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Effect of microwaves-induced plasma on some parameters of sperms, histopathological and sperm dna fragmentation in mice

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Using microwaves-induced plasma system of non-thermal atmospheric pressure operated with frequency 2.45 (GHz), microwave power of 800 W and used argon gas to generate the torch. The influence of microwaves-induced plasma (MIP) on male fertility by using the following tests (measurement hormone of testosterone level, sperm viability, motility and abnormalities), Sperm DNA fragmentation and on histo pathological changes of testes was studied in mice. Thirty-two adult male mice were divided into four groups one control group and three treated groups with microwaves plasma at times (20, 50, and 90 sec). The results showed significant increase ($P < 0.05$) in sperms motility and viability, decrease in the percentage of abnormalities and DNA fragmentation of sperms in mice treated with (MIP) compared with control group, while the results showed significant increase in diameters of seminiferous tubules and decrease of interstitial spaces in treated groups with (MIP) compared with control group.

Keywords: Microwave plasma, Sperms, DNA fragmentation, Histo pathological

INTRODUCTION

Plasma is commonly an ionized gas. It is combining of charge particles (electron, ion and molecules (Mazhir et al., 2016). The term ionized return to the existence of one or more free electron. Which are not required to an atom or molecules (Szabo et al., 2014; Mazhir et al., 2017). Microwave plasma is a type of plasma, that has high frequency electromagnetic radiation in the GHz range. Microwave generated plasma system are choose over other kinds of plasma sources because they are electrode less plasma, so the task of replacing or cleaning the electrodes and filaments is averted (Moisan and Zakrzewski 1991). Microwave discharges produce non-equilibrium plasma since the electrons can respond to the oscillations of the electric field whereas the ions are not able to respond due to

their large mass. So most of the microwave energy goes preferentially to the electrons, and then produce plasma far from thermodynamic equilibrium (Mazhir et al., 2018). Atmospheric microwave induced plasma (MIP) sources are inexpensive thanks to no need for vacuum system, strong to working for a huge time, and secure to be touched by living texture like the human body (Iza et al., 2008). The non-thermal MIP has been recognized as new tools in the biomedicine. MIP has proved their effectiveness to be used in bio-medical applications such as sperm parameters and histo pathological changes of testis in mice. in this study (Fridman et al., 2008). showed that these microwaves plasma increased; motility, viability, and count of sperms with normal morphology. Studies in animals have shown altered spermatogenesis, cytotoxicity and

degeneration of spermatocytes, Sertoli cells, and Leydig cells (Sussman et al., 198). The effects of microwaves plasma also documented in reproductive system. It induces cellular alteration in male gonads (testes) in human as well as in animals (Badri et al., 2000). But there is little documentation in literature regarding mode of action and mechanism of cell death on testes during proliferative stage of reproductive system after long term continuous exposure of microwaves plasma on an animal model. The effects of microwaves plasma also studied on mice skin when they were exposure to plasma for different times (Alyaa et al., 2017). The influence of microwave plasma on the Liver and DNA for mice cells were explained by using the comet score application and texture analysis image processing (Alyaa et al., 2018).

The aim of this work is to study the effects of microwaves plasma on some parameters of sperms, sperm DNA fragmentation and some histo pathological changes on mice tests.

MATERIALS AND METHODS

Experimental part

Albino mature male mice (30-35 gm) were bought from Biotechnology Research Center -Al-Nahrain University and kept on a 10:14-hours dark light cycle in the animal house treated and control mice were presented with food and water *ad libitum*. 32 mice were used in this study. The animals were placed in small plastic cages that were cleaned weekly with tap water and soap and sterilized with 70% ethyl alcohol the over the study period. Room temperature was kept at (24±2) °C.

The microwave plasma generated from this system were applied on mice which shows in figure (1) and worked with 2.45 GHz microwaves frequency, 175 Volt applied voltage, 2 L/min argon gas flow rate and 10 Mm discharge tube diameter .microwave plasma is exposed to mice on the back area after hair removal from this area. One week after arrival, males were randomly divided into four groups, containing 8 mice in each group, based on the design of the study:

Group1: Control group which were in experimental conditions, but the mice were not exposed to microwaves plasma.

Group 2: This group was exposed to microwaves plasma for twenty seconds.

Group 3: This group was exposed to microwaves plasma for fifty seconds.

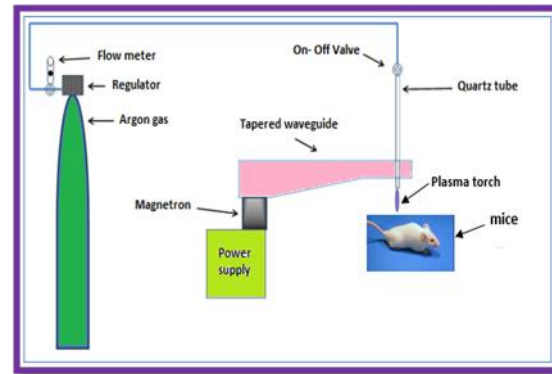


Figure (1) a schematic diagram of the MIPJ system applied on the mice.

Group 4: This group was exposed to microwaves plasma for ninety seconds.

After exposed to microwaves plasma for group (2, 3, 4). The animals were scarified in all groups by dislocation of cervical Vertebrae.

Samples collection

Blood samples were collected from mice by heart puncture and the serum was separated by centrifuge at 3000 rpm for 15 minutes. Serum was assayed either immediately or stored at -20 °C. After scarified the testes and epididymis were collected and placed in a sterile disposable container with (10%) formalin as fixative solution for (18-24) hours for histological study.

Microscopically examination

Spermatozoa were assessed by light microscopy (Olympus Co., Tokyo, Japan) according to WHO Laboratory manual WHO (1992) for viability, percentage dead/live spermatozoa , Motility and abnormalities.

Sperm Parameters

Sperm motility

The epididymis were separated and spermatozoa were taken from the two tails of epididymis, and was placed in a petri dish containing 1 ml PBS and minced into small pieces by sharp blade and forceps before being incubated at 37°C for 30 min and Take drops of solution by pipette and place them on the glass slides and covered by a cover slide to examination sperm motility.

The motility was calculated according to (Bearden et al., 1992) by using the following formula:

$$\text{Motility \%} = \frac{\text{No. of motile sperms}}{\text{Total No. of sperms}} \times 100$$

Sperm viability percent

The percentages of live sperms were measured as following according to the method reported by (Bearden et al., 1992). A drop of the sperm suspension was placed on the slide, and then a drop of eosin-nigrosine stain was added and mixed, the mixture was spread using a clean slide and left to dry. 100 sperms were counted, observation the spermatozoa which red stained (alive spermatozoa) can be distinguished from those unstained spermatozoa (dead spermatozoa), the percentage of live sperms was calculated using the following equations:

$$\text{Viability \%} = \frac{\text{Number of Living sperm}}{\text{Total Number of sperm}} \times 100$$

Abnormal sperm morphology percent

The abnormal sperms shapes were different from the normal sperm shapes and showed malformed head or tail or both. To calculate the abnormality of sperm by using the same slide which was used for sperm viability, according to (Bearden et al., 1992) formula?

$$\text{Abnormality \%} = \frac{\text{No. of abnormal sperm}}{\text{Total No. of sperm}} \times 100$$

Acridine Orange Test (AOT)

Acridine orange is a metachromatic fluorescence probe for demonstration of degree of sperm nuclear DNA susceptibility to in-situ acid-induced denaturation by distinction between native double-stranded DNA (green fluorescent) and denatured single-stranded DNA (red fluorescent). Briefly, the air-dried smears were fixed in Carnoy's solution (methanol/ glacial acetic acid, 3:1) at 4°C for at least 2 hrs. Each sample was stained by freshly prepared AO (0.19 mg/ml in McIlvaine phosphate-citrate buffer (pH=4) for 10 min (Al-Ahmed 2016). Smears were assessed on the same day using fluorescent microscope (Zeiss Co., Jena, Germany) with a 460-nm filter.

Testosterone assay

For determination of Testosterone hormone levels by using a ELISA kits (mono bind) and

reader of absorbance by ELISA reader (Human, Germany).

Histological study

The histological examinations were performed after the reproductive organs were isolated and cleaned from the surrounding adipose tissues. Then the organs were kept in formalin 10% for 24 hrs, transferred to 70% ethanol until they were used for histological preparation. And processed for routine paraffin embedding. The testes were cut into 5-µm sections. Three serial sections per testes were mounted on slides, deparaffinized, rehydrated, and stained with hematoxylin - eosin stain

Sections of the testes were examined by light microscope, seminiferous tubules and interstitial spaces were assayed in each tests using a calibrated micrometer with stage micrometer and Ocular lens were used 40X for examination. The mean value of each was calculated. The interstitial space observed between two consecutive seminiferous tubules by using the ocular micrometer (Taha et al., 1999).

Statistical analysis

All data were analyzed by using one-way analysis of variance (ANOVA- test) with significant differences ($P < 0.05$) and the data were presented as means $\pm$ SD.

RESULTS

Sperms parameters

Effects of microwaves plasma on Sperms Parameters are showed in table (1). The motility and viability were significantly ($P < 0.05$) increased in treated groups to microwave plasma at time (20, 50 and 90 sec) which shown in figure (2A, 3A and 4B). While the abnormalities was significantly decreased in treated groups to microwave plasma at time (20, 50 and 90 sec) which shown in figure (2, 3B and 4A). Table (1) shows three different sperms parameters such as life, abnormality and motility. The result were measured at control environment (before plasma treatment), the mice were exposed to microwave plasma at 20, 50 and 90 sec. The mean value was measured with its standard deviation. The motility of sperm at control was 63 ± 2.739 .

Table (1). Percentage of sperms motility, life sperms and sperms abnormalities in treated and control groups.

Groups	Motility of sperms (%) (mean $\pm$ SD)	Life of sperms (%) (mean $\pm$ SD)	Abnormalities of sperms (%) (mean $\pm$ SD)
Control	A 63 $\pm$ 2.739	A 67.779 $\pm$ 3.811	A 30.797 $\pm$ 2.85
20 sec	B 80 $\pm$ 3.536	B 70.568 $\pm$ 2.907	A 29.282 $\pm$ 2.734
50 sec	C 87 $\pm$ 2.739	C 78.170 $\pm$ 2.997	B 22.789 $\pm$ 2.667
90 sec	D 93 $\pm$ 2.739	D 80.846 $\pm$ 2.059	C 19.773 $\pm$ 1.857
LSD	3.769	1.453	1.236

Differences A, B,C and D are significant ($P < 0.05$) to compression row

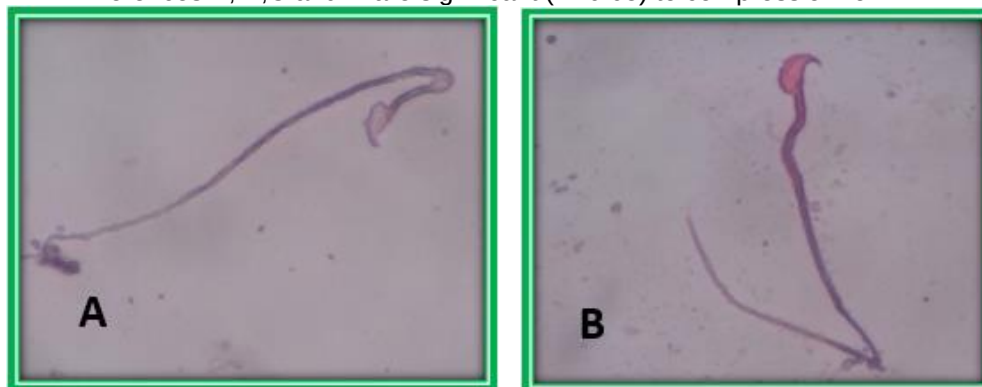


Figure 2. Showed A) live and abnormal sperm B) died and abnormal bent sperm treated with control group (E X40)

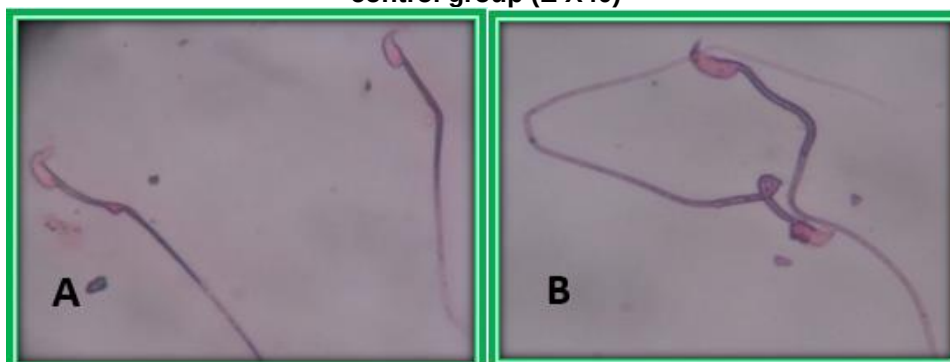


Figure 3. Shows A) live and normal sperms treated group with microwaves plasma to 90 sec, B) died and abnormal sperms (treated group with microwaves plasma to 20 sec (E X40)



Figure 4. Shows, A) died and abnormal double head sperms, B) died and normal sperms (treated group with microwaves plasma to 50 sec (E X40)

When exposed to microwave plasma, sperm motility increased to 80 ± 3.536 at 20 sec and at 50sec the value was 87 ± 2.739 , at 90 sec the motility reached to 93 ± 2.739 . All motility results at control, 20, 50 and 90sec were significant ($P < 0.05$). Sperm life has value of 67.779 ± 3.811 at control and increased to 70.568 ± 2.907 for 20 sec microwave plasma exposure. The sperm life values increased at 50 and 90 sec, the life value of sperm was 78.170 ± 2.997 and 80.846 ± 2.059 respectively. The sperm life mean results were all significant ($P < 0.05$). The abnormality for sperm starts at 30.797 ± 2.85 at control and this value decreases to 29.282 ± 2.734 at 20 sec exposure time to microwave plasma. At 50 sec of treatment the abnormality decrease to 22.789 ± 2.667 and at 90sec the abnormality also decreases to 19.773 ± 1.857 . The result of control and 20 sec was not significant. The other results of the abnormality was significant ($P < 0.05$).

Effects of microwaves plasma on DNA fragmentation and Testosterone level

Effects of microwaves plasma on DNA fragmentation and Testosterone level showed in table (2). The DNA fragmentation was significantly ($P < 0.05$) decreased in treated groups to microwave plasma at time

Table (2). Data of DNA fragmentation and Testosterone level in treated and control groups.

Groups	DNA fragmentation (mean $\pm$ SD)	Testo. (ng/ml) (mean $\pm$ SD)
Control	A 22.889 ± 2.613	A 6.667 ± 2.646
20 sec	B 18.489 ± 3.188	A 6.490 ± 1.155
50 sec	C 13.512 ± 2.351	A 7.059 ± 1.649
90 sec	D 9.757 ± 1.375	A 6.863 ± 1.063
LSD	1.347	2.225

Differences A, B,C and D are significant ($P < 0.05$) to compression row

(20,50 and 90 sec) which shown in figure(5B,C, 6B and 7B), while the Testosterone level has no significant in all groups. Table (2) shows the results of Test in ng/ml and DNA fragmentation of the sperms. The results were measured at control environment (before plasma treatment), when the mice exposed to microwave plasma at time 20,50 and 90 sec. The mean value was measured with its standard deviation. The DNA fragmentation of sperm at control was 22.889 ± 2.613 . When exposed to microwave plasma, the DNA fragmentation decreases to 18.489 ± 3.188 at 20 sec, and at 50sec the value was 13.512 ± 2.351 . At 90 sec DNA fragmentation reached to 9.757 ± 1.375 . All DNA fragmentation results at control, 20, 50 and 90 were significant ($P < 0.05$). Testo has value of 6.667 ± 2.646 at control and remains almost constant with value of 6.490 ± 1.155 for 20 sec microwave plasma exposure. Testo values at 50 and 90 sec were 7.059 ± 1.649 and 6.863 ± 1.063 respectively. All Testo mean results were no of significance Which tells that the microwave plasma has no effect on the test value. The results are shown in figure(8) with its bar error.

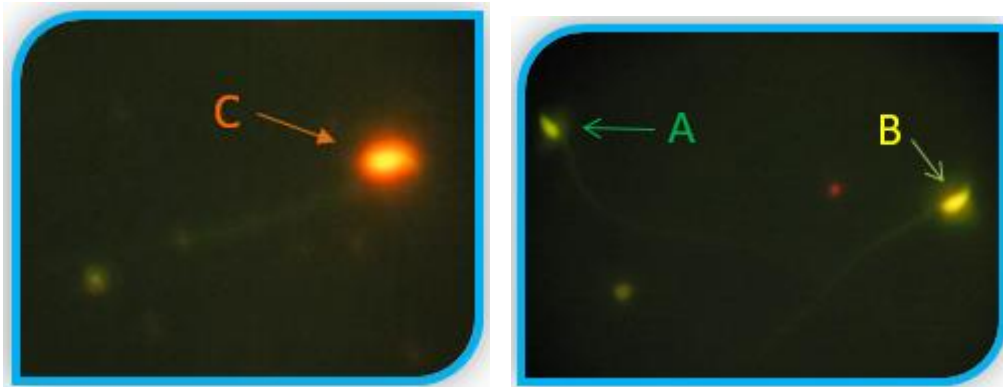


Figure 5. Shows (A) the sperm without fragmented (green), (B) the sperm fragmented (yellow), (C) the sperm fragmented (orange) treated with control group (E X40)



Figure 6. Shows (1-A the sperms without fragmented (green) treated group with microwaves plasma to 20 sec, (2-A the sperm without fragmented (green), (B) the sperm fragmented (yellow) treated group with microwaves plasma to 50 sec (E X40).

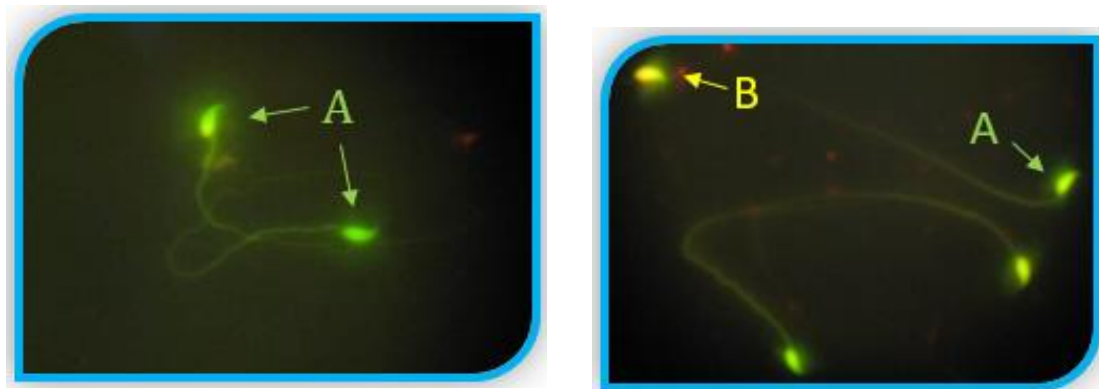


Figure 7. Shows(A) the sperms without fragmented (green), (B) the sperm fragmented (yellow) treated group with microwaves plasma to 90 sec (E X40).

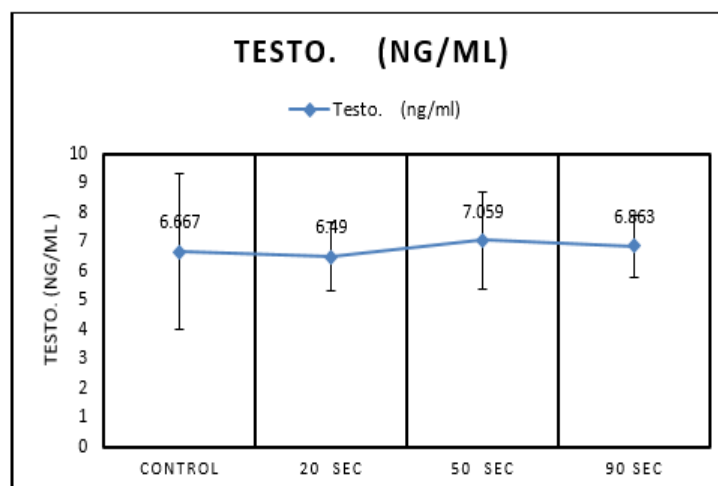


Figure 8. Shows the testosterone Harmon level in treated and control groups
Histological examination of the testes.

Table (3). Data of The diameters of seminiferous tubules and interstitial space in treated and control groups

Groups	Diameter of seminiferous tubules (μm) (mean $\pm$ SD)	Interstitial space (μm) (mean $\pm$ SD)
Control	A 443.28 $\pm$ 31.57	A 125.20 $\pm$ 18.91
20 sec	B 571.50 $\pm$ 42.52	B 74.98 $\pm$ 7.96
50 sec	C 689.60 $\pm$ 57.21	C 31.54 $\pm$ 2.42
90 sec	C 737.90 $\pm$ 60.90	C 24.60 $\pm$ 1.23
LSD	54.613	11.422

Differences A, B and C are significant ($P<0.05$) to compression row

The diameters of seminiferous tubules and Interstitial space

Effects of microwaves plasma on The diameters of seminiferous tubules and interstitial space are showed in table (3). The diameters of seminiferous tubules significantly ($P<0.05$) increased in treated groups to microwave plasma at time (20, 50 and 90 sec) which shown in figure (4.25, 4.26 and 4.27), while the Interstitial space was significantly ($P<0.05$) decreased in treated groups to microwave plasma at time (20,50 and 90 sec) which shown in figure(9, 10 and11). Table(3) Show the diameters of seminiferous tubules and Interstitial space. The result were measured at control environment (before plasma treatment) and when the mice exposed to microwave plasma at time 20,50 and 90 sec. the mean value was measured with its standard deviation.

The diameter of seminiferous tubules at control was 443.28 $\pm$ 31.57. When exposed to microwave plasma the diameters of seminiferous tubules increase to 571.50 $\pm$ 42.52 at 20 sec and at 50sec the value was 689.60 $\pm$ 57.21, at 90 sec the diameters of seminiferous tubules reach to737.90 $\pm$ 60.90. The result of 50 and 90 sec was no significant. The other result of the diameters of seminiferous tubules was significant ($P<0.05$).

The Interstitial space start at 125.20 $\pm$ 18.91 at control and this value decrease to 74.98 $\pm$ 7.96 at 20 sec exposure time to microwave plasma. At 50 sec of treatment the Interstitial space decrease to 31.54 $\pm$ 2.42 and at 90sec the interstitial space also decreases to 24.60 $\pm$ 1.23. The result of 50 and 90 sec was no significant. The other result of the Interstitial space was significant ($P<0.05$).

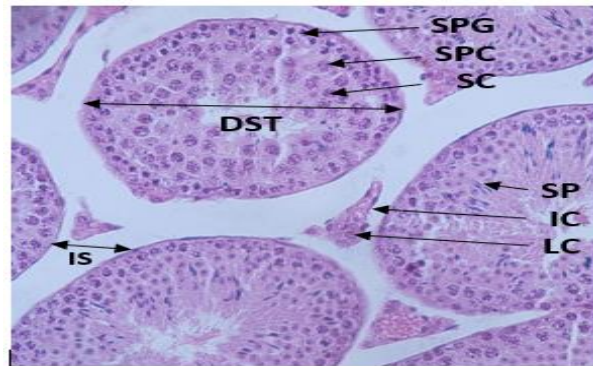


Figure 9. A cross section of testes in mice (control group), (SPG) spermatogonea (SPC) spermatocytes (SC) Sertoli cells (SP) Spermatids (IC) interstitial cell (LC) Leydig cells. Showing decrease in diameter of seminiferous tubules (D.S.T.) , and increase in interstitial space(I.S.), (H& E) stain, 40X.

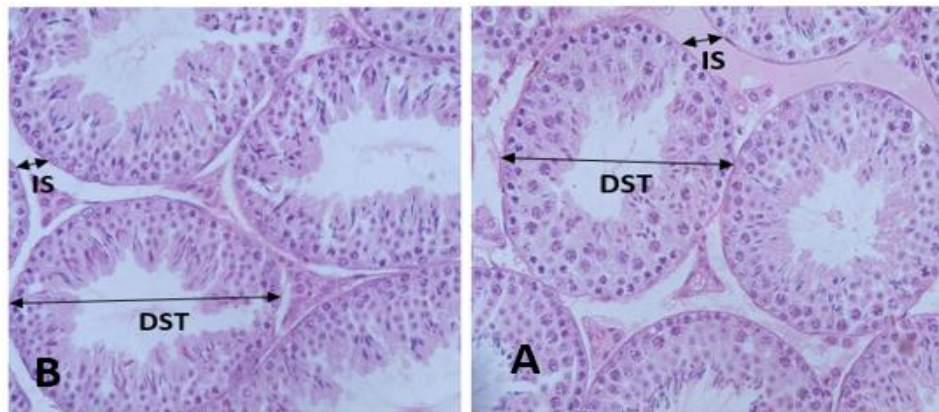


Figure 10. A cross section of testes in mice (treated group with microwaves plasma to (A) 20 sec and (B) 50 sec) (H& E) stain, 40X.

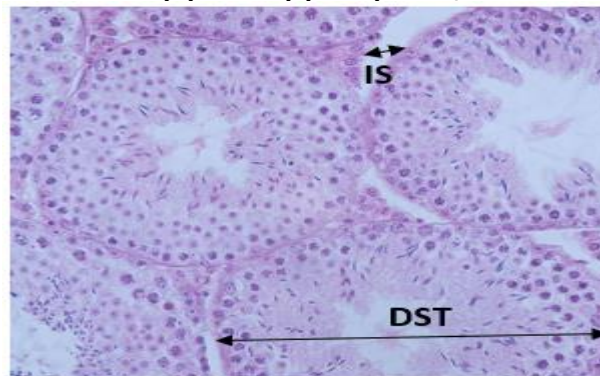


Figure 11. A cross section of testes in mice (treated group with microwaves plasma to 90 sec), showing increase in diameter of seminiferous tubules (D.S.T.) , and decrease in interstitial space(I.S.), (H& E) stain, 40X.

DISCUSSION

The results showed percentage of sperm positive and significantly increase in motility and live while

significantly decrease in abnormalities in treated groups compared with control group, as well the effect of microwaves plasma increases the energy in the mitochondria. As well as the temperature of

the electron that works to increase the movement of sperm. The results of DNA fragmentation by acridine orange test and of motility provide evidence that microwaves plasma exposure time results in less DNA fragmentation and improvement of motility. In studies which are carried on animals they displayed that microwaves plasma exposure time leads to sperms parameters improvement, Dobrin et al., (2015) showed that ram sperm with microwaves plasma leads to an increase of the viability, motility and functional integrity of the sperm membrane. Similar results were found on turkey semen in which microwaves plasma resulted in an increase in the parameters quality (Iaffaldano et al., 2005; Hu et al., 2007). (Ocana- Quero et al., 1997) established that microwaves plasma lead to a decrease mortality of spermatozoa and an increase in acrosome reaction after microwaves plasma exposure time. Corral - Baqués et al., (2005) reported that the microwaves plasma of dog sperm leads to improvement the progressive motility and velocity of the dog sperm after exposure time. (Breitbart et al., 1996) and Cohen et al., (1998) found the same results in mouse sperm microwaves plasma (Cohen et al., 1998), the intracellular movement of Ca^{2+} controls acrosome reaction, sperm capacitation and motility (Darszon et al., 2007). Another mechanism through which the microwaves plasma exposure time exerts its effect through stimulating the light-sensitive cytochrome c oxidase complex in the mitochondria and this leads to increase in ATP production (Koppers et al., 2008). From these results it was concluded that using the microwaves plasma as a method of may exert beneficial effects in the improvement of sperm motility and preservation of DNA integrity of the sperm. The results showed the effect of microwaves plasma on the diameters of seminiferous tubules and Interstitial space, the results were positive and significantly increase in the diameters of seminiferous tubules and significantly decrease in Interstitial space, In addition to the temperature of the electron that is working on the increase, and this study better than kim et al., (2007), which use microwaves radiation the results were decrease in diameters of seminiferous tubules. These results indicate that a time exposure of microwave plasma exerts a positive impact on male reproductive system function and fertility, also results indicate that the 90 sec microwave plasma exposure are more effective as compared to control, 20 and 50 sec.

CONCLUSION

The effect microwaves plasma on sperm parameters caused increase in motility and viability with time exposure to reach maximum value at 90 second, while decrease in abnormalities with time exposure. The sperm DNA fragmentation decreased in treated groups to microwave plasma at time (20, 50 and 90 sec) compared with control group. While the microwave plasma has no effect on the testosterone Harmon level. The results showed significant increase in diameters of seminiferous tubules and decrease of interstitial spaces in treated groups with (MIPJ) compared with control group.

CONFLICT OF INTEREST

The authors declared that present study was performed in absence of any conflict of interest.

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AUTHOR CONTRIBUTIONS

SNM designed the plasma system. SNM and NAA and AHA worked on the Physical part. HIA and NAA worked on biological part. All authors reviewed and approved the final version.

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