealing negative reaction due to absent cells. (Activated Caspase, X400)

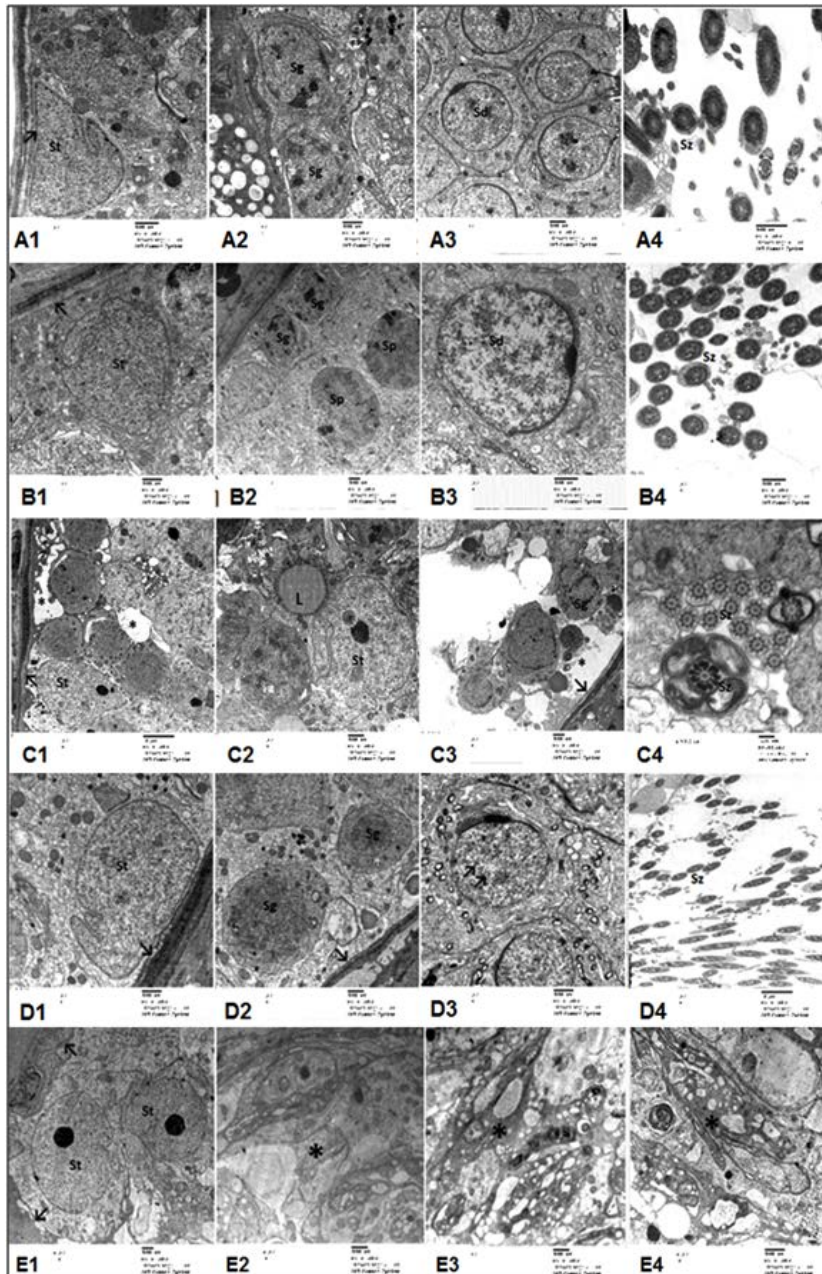


Fig. 4. Electron micrographs of rat testis. A: Normal rats showing seminiferous tubules surrounded by normal basal lamina (+) and lined with Sertoli cells (St) and normal spermatogenic cells in different stages of maturation, spermatogonia (Sg), spermatids (Sd) and sperms (Sz). B: Rats received PJ only showing similar to (normal rats in A) with numerous normal mature sperms (Sz). C: MSG treated rats showing seminiferous tubules surrounded by abnormal basal lamina (*) and lined with Sertoli cells (St) and degenerated spermatogenic cells spermatogonia (Sg) and few sperms (Sz) surrounded by abnormal mitochondrial sheath. D: PJ concomitant with MSG rats showing seminiferous tubules surrounded almost normal basal lamina (+) and lined with normal Sertoli cells (St). Spermatogonia (Sg), primary spermatocytes (Sp), round and elongated spermatids (++) and adequate number of normal sperms (Sz). E: MSG withdrawal RATS showing severely damaged seminiferous tubules surrounded by thick basal lamina (+) and lined with Sertoli cells only (St) and contain vacuolated material (Va) and remnant of degenerated spermatogenic cells (*)

spermatogenic cells and Sertoli cell that rest on a basal lamina (Figure 1 A&B). The lumen could be easily delineated in almost all tubules and the majority of them are occupied by the late spermatids that acquired darkly stained elongated heads and long tails. Sertoli cells were clearly identified with characteristic oval nuclei (Figure 2 A&B). Group II sections revealed evident increase in the number of sperms in the lumen of the tubules (Figure 1 B). Each tubule is surrounded by normal basal lamina forming the boundary tissue (Figure 2 B). Group III stained sections showed severe to moderate damaged of seminiferous tubules manifested as hyaline material and widening of the spaces between seminiferous tubules. Some seminiferous tubules manifested grade damage that included disorganization of spermatogenesis cells and edematous with congestion of interstitial tissues. Damaged germ cells which separated from the basal lamina and contains vacuoles within the spermatogonia with loss of late spermatids. Sloughing and exfoliation of spermatocytes, spermatids and immature germ cells appeared into the lumen of the seminiferous tubules. Moreover, the tubules were surrounded by thin irregular, destructed and a split basal lamina (Figure 1 C-F and Figure 2 C&D). Group IV revealed more or less normal architecture of seminiferous tubules except focal and mild vacuolation of spermatogenic cells and Sertoli cells. The tubules were surrounded by well-defined basal lamina with evident amelioration of degenerative changes (Figure 1 G&H and Figure 2 E). Group V revealed shrunken, complete destruction and degeneration of spermatogenic cells as evident by loss of germ cells, necrotic tissues were present in most of the seminiferous tubules and vacuolated

hyaline material involving interstitial tissue. Destruction of most spermatogenic cell layers with absence of spermatozoa was clearly recognized. The seminiferous tubules contain Sertoli cells only resting on thick and darkly stained basal lamina (Figure 1 I&J and Figure 2 F).

Immuno histochemical

Examination of immunohistochemical stained sections of the MSG treated rats (group III) the relative intensity of activated Caspase -3 expression was significantly higher in the testicular germ cells, primary spermatocytes and predominantly in the spermatogonia when compared with the control groups (Figure 3). In the protected group (group IV) sections revealed more or less normal reaction as compared with the control group (Figure 3 D). Atrophied tubules lined with Sertoli cell only, and with complete degeneration of spermatogenic cells were seen in the testes of withdrawal group (Figure 3 E).

Ultrastructural

Ultrastructural examination of the testes of the control rats (Group I) showed that germ cells had cellular characteristics typical of those seen in active spermatogenesis. Sertoli cell of the control rats showed, distinct nuclear and cytoplasmic characteristics consistent with an active secretory state. The nuclei were irregular with deep indentation. Adjacent to the nuclei, the cytoplasm was characterized by cisternae of RER in close association with mitochondria and ribosomes scattered throughout the cytoplasm. The cell membranes showed close association with adjacent spermatocytes and spermatids. The spermatogonia displayed round or oval configuration with prominent nuclei. The adluminal compartment of seminiferous tubules contained round spermatids

Table 1. Sperms structure and function scores in different studied groups

Sperm character	I- Control	Group II (PJ)	Group III (MSG)	Group IV (MSG&PJ Protected)	Group V MSG withdrawal
Sperm Count	533 ± 221.4	905 ± 90.1	5.6 ± 4.7*	43.2 ± 2.7*	0.51 ± 0.7*
Motile Sperms	10.8 ± 2.4	13.7 ± 2.4	1.4 ± 1.6*	2.0 ± 1.6*	1.1 ± 1.1*
Sperm Tail Defect	17.6 ± 3.0	20.5 ± 2.2	31.5 ± 23.1 *	12.9 ± 2.2	22.0 ± 23.4
Sperm Vitality	76.3 ± 4.3	71.4 ± 3.8	4.1 ± 3.0*	15.9 ± 2.7*	4.4 ± 4.9*
Normal Sperms	17.7 ± 2.3	23.0 ± 5.1	1.8 ± 1.6*	4.8 ± 1.3*	0.9 ± 1.1*

Values are means ± SD (n=10). *: significant at p < 0.001 compared to control. °: X10³

characterized by well-defined nuclei with distinct nuclear membranes. The lumen of seminiferous tubules revealed normal mature sperms as evident by normal heads and tails structure surrounded with mitochondrial sheath (Figure 4 A1-4). Group II revealed under TEM examination revealed almost similar structure as in control group, but the number of sperms was evidently increased (Figure 4 B1-4). Electromicrograph of MSG treated group (III) showed degenerative changes in seminiferous tubules. The cytoplasm of Sertoli cell showed evident cytoplasmic vacuolation and large lipid droplet. Spermatogonia and spermatocytes showed morphological changes typical of apoptosis as evident by cell shrinkage, excessive chromatin condensation, cytoplasmic vacuolation and organelles were relatively sparse. Abnormal spermatids were also common in monosodium glutamate treated rats as evident by abnormal shape nuclei with acrosomal defects, nuclear degeneration and sparse cytoplasmic organelles. The lumen of the tubules revealed few abnormal sperms surrounded by abnormal mitochondrial sheath (Figure 4 C1-4). MSG and PJ treated group (IV) electron images examination of seminiferous tubules showed a significant improvement as evident by normal Sertoli cells resting on almost normal basal lamina, normal spermatogonia and spermatocytes, normal round spermatids with intact acrosomal cap, and the lumen of the tubules illustrating normal and adequate sperms (Figure 4 D1-4). Withdrawal group ultrastructural examination of seminiferous tubules revealed atrophy of seminiferous tubules lined with Sertoli cell resting on thick irregular basal lamina and filled with vacuolated remnant of degenerated spermatogenic cells (Figure 4 E1-4).

Semen analysis

Seminal fluid assessment for sperm morphology, count and motility in different groups is an important step in the evaluation of testicular function and structure in male rats. Analysis showed variations in the sperm count in different groups compared to control group I. An average increase in sperm count was seen in PJ supplemented group (group II) while both MSG treated group (group III) and MSG-withdrawal group (group V) showed a marked significant decrease in sperm count. Supplementing MSG group with PJ (group IV) has improved sperm count albeit very low compared

to control group (group I). Statistical significant difference is present between all groups with p value < 0.001 . Multiple comparisons showed that Group IV shows statistical significant increase in sperm count compared with groups III and V, with p value < 0.001 (Table 1). Sperm motility results have shown that PJ supplemented group (group II) had increased significantly in sperm motility compared to control (group I), while all groups treated with MSG (group III, IV and V) showed significant decrease in sperm motility, and the difference was statistically significant with p value < 0.001 . Sperm tail defect count results showed variations among all groups with the highest increase was among the MSG treated group (III), and the lowest score was among the MSG group supplemented with PJ (group IV). However, there was no significant difference between all groups with (Table 1). Sperm vitality results showed significant variations among all groups with highest values among PJ supplemented rats (II) and the lowest was among MSG treated groups (III, IV, V). PJ supplemented MSG group (IV) showed better values compared with MSG model groups, and a significant difference was measured between all groups with value < 0.001 (Table 1). It can be seen that histological findings were supported by seminal fluid assessment in the form of abnormal shape and significant decreased in number of sperms in both MSG treated groups, p value < 0.001 (Table 1).

DISCUSSION

MSG has been reported to cause toxic effects on several organs functions such as ovary, testis, brain, pancreas, kidney and liver, however, these effects varies in different studies depending on the route of administration, dosage and animal model used³⁷. Effects include metabolic disturbances in different chemicals as serum insulin, fatty acids and triglycerides, transaminases and pathological changes in ovaries and fallopian tube and testis^{9, 13-18, 20, 21}. In human, MSG intake was associated with mild disturbances contrary to preclinical animal studies]38[. Histological findings in this study were comparable with other studies that were carried out on the testes of different animals treated with MSG. For example, administration of MSG for two months in rats

caused degeneration in testicular tissue¹⁵. MSG six weeks consumption produced changes in fertility parameters, such as testicular tissue and sex related hormones¹⁷. A one-month duration of low doses of MSG did not result in variations in sex hormones and antioxidant hormones in rodents¹⁸. Also, in male rats MSG resulted in increased abnormal sperms and reduced its count²⁰. Our results showed very low sperm count which could be explained in part by direct toxic effect of MSG and in part by a decrease testosterone production which lead into decrease spermatogenesis³⁹. MSG toxic effect over testicular tissue was suggested to be mediated by three pathways; neural pathway including hypothalamus and pituitary gland that lead to disturbance in sex hormone production⁴⁰ or via directly targeting corresponding receptors in testis⁴¹ or via the production of reactive oxygen species (ROS) as a result of low vitamin C which leading to injury to testicular tissue⁴².

It has been found that the principal cause of idiopathic male infertility is an underlying pathological condition known as oxidative stress³². ROS produced by sperms are necessary factors during the process of developing sperm ability to fertilize oocytes, however, imbalances in ROS metabolism will lead to increase in lipid peroxidation cause membrane damage and decreased energy production and subsequently leading to abnormal sperms structure and function^{43,44}. However, testicular tissue contains numerous intrinsic antioxidant enzymes such as peroxidases and dismutase's, additionally, antioxidant chemical factors such as vitamins C and E to protect its spermatogenic function from oxidative stress damage⁴⁵⁻⁴⁷. The current study demonstrated that monosodium glutamate induced a reduction in the concentrations of all spermatogenic cell types, including spermatogonia, spermatocytes and spermatids without any reduction in testicular tissue weight, which may indicate that ROS were the most important mechanism for this pathology⁴⁸. On the other hand, the daily consumption of PJ over a period of one month resulted in a significant increase in the spermatogenic cell concentration, which could be due to a reduction in the ROS parameters. PJ possesses strong antioxidant properties due to its high content of polyphenols particularly ellagitannins (EA) which can easily pass through the mitochondrial membrane⁴⁹⁻⁵².

Animal study revealed that the daily consumption of PJ a source of anti-oxidative stress over a period of 50 days caused a dropping in ROS parameters and an improvement in normal sperms parameters⁵³. Another *in vivo* study demonstrated that a daily consumption of PJ at various doses for six weeks period resulted in a significant increase in serum glutathione peroxidase (GSH-Px) and catalase activities in rats and a significant improvement in epididymal sperm function and structure parameters⁵⁴.

CONCLUSION

The current experimental investigations have shown that MSG are capable of producing degenerative changes in rats testicular tissue, as well as causing a decrease in number and vitality of testicular sperms, leading to infertility. However, daily consumption of PJ counteracts MSG degenerative changes, cause recovery of testicular tissue, and therefore improving male infertility.

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