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EFFECT OF OZONE THERAPY ON SPERMATOGENESIS IN EXPERIMENTALLY GENTAMYCIN-INDUCED TESTICULAR LESION IN ADULT MALE ALBINO RAT

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ABSTRACT

Background: Spermatogenesis is a complex process through which spermatogonial germ cells undergo mitosis, meiosis and cell differentiation to generate mature spermatozoa. Gentamycin (GM) is an aminoglycoside antibiotic that has negative effect on spermatogenesis. **Objective:** Evaluation of the possible effect of medical ozone on spermatogenesis in experimentally gentamycin-induced testicular lesion in male albino rat. **Materials and Methods:** Forty adult male albino rats of a local strain were chosen as a model for the present study. They were divided into equal four groups: Group I (Control group), Group II (Gentamycin-treated group), GroupIII (Ozone and gentamycin-treated group), and Group IV (Gentamycin followed by ozone-treated group). At the end of the experimental period, blood samples were obtained for determination of malondilaldehyde (MDA) and catalase (CAT). Testicular samples were obtained for transmission electronmicorsocpic study. **Results:** Gentamycin induced oxidative stress and reduced spermatogenesis. Medical ozone produced partial improvement in redox homeostasis and spermatogenesis when used in combination or after gentamycin. **Conclusion:** Medical ozone can be used to attenuate harmful effects of GM on testicular functions. Further studies can be done to evaluate the effect of different doses and different durations of medical ozone on different systems of the body.

INTRODUCTION

Spermatogenesis is a very complex, highly orchestrated and tightly regulated process by which spermatogonia developed into mature spermatozoa ***(Lee et al., 2009; Olayemi, 2010 and Surech et al., 2015).*** It can be affected by many factors and diseases such as diabetes mellitus ***(Jangir and Jain, 2014)***, endocrinal disorders ***(Schlatt and Ehmcke, 2014)***, environmental factors ***(Liu et al., 2007 and Liu, 2010),*** senility ***(Beattie et al., 2015)***, and drugs ***(Khaki, 2015)***.

Gentamycin is commonly used in management of septicemia and other infections caused by gram negative bacteria ***(Zahedi et al., 2012)***. It has negative effects on spermatogenesis in human and rat (**Khaki, 2009**).

The genotoxic effect of gentamycin may be caused by oxidative stress which is induced by the drug or its metabolites ***(Kim et al., 2014 and Khaki, 2015)***.

Medical ozone is a pharmaceutical compound which consists of a mixture of gases containing not less than 5% of ozone (O_3) and not more than 95% of pure oxygen (O_2). It has the ability to pro tect the body against pathological conditions caused by oxidative stress ***(Sagia & Bocci, 2011, Helal et al., 2013 and Bocci & Valacchi 2015)***.

So, this work was designed to evaluate the effect of medical ozone on spermato-genesis in experimentally gentamycin-induced testicular lesion in adult male albino rat.

MATERIALS AND METHODS

This work was carried out on 40 adult male albino rats of a local strain weighing 110-130 g (average weight was 120). Rats were purchased from Nile Pharmaceutical Company. They were kept in cages (30x25x30 per 4 rats) at normal temperature with natural dark and light cycle. They were maintained on balanced diet with free water supply. They were kept 2 weeks for adaptation to the new environment before starting of the experiment in Taymor unit for medical ozone in Physiology Department, AL-Azhar Faculty of Medicine. They were divided into four equal groups:

- **Group I (Control Group) received** 1 ml distilled water intra-peritoneally (IP).
- **Group II** received gentamycin (5mg/kg) IP for ten days.
- **Group III** received medical ozone (5ml of 25ug/mL) by rectal insufflation in combination with gentamycin (5 mg/kg) IP for 10 days.
- **Group IV** received gentamycin (5mg/kg) IP for ten days, and then received medical ozone (5ml of 25ug/mL) by rectal insufflations for two weeks.

Ozone concentration was measured using an ultraviolet spectrophotometer at 254 nm. Rats received 1 mg/kg of medical ozone with concentration of 25µg/ml slowly by rectal insufflation after induction of defecation ***(Al-Gendy, 2010).***

Induction of testicular lesion was done by IP injection of gentamycin (5 mg/kg) for ten days ***(Ghosh and Dosgupta, 1999).***

Biochemical assay: At the end of the experimental period, blood samples were withdrawn from the retro-orbital plexus into test tubes. Serum was separated and stored frozen at-20^{o}C until assayed for serum malondialdehyde (MDA) ***(Erdelmeier, 1997)*** and catalase (CAT) ***(Zamocky and Koller, 1999)***.

Transmission electron micro-scopic study: At the end of experiment, and immediately after withdrawal of blood samples, rats were anesthetized and testes were obtained. Ultrasections were obtained for examination by transmission electron microscopy (Jeol-Jem-2010) at the Regional center for Mycology and Biotechnology (RCMB), Al-Azhar University.

Approval of ethical committee was obtained.

Statistical analysis: Data input and analysis were done using SPSS computer program. All results were expressed as Mean ± Standard error. Mean value of the different groups were compared using a one-way analysis of variance (ANOVA). Least significant difference (LSD) posthoc analysis was used to identify significantly different mean values. P value < 0.05 was accepted to denote a significant difference.

RESULTS

Biochemical changes:

The data of present work revealed that IP injection of GM in group II led to significant increase in MDA level from 11.2 ± 1.4 in control group (group I) to 30.07 ± 4.4 (+ 168.4%), and significant

decrease in CAT level from 30.8 ±1.8 in control group to 24.9 ± 2.2 (-19.15%) (Table 1).

Concomitant use of medical ozone with GM in group III led to significant decrease in MDA 22±3.8 (-12.35) and insignificant decrease in CAT level 22.6 ± 3.4 (-9.23%) when compared with the group that received GM alone (group II) (Table 2).

Also, use of medical ozone after treatment by GM in group IV led to significant decrease of MDA 21.6 ± 3.3 (-24.7), and insignificant increase in CAT level 25.6 ±2.8 (+ 2.8) when compared with GM treated group (Table 2).

There were insignificant decrease in MDA level and significant increase in CAT level in group IV when compared with group III (Table 3).

These results when compared with control group indicated that medical ozone did not reverse the toxic effect of GM completely (Table 1).

Table (1): Changes in MDA and CAT in studied groups in comparison to group I **(Mean ± S.E.)**.

Groups / **Parameters**	**Group I**	**Group II**	**Group III**	**Group** IV
MDA (µmol/L)	11.2 ± 1.4	30.07*±4.4	22 ±3.8*	21.6 ± 3.3*
% changes		+ 168.4%	+ 49%	+ 92.8
CAT (U/mL)	30.8 ±1.8	24.9 ±2.2*	22.6 ± 3.4*	25.6 ± 2.8*
% change		- 19.15%	- 26.62%	-16.88%

Group I : Control
Group II : Gentamycin treated
Group III : Ozone in combination with gentamycin
Group IV : Gentamycin followedby ozone
***Significant**

Table (2): Changes in MDA and CAT in studied groups in comparison to group II (**Mean ± S.E.).**

Groups / **Parameters**	**Group II**	**Group III**	**Group** IV
MDA (µmol/L)	30.07 ± 4.4	22 ±3.8*	21.6 ± 3.3*
% changes		-26.8%	-28.16%
CAT (U/mL)	24.9 ± 2.2.	22.6 ± 3.4	25.6 ± 2.8
% change		-9.235	+2.81

Group II : Gentamycin treated
Group III : Ozone in combination with gentamycin
Group IV : Gentamycin followedby ozone.
*** Significant.**

Table (3): Changes in MDA and CAT in group IV in comparison to group III **(Mean ± S.E.).**

Groups / Parameters	Group III	Group IV
MDA (µmol/L)	22 ±3.8	21.6 ± 3.3
% changes		-1.81%
CAT (U/mL)	22.6 ± 3.4	25.6 ± 2.8*
% change		+13.27%

Group III : Ozone in combination with gentamycin
Group IV : Gentamycin followed by ozone
*Significant

Transmission electron microscopic examination

Group I (Control group**)** revealed normal picture of seminiferous tubules. There was regular intact basal lamina with normal myoid cells. Sertoli cells were found with large intended nuclei, (arrow), abundant mitochondria and smooth endoplasmic reticulum in its cytoplasm, Spermatogonia type A with large pale oval nuclei and spernatogonia type B were noticed (fig.1&2).

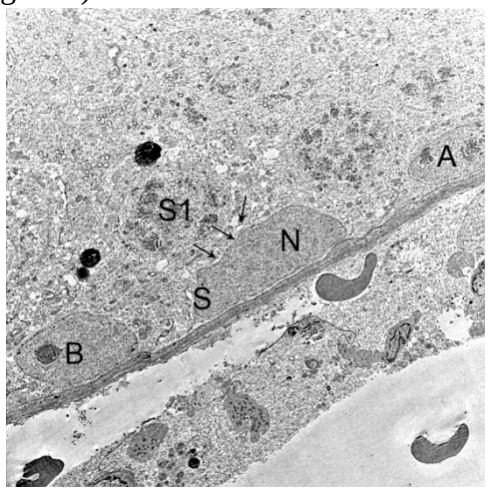

Figure (1): Control group(X 3000)
A: Spermatogonium type A
B: Spermatogonium type B
N: Nucleus
S: Sertoli cell
S1: Primary spermatocyte

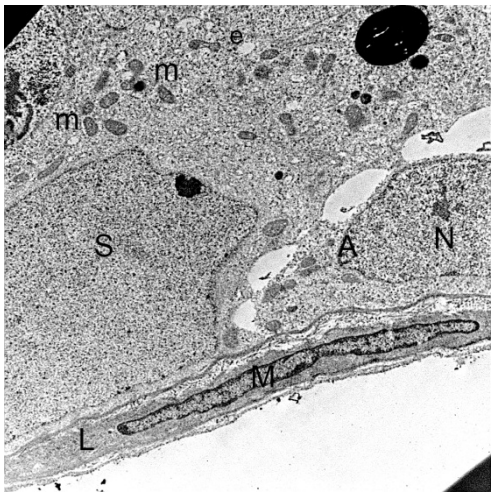

Figure (2): Control group(X 8000)
A: Spermatogonium type A
e: Smooth endoplasmic reticulum
L: Basal Lamina
M: Myoid cell
m: Mitochondria
N: Nucleus
S: Sertoli cell

There were early rounded spermatids with spherical nuclei which showed different stages of acrosome formation; Golgi apparatuses, proacrosomal granules, acrosomal vesicles and acrosomal cap. Mitochondrial aggregation was noticed at the periphery of plasma membrane of spermatids (fig.3).

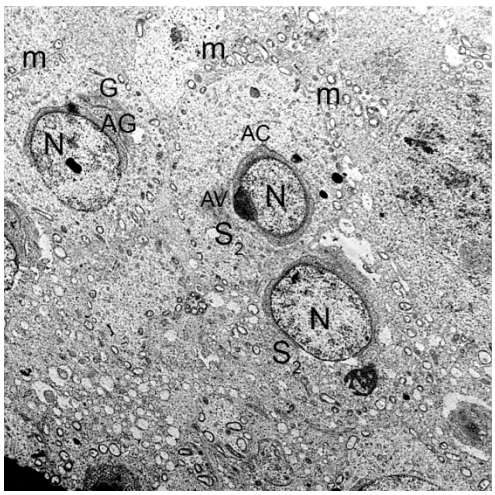

Figure (3): Control group(X 5000)
AC: Acrosomal cap
AG: Acrosomal granule
AV: Acrosomal vesicle
G: Golgi apparatus
m: Mitochondria
N: Nucleus
S2: Rounded spermatid

Other view showed longitudinal section of elongated spermatid closely related to sertoli cell. It has elongated nucleus with dark condensed nucleoplasm and covered by acrosome.The manchette of this spermatid originated at the base of acrosome. Completely formed sperm was noticed in the same view (fig.4).

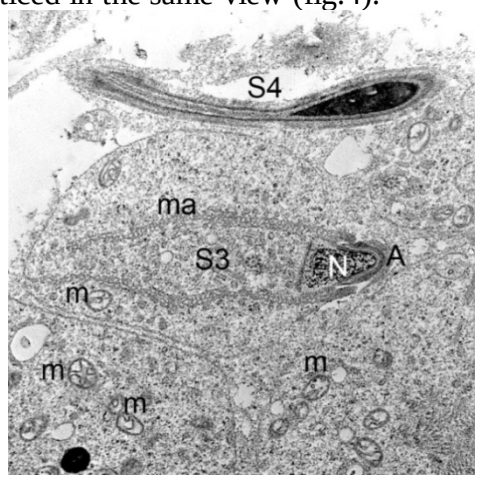

Figure (4): Control group(X 15000)
A: Acrosome
m: Mitochondria
ma: Manchette
N: Elongated nucleus
S3: Elongated spermatid
S4: Spermatozoa

Group II (Gentamycin treated group**)** showed marked alteration in the normal structure of the seminiferous tubules. There was thickened fibrotic, irregular basal lamina with myoid cell. There were degenerated spermatogonia. Primary spermatocytes showed vacuolated mitochondria and stack of rough endoplasmic reticulum in there cytoplasm (fig.5).

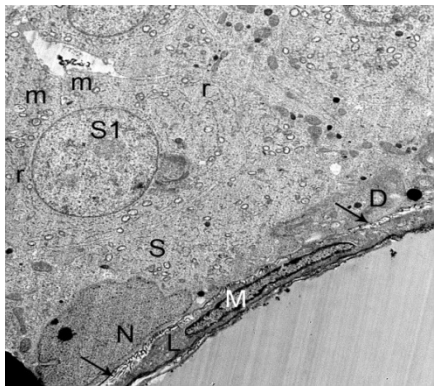

Figure (5): Group II(X5000)
Black arrow: Fibrotic basal lamina
D: Degenerated spermatogonium
L: Basal Lamina
M: Myoid cell
m: Mitochondria
N: Nucleus
r: Rough endoplasmic reticulum
S: Sertoli cell
S1: Primary spermatocyte

Sertoli cells had large indented nuclei with focal rupture of their nuclear membrane (thick arrow). The cytoplasm of them has abnormal forms of mitochondriae and large vacuoles (fig. 6).

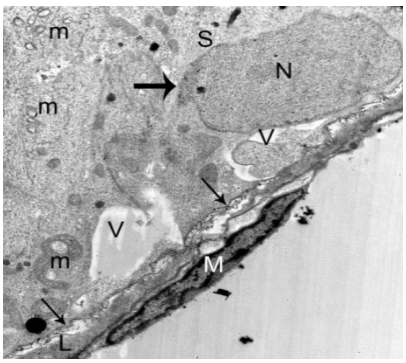

Figure (6): Group II(X8000)
Black arrow: Fibrotic basal lamina
L: Basal Lamina
M: Myoid cell
m: Mitochondria
N: Nucleus
S: Sertoli cell
Thick arrow: Focal rupture of nuclear membrane
V: Vacuoles

There were rounded spermatids without any evidences of acrosome formation. Degenerated spermatids were noticed in the same view (fig 7).

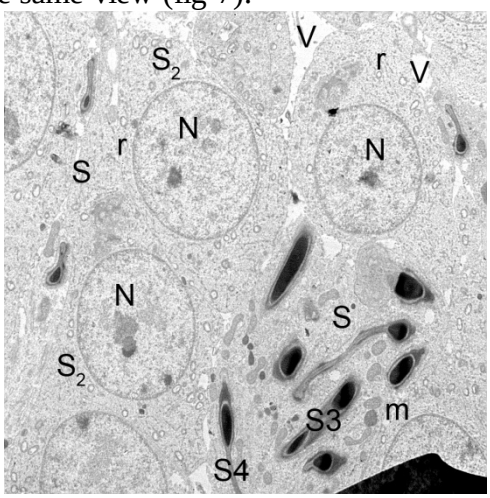

Figure (7): Group II(X5000)
S: Sertoli cell
S2: Rounded spermatid
S3: Elongated spermatid
S4: Spermatozoa
m: Mitochodria
N: Nucleus
r: Rough endoplasmic reticulum
V: Vacuoles

Group III

(Ozone in combination with gentamycin treated group**)** revealed thickened regular fibrotic basal lamina. Spermatogonia type A has large pale oval nucleus with peripheral clumps of heterochramatin (arrow), and focal rupture of their nuclear membranes (thick arrows). The cytoplasm of these cells contained abnormal forms of mitochondria and extensive vacuolation (fig.8).

Figure (8): Group III(X 6000)
A: Spermatogonium type A
Black arrow: peripheral clumps of heterochramatin
L: basal lamina
m: Mitochondria
N: Nucleus
S1: Primary spermatocyte
Thick arrow: Focal rupture of nuclear membrane
V: Vacuoles

There were early rounded spermatid with spherical nuclei, and different stages of acrosome formation; Golgi apparatus, proacrosomal granule and acrosomal cap. Aggregation of mitochondria at the periphery of the plasma membrane of this spermatid was noticed (fig.9).

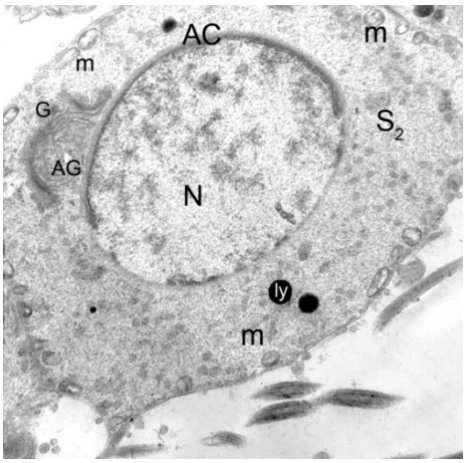

Figure (9): Group III(X 10000)
AC: Acrosomal cap
AG: Acrosomal granule
G: Golgi apparatus
ly: Lysosoma
m: Mitochondria
N: Nucleus
S2: Rounded spermatid

Cytoplasm of degenerated sertoli cell contained abnormal forms of elongated spermatid and degenerated-mitochondria (fig.10).

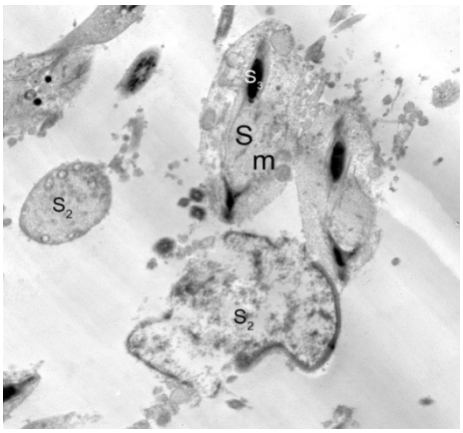

Figure (10): Group III(X 6000)
m: Mitochondria
S: Sertoli cell
S2: Rounded spermatid
S3: Elongated spermatid

Group IV (Gentamycin followedby ozone-treated group**)** showed thick, fibrotic, irregular basal lamina. Sertoli cell has small intended nucleus, abnormal forms of mitochondriae, and extensive vacuolation in there cytoplasm (fig.11).

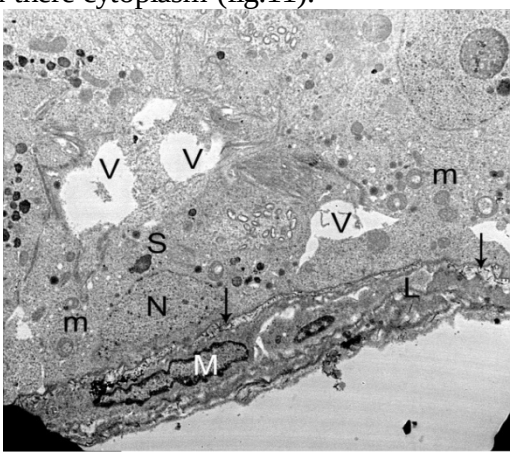

Figure (11): Group IV(X 5000)
L: Basal Lamina
Black arrow: Fibrotic basal lamina
M: Myoid cell
m: Mitochondria
N: Nucleus
S: Sertoli cell
V: Vacuoles

Both spermatogonia A and B have irregular nuclei with peripheral clumps of heterochromatin (arrow), and extensive vacuolation in there cytoplasm (fig.12).

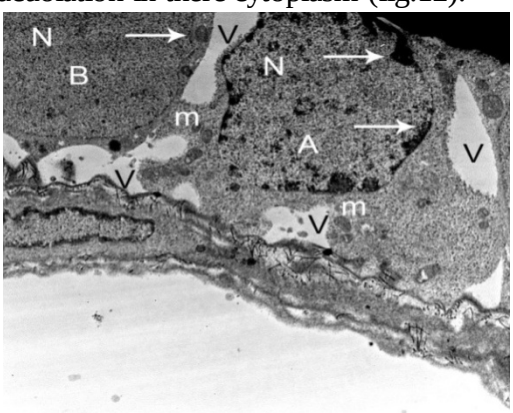

Figure (12): Group IV(X8000)
A: Spermatogonium type A
B: Spermatogonium type B
m: Mitochondria
White arrows: Peripheral margination of heterochromatin
V: Vacuoles

There was rounded spermatid with different stages of acrosome formation (fig.13).

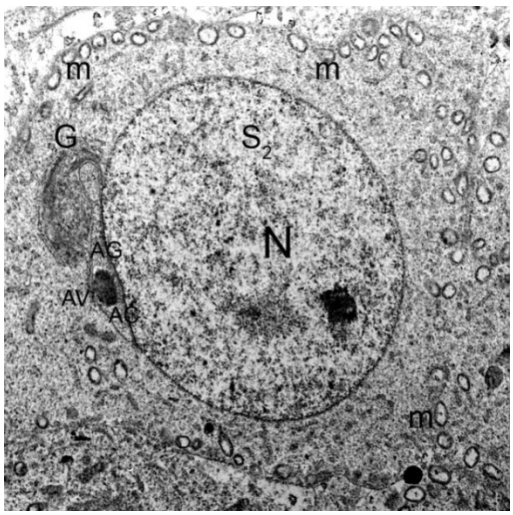

Figure (13): Group IV(X 10000)
AC: Acrosomal cap
AG: Acrosomal granule
AV: Acrosomal vesicle
G: Golgi apparatus
N: Nucleus
S2: Rounded spermatid

DISCUSSION

Gentamycin is an aminoglycoside antibiotic that commonly used in management of septicemia and other infections caused by gram negative bacteria ***(Zahedi et al., 2012)***. It has a good result, but its use is often limited due to its complications which include ototoxcity, nephro-toxicity and genotoxicity ***(Narayana et al., 2008)***.

So, many researches were designed to organize strategies to diminish side effects of antibiotic while preserving their therapeutic efficacy ***(Nouri et al., 2009; Gul et al., 2012; Lee et al., 2012 and Kim et al., 2014).*** One of these strategies is ozone therapy ***(Saleh et al., 2104 and Bocci & Valacchi, 2015).***

The present study was designed to investigate the possible effect of medical ozone on spermatogenesis in GM-induced testicular lesion by evaluating status of redox homeostasis and histopathological changes of seminiferous tubules. MDA is one of thiobarbituric acid reactive substances (TBARs) that indicates oxygen free radical dependent lipid peroxidation, while CAT is one of the most important antioxidant enzymes that keeps H_2O_2 at optimal level by converting its excess to H_2O (**Srivastava, 2015**). Both of them were measured to evaluate oxidative status in this work.

The results of this study showed that IP administration of GM induced oxidative stress by increasing MDA and decreasing CAT resulting in structural and cytotoxic changes in testis.

In comparison with GM- treated group, the results of this work showed that concomitant treatment with medical ozone attenuated the oxidative stress caused by GM as evident by significant decrease in MDA in this group, the percentage change was -12.35%. However, more reduction of MDA was obtained with medical ozone after GM treatment, the percentage change was -24.70%. These different degrees of medical ozone' effect were reported by **Alpcan et al. (2014)** who suggested that ozone therapy plays its role depending on the pathological status of tissues. It was found to be protective against oxidative stress under pathological conditions, while it obviously seemed to cause some harmful effects when used in healthy condition.

These results were consistent with ***Chang et al. (2011)*** who proved that GM can produce large amounts of reactive oxygen species (ROS) and reactive nitrogen species (RNS) in vivo and vitro. Such compounds enhance oxidative degradation of polyunsaturated fatty acids

and formation of large amount of MDA ***(Goel et al., 2005).***

Safwat et al. (2014) and ***Tolga et al. (2014)*** reported that ozone therapy acts as an effective oxidative stress regulator, mainly by stimulating the antioxidant system of the cells and scavenging ROS as well as lipid oxidation products.

Cakir (2014) and **Elkotb & Galal (2014)** demonstrated that medical ozone was able to reduce prooxidant substance including MDA.

Medical ozone may inhibit the growth of gram -ve bacteria which are the main source of endotoxins. Reduction of such substances prevents further formation of prooxidant substances including MDA ***(Roa et al., 2004 and Azarpzhooh & Limeback, 2008).***

As regard to CAT, there was significant decrease in its level in GM-treated group in comparison with control group and insignificant changes in groups treated by medical ozone when compared with GM group. However, there was significant increase in CAT level in group IV (Gentamycin followed by ozone-treated group) when compared with groupIII (Ozone in combination with gentamycin treated group).These different degrees of medical ozone, effect on CAT level could be explained by the finding of **Alpcan et al. (2014)** as discussed before.

Low level of CAT in this study confirmed the results of **Ghosh et al. (2002)** who reported that GM inhibits tissue expression of antioxidants enzymes including CAT. Also, MDA can react with most cellular macromolecules, inactivating enzymes or denaturing proteins causing DNA damage with mutation of different gens including those expressing CAT (**Srivastava, 2015**).

The cytotoxic changes of seminiferous tubules in GM-treated group denoted severe form of necrosis. There was degeneration of sertoli cells with focal rupture of their nuclear membrane, degeneration of germinal epithelial cells, especially spermatogonia, with abnormal forms of mitochondria. Different types of cells inhabiting seminiferous tubules showed large vacuoles in their cytoplasm that indicated degeneration of rough endoplasmic reticulum. In all the studied groups, spermatogonia were mostly resting on the basal lamina except in group II in which spermatogonia were either degenerated or replaced by large vacuole.

The basal lamina was thick and fibrotic as a result of cooperation between Sertoli and Myoid cells. This thickness is trying to protect germ cells against toxic effect of GM **(Murphy and Richburg, 2015)**.

However, electromicroscopic studies of rats treated by GM and medical ozone at the same time, as well as rats treated with ozone after induction of testicular atrophy by GM revealed that medical ozone attenuated mainly the pathological effects of GM on distal groups of cells in somniferous tubules (spermatids).

Oxidative stress, elevated MDA and the alteration of membrane properties can explain testicular architecture abnormalities caused by GM. MDA molecule can bind with phospholipids in the cell membrane leading to its destruction ***(Narayana, 2008).*** Also, it has the ability to oxidize the cellular macromolecules, i.e. proteins as well as

DNA and lipids in different testicular cells. Such modification can provide a strong pro-apoptotic signals leading to necrosis of different germ and sertoli cells (**Kim et al., 2014**).

The spermatozoa are more liable to oxidative damage because there cell membrane contain large amount of phospholipids ***(Zahedi et al., 2012).***

Similar results were reported by ***Nouri et al. (2009)*** who suggested that oxidative stress plays an important role in sperm dysfunction and testicular damage by GM.

The cytotoxic changes of seminiferous tubules in our work could be attributed to the direct effects of GM which included germ cell DNA damage, defective acrosomal formation, defective protein synthesis and somatic cell damage in the testis **(Naryana, 2008 and Kim et al., 2014).**

The failure of germ cells to regenerate may be caused by low level of testosterone that occurred as a result of toxic effect of GM on leydig cell **(Ramsawamy & Weibauer, 2015 and Khattab, 2016)**.

Medical ozone did not reverse the toxic effects of GM on sertoli cells because the ability of these cells to proliferate after puberty is low ***(Hayrabedyan et al., 2012).*** Also, the early stages of germ cells in seminiferous tubules were not responding to ozone therapy because it is severely damaged by GM toxicity ***(Khaki et al., 2015).*** The previous data explained why improvement effect of medical ozone in this work was mainly on spermatids.

The beneficial effect of medical ozone could be explained by the finding of **Aydos**and his colleagues (**2014).** They found that medical ozone was able to reduce some apoptotic markers such as tumor necrosis factor receptor1 (TNFR1), caspace 3 and caspace 8. **Khattab (2016)** also found that medical ozone was able to increase testosterone secretion from leydig cell that affected by GM toxicity. This hormone is essential for all stages of spermatogenesis **(SEAL, 2013)**.

Medical ozone has a good solubility in both lipid and aqueous environment. So can readily cross blood testicular barrier and protect spermatids by improvement and reestablishment of redox homeostasis that disturbed by GM ***(Smith et al., 2015).***

Medical zone has the ability to improve microcirculations and enhance erythrocytes metabolism **(Sagia and Bocci, 2011).** By this way, it can promote the oxygen carrier capacity of hemoglobin for spermatid and increased its ability to resist GM toxicity. Medical ozone can energize spermatids by stimulating Krebs' cycle and enhancing the carboxylation of pyruvate. In addition, it can stimulate the proliferation of mitochondria and production of ATP ***(Di Filippo et al., 2015).*** This was approved by electron microscopic examination that showed different stages of acrosome formation and numerous mitochondria in spermatids.

CONCLUSION

Medical ozone has a protective effect against the disaster of GM on some testicular cells (spermatid). This can direct the attention of medical ozone in alleviating the side effects of GM and help its use in treatment.

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دراسة تأثير العلاج بالأوزون الطبي علي تكوين الحيوانات المنوية في ذكور الفئران البيضاء البالغة المصابة بتلف الخصية المحدث تجريبيا

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خلفيـة البحـث: تكـوين الحيوانـات المنويـه عمليـة تتـأثر بعوامـل عـدة ومنهـا العقـاقير . ويعـد إسـتخدام عقـار الجنتاميسين أمراً شائعاً لدي الأطباء ولكن إستخدامه بجرعات كبيرة ولفترات طويلة يؤدي إلي الكثير من المضاعفات ومنها تلف بالخصية عن طريق زيادة الشوارد الحرة. ويعتبر الأوزون الطبي من طرق العلاج الغير تقليدية التي تحدث تحسنا ملحوظا في كثير من الامراض التي حدثت بسبب زيادة هذه الشوارد.

الهدف من البحث: دراسة تأثير العلاج بالأوزون الطبي علي تكوين الحيوانات المنوية في ذكور الفئران البيضاء البالغة المصابة بتلف الخصية .

موارد وطرق البحث: أجري هذا البحث علي اربعين من ذكورالفئران البيضاء البالغة من سلالة محلية كنماذج للدراسة. وقد قسمت هذه الفئران الي أربعة مجموعات متساوية كالأتي:

المجموعة الاولي:أعطيت الماء المقطر فقط عن طريق الحقن البريتوني.

المجموعة الثانية: أعطيت عقار الجنتاميسين فقط عن طريق الحقن البريتوني بجرعة 5مجم/كجم، لمدة عشرة ايام لاحداث تلف بالخصية.

المجموعة الثالثة: أعطيت عقار الجنتاميسين عن طريق الحقن البريتوني بجرعة 5مجم/كجم، وفي نفس الوقت تم أعطاؤها غاز الأوزون الطبي عن طريق الشرج لمدة عشرة أيام.

المجموعة الرابعة: أعطيت عقار الجنتاميسين عن طريق الحقن البريتوني بجرعة 5مجم/كجم لمدة عشرة أيام ثم أعطاؤها غاز الاوزون الطبي عن طريق الشرج لمدة اسبوعين.

وفي نهاية التجربة تم أخذ عينات من الدم لقياس كل من مالونيل داي ألدهيد والكاتاليز، كما تم أخذ عينات من الخصية لفحصها باستخدام الميكروسكوب الإلكتروني النافذ.

النتائج :أظهرت التحاليل الإحصائية إرتفاعا ذا دلالة إحصائية في نسبة المالونيل داي الهيد وانخفاضا ذا دلالة إحصائية في نسبة الكاتاليز في كل المجموعات مقارنة بالمجموعة الأولي وعند مقارنة هذه النتائج بالمجوعة الثانية وجد إنخفاضا ذا دلالة إحصائية في نسبة المالونيل داي الهيد وتغيرات ليست ذات دلالة إحصائية في نسبة الكاتاليز.

كما أوضح الفحص باستخدام الميكروسكوب الإلكتروني النافذ ضموراً واضحاً في خصي فئران كل المجموعات مقارنة بالمجموعة الأولي كما أظهر الفحص تحسنا ملحوظا في خصي فئران المجموعات التي تم معالجتها بالأوزون الطبي مقارنة بالمجموعة الثانية.

الاستنتاج:أحدث العلاج بالأوزون الطبي تحسنا ملحوظاً في بعض خلايا الحيوانات المنوية في الخصية المتلفة تجريبيا في ذكور الفئران البيضاء نتيجة قدرة الأوزون الطبي علي تقليل الشوارد الحرة.