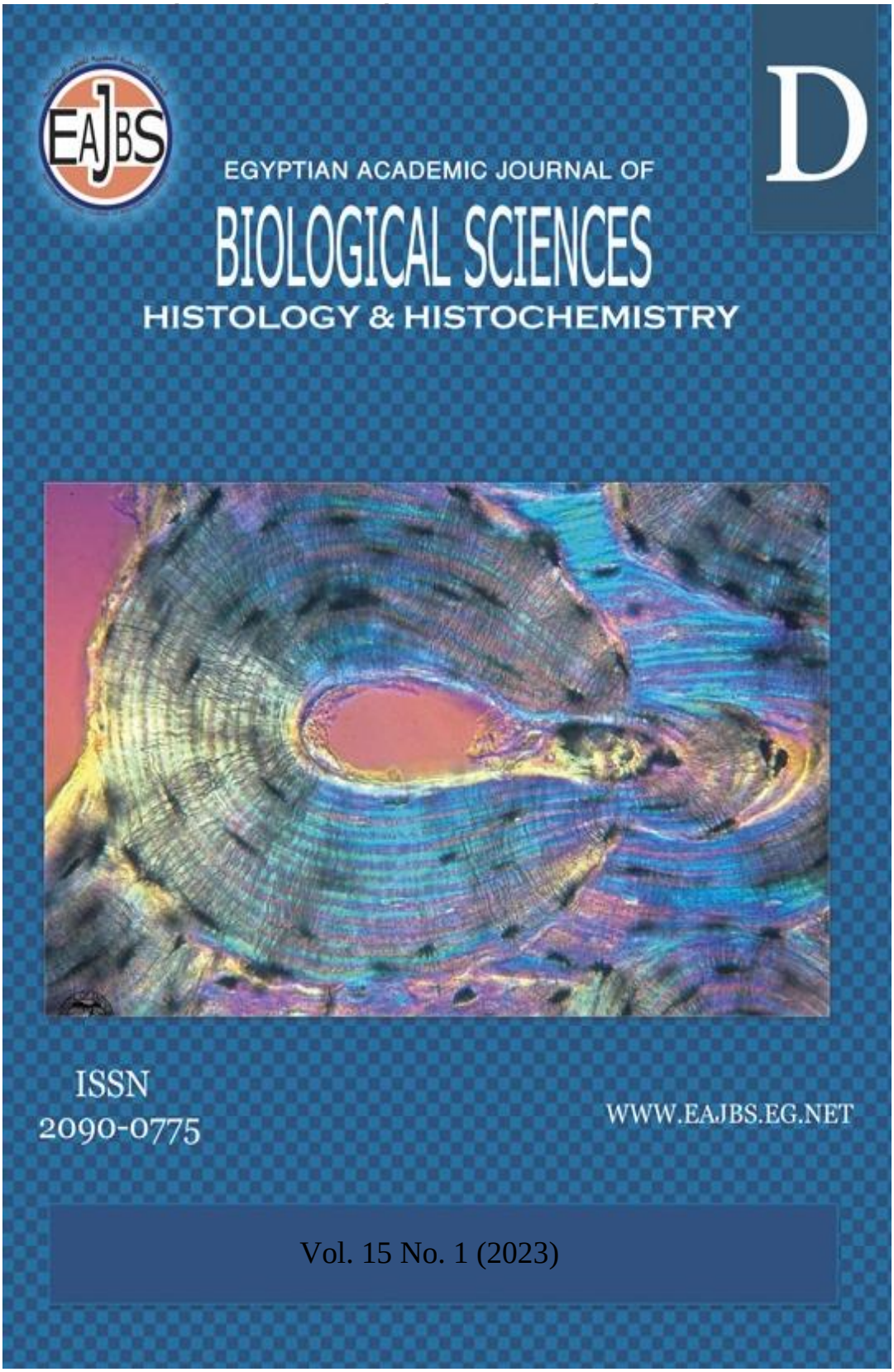
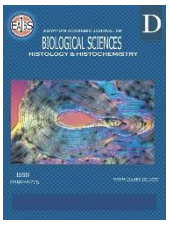


Citation: Egypt. *Acad. J. Biolog. Sci. (D-Histology and histochemistry) Vol.15(1) pp79-99(2023)*
DOI: 10.21608/EAJBSD.2023.286097

Egypt. Acad. J. Biolog. Sci., 15(1): 79-99 (2023)

Egyptian Academic Journal of Biological Sciences
D. Histology & Histochemistry
ISSN 2090 – 0775
http://eajbsd.journals.ekb.eg



Reproductive Toxicity of Sodium Nitrite and Its Modulation by Ascorbic Acid as An Antioxidant in Male Mice

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ARTICLE INFO
Article History
Received:2/1/2023
Accepted:16/2/2023
Available:22/2/2023

***Keywords*:**
$NaNO_2$ reproductive toxicity, male mice, sperm, testis, PCNA

ABSTRACT

Sodium nitrite ($NaNO_2$) is a main colorant and food preservative in the food industry. Besides the variety of medicinal and industrial applications, toxicity for animals and humans is well documented after overexposure of nitrite. This study aimed to investigate the reproductive toxicity of sodium nitrite and its modulation by ascorbic acid as an antioxidant in male mice. 60 adult male albino mice were used and divided into (6) groups. *Gp(I)* control. *Gp(II)* administered 100 mg/kg/d of ascorbic acid. *Gp(III & IV)* were received **(**16 and 32 mg/kg/day) sodium nitrite. *Gp(V and VI)* received (16 and 32 mg/kg/d) $NaNO_2$ and 100 mg/kg/d of ascorbic acid. Male mice were orally administered $NaNO_2$ by gastric intubation for 35 days**.** The study demonstrated that ascorbic acid ameliorates the alterations induced by sodium nitrite in reproductive performance, gonadosomatic index, testicular weight, sperm morphology, motility and count and repairing in the recorded histopathological lesions in the testis tissues. It could be concluded that ascorbic acid treatment has ameliorative effects against sodium nitrite reproductive toxicity

INTRODUCTION

Sodium nitrite (E250) has a molecular mass of 69.00 g/mol and the molecular formula $NaNO_2$. Its EC number or EINECS is 231-555-9. The most widely used curing chemical agent in the meat industry is nitrite, which is known to have human negative health effects. Nitrites improve the flavour, taste, and scent of the meat while preserving its reddish-pink hue and reducing the chance of bacterial infection, particularly from *Clostridium botulinum*. Sadly, recent study has shown that this method can have certain negative impacts (Ferysiuk & Wójciak, 2020). It is used as a color fixative and preservative in the food sector for fish and meat products (E250). As a laboratory reagent and corrosion inhibitor in photography, it is also employed in the creation of diazo dyes, nitroso compounds and other organic compounds. Metal coatings for phosphatizing and detaining are used in the manufacturing of rubber chemicals. Sodium nitrite has been used as a vasodilator, bronchial dilator, intestinal relaxant, and antidote for cyanide poisoning in both human and veterinary medicine (National Toxicology Program, 2001). Yet, industrialization and the careless use of nitrate/nitrite salts have led to high levels of $NaNO_2$ exposure in humans. The mouth is the most typical method of nitrite exposure.

The gastro-intestinal tract and small intestine are mostly affected by nitrite, which is consumed through unclean food or drinking water (Ansari *et al*., 2017). Acute exposure to high nitrite levels has been linked to death, primarily owing to methemoglobinemia (Chui *et al*., 2005). Chronic exposure to low amounts of nitrite causes birth abnormalities, respiratory tract diseases, nervous system damage, and paralysis, among other things. Long-term nitrite exposure can also be carcinogenic and mutagenic (Ansari *et al*., 2017). One of the main mechanisms through which nitrite exerts its toxicity is oxidative damage. Changes in the testis' hormonal profile (Farias *et al*., 2008) and vascularization (Farías *et al*., 2005) have been documented.

Vitamin C, also known as ascorbic acid, is a powerful antioxidant found in biological systems (Duarte *et al*., 2005). It's a water-soluble vitamin that's easily absorbed by the gastrointestinal tract and essential for a variety of biological processes and biochemical reactions in both humans and animals (Li and Schellhorn, 2007). It is a vital component of the human body. Vitamin C, has been discovered to have beneficial effects on the weight of the male reproductive system (testicles and epididymis) as well as sperm parameters such as progression, count, viability, motility and abnormalities as an antioxidant (Shabanian *et al*., 2017). It has been shown to protect spermatogenesis, plays a key function in fertility and semen integrity in both animals and men (Agarwal *et al*., 2005; Eskenazi *et al*., 2005), boost testosterone levels (Sönmez *et al*., 2005), and inhibit sperm agglutination (Sönmez *et al*., 2005). Vitamin C was found to be an antioxidant in the reproductive environment (Nazirolu, 2003). Data about its effects on the reproductive system are debated. So the present study aimed to examine the reprotoxic effects of two sub-lethal doses of sodium nitrite on male mice and the possible ameliorative effect of ascorbic acid.

MATERIALS AND METHODS

Experimental Animals:

. This study approved by Fayoum University Institutional Animal Care and use Committee (FU-IACUC) Code No. of proposal: AEC 2226, at Jan.16, 2023. Sixty adult male albino mice weighing 28±2 grams were purchased from VACSERA at Helwan, Egypt. Male mice were acclimatized to the animal house in Zoology Department, Faculty of Science, Fayoum University, Egypt in accordance with standard laboratory conditions with access to food and water ad libitum, with 12 h dark and light cycles**.** Male mice were divided randomly into six groups of 10 mice per group, orally and daily administered two doses of sodium nitrite and/or ascorbic acid for 35 days under the similar conditions. The control (G1) was administered distilled water for 35 days. Ascorbic acid (G2) was administered 100 mg/kg/day of ascorbic acid for 35 days. Low sodium nitrite dose (G3) administered 1/8 of $NaNO_2$ LD_0 (16mg/kg/d) for 35 days. High sodium nitrite dose (G4) administered 1/4 of LD_0 (32 mg/kg/d) for 35 days. G5: administered the low dose of $NaNO_2$ (16 mg/kg/d) in combination with 100 mg/kg/day of ascorbic acid for 35 days. G6: administered a high dose of $NaNO_2$ (32 mg/kg/d) in combination with 100 mg/kg/day of ascorbic acid for 35 days.

Reproductive Performance Study:

In a study of fertility, ten males per concentration for each group were mated with untreated female mice from the same strain (1:1) for 10 days (period for completion of two estrous cycles). Vaginal plugs were checked every day, and the day when the plugs were found was deemed the first day of pregnancy. The number of males who mated and produced a vaginal plug multiplied by 100, along with the number of males who coexisted with females, is the mating index %.

Body and Testicular Weight measurements and Gonadosomatic Index:

The weight of each mouse was measured at the start of the investigation, and then the body weight of the mice was recorded twice a week throughout the trial. The animals were slaughtered at the end of the trial (35 days), and the weights of the left and right testis were determined, from which the gonadosomatic index (GSI) was estimated and computed by dividing the testis weight / the animal's body weight.

Sperm Analysis:

After the last dose, the mice were euthanized 24 hours later. The epididymis tissues were removed and incubated at 37°C for 15 minutes in a pre-warmed petri dish containing 0.2 ml of calcium and magnesium-free Hank's solution. The epididymis was examined for sperm viability, count, motility, and abnormalities

Sperm Motility:

According to Ekaluo *et al*. (2013), sperm motility was assessed by placing two drops of sperm solution on a microscope slide and covering it with a cover slip. The total number of spermatozoa counted under lens (40 X) / the number of progressively motile cells and expressed as a %.

Sperm Viability:

Eosin-Nigrosin staining was used to test the viability of the sperm (Bjorndahl, 2003). Each sample was made by mixing a fraction of the sperm suspension with an equal volume of Eosin-Nigrosin, staining, and air drying smears on a glass slide. The viability of the slides was measured in percentages. Dead sperm cells took up satin and appeared reddish, whereas normal healthy sperm cells appeared whitish. The number of the living sperm cells out of the total number of detected cells was used to compute the % viability.

Sperm Count:

The improved Neubauer hemocytometer was used to get the epididymal sperm count, and the heads were manually counted under a light microscope. The total number of sperm/ml was used to represent the data (Ekaluo *et al*., 2008).

Sperm Abnormality:

For the abnormality test of the sperm head, a quantity of sperm suspension was combined with 1% eosin Y (10:1) for thirty minutes and air dried smears were made on glass slides. Every 200 spermatozoa observed on each slide for each sample was analyzed for percentages of sperm abnormalities (head, mid-piece, and tail). According to Ekaluo *et al*. (2009), the proportion of sperm head abnormalities was calculated.

Morphometric Analysis:

For each mouse testis, 30 profiles of seminiferous tubes that are round or nearly round were randomly selected and measured. Using an Olympus BX-40 microscope and the Image j software, the longitudinal and transverse tubular diameters were measured and expressed in m at 100 X magnification. Also, using the same tubules that were used to measure tubular diameter, the height of the germinal epithelium and its lumen were measured. From the basement membrane to the most advanced stage of germinal cells, the spermatids, the germinal epithelium was thought to exist. Also measured and represented as a mean value were the blood vessel diameters of the testes sections (Batra *et al.*, 2001),

Histological Examination:

The entire left testis and parts of the right testis were fixed in 10% neutral buffer formalin and cut into 4-6 µm thick paraffin sections using a rotary microtome. These sections were then stained with hematoxylin and eosin and examined under a light microscope (Sheehan and Hrapchak, 1987). Additional immunohistochemistry techniques were applied on additional paraffin sections.

Immunohistochemical Technique: **PCNA Immunohistochemistry:**

Dewaxing and rehydrating the 5 µm paraffin tissue sections in xylene and distilled water. Following that, antigen

retrieval was carried out in a microwave. Endogenous peroxidase activity in sections was inhibited for 30 minutes at room temperature with 3% hydrogen peroxide before the sections were blocked for 15 minutes with 5% bovine serum. After that, the sections were incubated with particular primary antibodies directed against PCNA (GB13030; Wuhan Saiweier Biological Technology, China) for an extended period of time at 4 °C in a humid chamber. After washing with PB, the sections were then treated with a secondary antibody and stained by adding 3, 3′-diaminobenzidine. Hematoxylin was used as a counterstain, and tap water was used to rinse the sections. A microscope was used to view the IHC micrographs. PCNA is visible as a brown colour in spermatogonia that are mitotically divided.

RESULTS

Reproductive Performance:

Reproductive performance of male mice was decreased significantly (zero) in the case of the mice treated with (low) 16 and (high) 32 mg/kg sodium nitrite groups in comparison with the control (90%) and ascorbic acid treated group (90%). While the co-administered ascorbic acid (100mg/kg) with low (16mg/kg) and high (32 mg/kg) doses of sodium nitrite showed more or fewer percentage levels similar to the control group (85% and 80%, respectively). The fertility index percent decreased in sodium nitrite-treated groups (low and high doses) and reached zero percentage compared to that of the control group (100%), however, mice treated with sodium nitrite at the two studied levels (16 and 32 mg/kg) in combination with the antioxidant, ascorbic acid (100 mg/kg) increased the fertility index percentage to levels less similar to that of normal, 90% and 85%respectively (Table 1).

Table 1: Reproductive performance in male albino mice treated with sub-lethal doses of sodium nitrite individually or co-administered with ascorbic acid as an antioxidant for 35 days.

Animal groups	Number of females	Mating index % A	Fertility index % B
Control	10	90	100
Ascorbic acid (100 mg/kg/day)	10	90	100
$NaNo_2$ low dose (16 mg/kg/day)	10	0	0
$NaNo_2$ high dose (32 mg/kg/day)	10	0	0
$NaNo_2$ low dose + Ascorbic acid	10	85	90
$NaNo_2$ high dose +Ascorbic acid	10	80	85

A) No. of males which mated resulting in the vaginal plug or pregnant female / No. of males cohoused with females × 100.
B) No. of males which sired a litter / No. of males resulting vaginal plug or pregnant female × 100.

Body and Testicular Weight and Gonado-Somatic Index (GSI):

The body weights were recorded (as mean ± stander error SE) on the first day of the experiment and at the end of the treatment period, 35 days as shown in Table 2. Body weights gain in male mice treated with 16mg/kg sodium nitrite for 35 days showed a slightly significant increase ($p<0.05$) by 2.17% in and showed reduction by 2.04% in mice treated with 32 mg/Kg in comparison with the control which showed a significant increase in their body weight gain percent by 7.48%. On the other hand, ascorbic acid only was given to

animals and showed a significant increase in the body weight gain percent by 6.71%. Also, there was a marked improvement in the percent of body weight gain after treatment with ascorbic acid and low and high doses of sodium nitrite by 7.39% and 5.97%, respectively.

Testes of mice decreased significant in weight in the group administered the low dose of sodium nitrite (0.21±0.02) and a highly significant decrease in a high dose of sodium nitrite administered mice (0.17±0.02) in comparison with control (0.25±0.02) and ascorbic group (0.24±0.02). On the other hand, the testes weights of mice administered the low and high doses of $NaNO_2$ concurrent with ascorbic acid (100 mg/kg) were more or less similar to that of the control one and non-statistically significant as (0.23±0.02 and 0.23±0.02, respectively).

The percent of gonadosomatic index (GSI) showed a significant decrease in sodium nitrite administered groups (0.72±0.06 in case of mice administered 16mg/Kg), showed a highly significant decrease in sodium nitrite administered groups (0.58±0.07 in case of mice administered (32mg/kg) in comparison with the control (0.81±0.08) and ascorbic acid administered groups (0.82±0.07). On the other hand, mice treated with ascorbic acid (100 mg/kg) concurrent with a low and high dose of sodium nitrite, showed the percent of gonadosomatic index with values (of 0.75±0.06 and 0.75±0.07, respectively) (Table 3).

Table 2: Initial weight, final weight and the percent of body weight changes in male albino mice treated with two sublethal doses of sodium nitrite individually or co-administered with ascorbic acid both for 35 days.

Animal groups	Initial body weight (g)	Final body weight (g)	Body weight change (g)	Body weight change%
GP1: Control	28.60 ± 1.17	30.74±1.07	2.14 ± 0.70	7.48
GP2: Ascorbic acid (100 mg/kg/day)	28.30±1.14	30.20 ± 1.12	1.90 ± 0.60	6.71
GP3:$NaNo_2$low dose (16 mg/kg/day)	28.50 ± 1.18	29.12± 0.30**	0.62± 0.72 **	2.17 **
GP 4: $NaNo_2$ high dose (32 mg/kg/day)	28.52±1.14	28.10 ± 0.11**	-0.42± 0.43 **	-1.47**
GP 5: $NaNo_2$ low dose +Ascorbic acid	28.53 ± 1.16	30.64±0.53	2.11 ± 0.54	7.39
GP6: $NaNo_2$ high dose +Ascorbic acid	28.63 ± 1.23	30.34 ± 0.49	1.71 ± 0.52	5.97

Data are represented as the mean of 10 samples ±SE.

The means with the same letter for each parameter in the same column are not significantly different, otherwise, they do (Duncan multiple range test).

*P < 0.05 Significant. ** P<0.01 Highly Significant.

Table 3: showed left, right, total testes weights, final body weights and Gonadosomatic index % of male albino mice treated with two sublethal doses of sodium nitrite individually or co-administered with ascorbic acid both for 35 days.

Animal group	Left testes weight (g)	Right testes weight (g)	Total testes weight (g)	Final body weight (g)	Gonado-somatic index %
GP1: Control	0.12 ± 0.01	0.13 ± 0.01	0.25 ± 0.02	30.74± 1.07	0.82±0.08
GP 2: Ascorbic acid (100 mg/kg/day)	0.11 ± 0.01	0.13± 0.01	0.24 ± 0.02	30.20± 1.12	0.79±0.07
GP 3: $NaNo_2$ low dose (16 mg/kg/day)	0.10 ± 0.01**	0.11 ± 0.01**	0.21 ± 0.02**	29.12± 0.30	0.72±0.06*
GP 4: $NaNo_2$ high dose (32 mg/kg/day)	0.09 ± 0.01**	0.08 ± 0.01**	0.17 ± 0.02*	28.10± 0.11	0.60±0.07 **
GP 5: $NaNo_2$ low dose +Ascorbic acid	0.11 ± 0.01	0.12 ± 0.01	0.23 ± 0.02	0.53±30.64	0.75±0.06
GP6: $NaNo_2$ high dose +Ascorbic acid	0.11 ± 0.01	0.12 ± 0.01	0.23 ± 0.02	30.34± 0.49	0.75±0.07

Data are represented as the mean of 10 samples ±SE.

This means with the same letter for each parameter in the same column is not significantly different, otherwise, they do (Duncan multiple range test).

*P < 0.05 Significant. ** P<0.01 Highly Significant.

Sperm Analysis:

Sperm Count:

Figure 1A showed that the epididymal sperm counts were high significant decreased in mice administered 16 and 32mg/kg/d of $NaNO_2$ with 56.54±0.99 and 57.36±1.0×10^6/ml respectively, at p<0.01 compared with 68.5±1.1×10^6/ml in control group mice and 73.48±1.1×10^6/ml in ascorbic acid group. Moreover, mice in groups V &VI that were treated with low and high doses of sodium nitrite in combination with 100 mg/kg ascorbic acid exhibited a significant increase in sperm count with 66.92±0.8 and 67.10±0.8×10^6/ml respectively compared to sodium nitrite treated groups.

Sperm Motility:

The results demonstrated that mice treated with ascorbic acid had the highest motile sperms with a percentage of 75.98 ±0.8. However, mice treated with sodium nitrite only (16 and 32mg/kg $NaNO_2$) have a highly significant lowest percent of motile sperms (45.44±0.9% and 41.28±0.96%) compared to other studied groups. Moreover, mice treated with 16mg/kg/d (low dose) and 32mg/kg/d (high dose) of sodium nitrite in combination with the studied ascorbic acid dose showed significant advancement (p<0.05) in motility of sperm (70.12±1.0% and 69.3±0.95%) (Fig.1B).

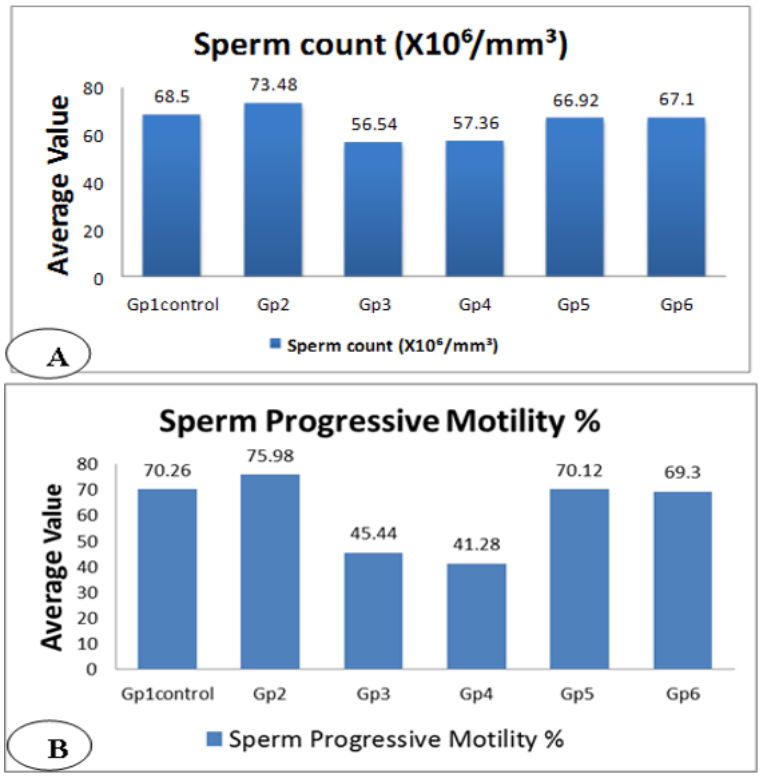

Fig.1: Sperm count (A) (X 10^6/mm^3) and Motility (B) of male albino mice treated with sublethal doses of sodium nitrite individually or co-administered with ascorbic acid as an antioxidant for 35 days.

Sperm Viability:

Examination of sperm smears showed that normal live sperms appeared whitish unstained, while dead sperms were attained and appeared pinkish (Fig.2 A & B). It is evident from the figure that low and high doses of sodium nitrite are toxic to the mice sperms and resulted in decreased sperm viability by a percent of 57.1±1.1% and 56.06 ±0.8% respectively, compared to that of control and ascorbic acid treated groups (71.5±0.9% and 79.22±1.4%, respectively). However, treatment with ascorbic acid together with low and high doses of sodium nitrite (Gps. 5 & 6) increased sperm viability to 70.14±0.4% and 69.36±1.2% for both groups, respectively (Fig.3).

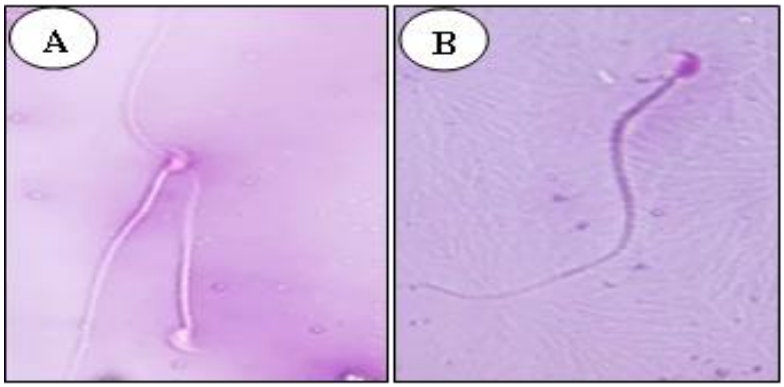

Fig. 2: A photograph shows unstained live sperm (A) and stained dead sperm (B).

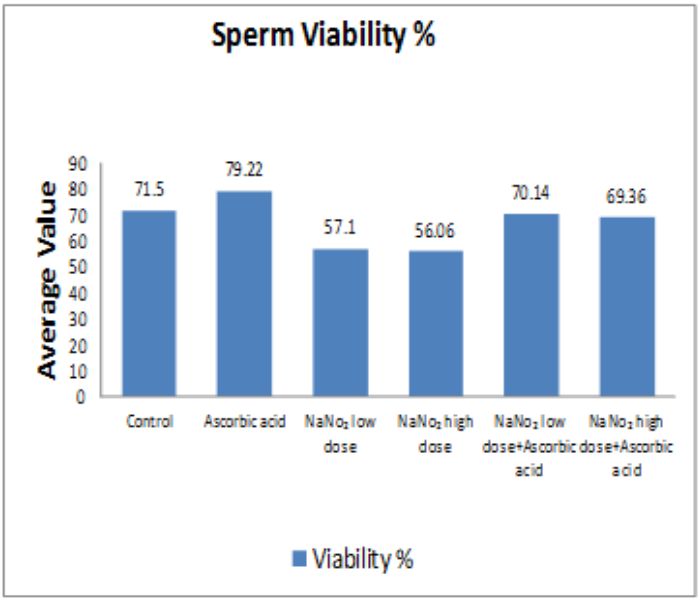

Fig. 3: Sperm viability of male albino mice treated with sublethal doses of sodium nitrite individually or co-administered with ascorbic acid as an antioxidant for 35 days.

Sperm Abnormalities:

The recorded sperm abnormalities in sodium nitrite administered groups included in head, mid-piece and sperm tail in comparison with the normal sperm seen in Table 4. The total abnormalities of the sperm and their percentages presented showed a highly significant increase ($P<0.01$) in case of sodium nitrite treated groups (16 and 32 mg/Kg/d) (69.64±7.49% and 78.28±8.37%) in comparison with their respective control (16.73±6.74%) and ascorbic acid (16.07±6.39%) groups. However, mice administered ascorbic acid (100 mg/kg/day) in combination with low (16 mg/kg/d) and high (32 mg/kg/d) doses of sodium nitrite showed a significant increase in normal sperm count and decrease in the abnormality incidence (19.63±6.27% and 21.57±7.12%) comparing with $NaNO_2$ groups.

Head Abnormalities:

Table (4) showed that the forms of head abnormalities caused by high and low doses of sodium nitrite treatments included amorphous head (20.46±0.80% and 23.44±0.90) in comparison with control (2.46±0.57%) and ascorbic acid (2.92±0.51%) while treatment with ascorbic acid in combination with low and high doses of sodium nitrite showed betterment of this abnormality to the percentage (3.42±0.56% and 3.50±0.73 %).

Hookless sperm head was observed in low (16 mg/kg) and high (32 mg/kg) doses of $NaNO_2$ treated groups (14.63±0.70 and 16.49±0.70%) in comparison with that of control (1.63±0.73%) and ascorbic acid (1.74±0.73%) treated groups. Treatment with sodium nitrite at doses of 16 or 32 mg/k/d, together with 100 mg/kg of ascorbic acid, resulted in significant improvements of 2.64 0.64 % and 2.87 0.73 %. Hammer-shaped head abnormality was significantly increased in low and high doses of sodium nitrite (16.63±0.70% and 15.49±0.70%), respectively in comparison with control (2.21±0.43%) and ascorbic acid (1.89±0.41%) treated groups, whereas treatment of ascorbic acid with both sodium nitrite doses showed enhancement with a percent of 3.17±0.12% and 3.77±0.15%, respectively. Pin-like head sperms were pronounced and markedly increased in low and high doses of sodium nitrite treated groups (3.18±0.33% and4.08±0.42%) in comparison with control (0.10±0.31%) and ascorbic acid (0.10±0.30%) groups. However, treatment of ascorbic acid with both 16 and 32mg/kg/d $NaNO_2$ doses showed improvement of these abnormalities to

percentages 0.45±0.16% and 0.52±0.14, respectively (Table 4).

Mid piece Abnormalities:

Midpiece abnormalities observed in the present study showed different forms including deformed mid-piece, bent, and coiled mid-piece and mid-piece with cytoplasmic droplet. The percentage of deformed mid-piece was increased in the case of mice administered both studied $NaNO_2$ doses by 0.79±0.29% and 0.82±0.46%, respectively in comparison with control (0.52±0.42%) and ascorbic acid 0.50±0.38%) treated groups while treatment of ascorbic acid concurrently with the low and high sodium nitrite doses showed marked betterment (0.58±0.23% and0.63±0.45%). Also, a bent abnormality was markedly increased in 16 and 32 mg/kg/d $NaNO_2$ treated groups with 1.40±0.59% and 2.55±1.40% compared with the control (0.87±0.38%) and ascorbic acid (0.65±0.45%). Meanwhile, treatment of ascorbic acid concurrently with sodium nitrite doses showed marked betterment by 0.89±0.42% and 1.02±0.45%. The percent of coiled mid-piece abnormality was increased in the case of mice treated with 16 and 32mg/kg/d doses of sodium nitrite (0.68±0.43% and 0.72±0.33%) in comparison with control (0.44±0.45%) and ascorbic acid (0.51±0.42) groups while treatment with ascorbic acid concurrently with 16 and 32mg/kg/d doses of sodium nitrite showed an improvement (0.41±0.73% and 0.59±0.75%) (Table 4). The percent of mid-piece with cytoplasmic droplet was markedly increased in sodium nitrite treated groups with low (1.10±0.39%) and high (1.59±0.33%) doses compared with the control (0.51±0.60%) and ascorbic acid (0.42±0.65%). Meanwhile, treatment of ascorbic acid concurrently with low (16mg) and high (32mg) doses of sodium nitrite showed marked improvement (0.60±0.43% and 0.76±0.85%).

Tail Abnormalities:

Several abnormalities in the sperm tail were shown by the microscopical examination of sperm smears from mice administered low and high doses of $NaNO_2$ (Table 4.). These abnormalities included a double tail, short tail and coiled sperm tails. The percentage of doubled tails was increased in the case of mice treated with low (3.45±1.12%) and high (3.9±1.13%) doses of sodium nitrite in comparison with control (3.18±1.09%) and ascorbic acid (2.19±0.88%) administered groups while treatment of ascorbic acid concurrently with 16 and 32mg/kg doses of $NaNO_2$ showed marked betterment by 2.26±1.10% and 2.34±1.12%. Also, a short tail abnormality was markedly increased in sodium nitrite-treated groups with the low and high doses (1.77±0.69% and 2.10±0.68%) compared with the control (1.61±0.70%) and ascorbic acid (1.39±0.75%). Meanwhile, treatment of ascorbic acid concurrently with both low and high sodium nitrite doses showed obvious improve (1.68±0.69% and 1.73±0.69%). The percent of coiled tail abnormality was increased in the case of mice treated with low and high doses of sodium nitrite (1.75±0.71% and 2.5±0.56%) in comparison with control (1.60±0.51%) and ascorbic acid (1.65±0.41) groups while treatment with ascorbic acid concurrently with 16 and 32mg/kg/d of nitrite showed an improvement by 1.63±0.45% and 1.74±0.51% respectively, (Table 4). The percent of doubled tail abnormality was increased in the case of mice treated with low (3.45±1.12%) and high (3.90±1.15%) doses of sodium nitrite and in comparison, with control (3.18±1.09%) and ascorbic acid (2.20±0.88) groups while treatment with ascorbic acid concurrently with low and high doses of sodium nitrite showed distinct improve (2.26±1.10% and 2.34±1.12%).

Table 4: Types of the recorded sperm abnormalities in male albino mice treated with low and high doses of sodium nitrite individually or co-administered with ascorbic acid both for 35 days.

Treatment and doses	Types and Percent of sperm abnormalities												Types and Percent of sperm abnormalities
	Percent of head abnormalities				Percent of mid piece abnormalities				Percent of tail abnormalities				
	Amorphous	Hammer shape	Hook-less	Pin-like	Deformed	Bent	Coiled	Cytoplasmic droplet	Coiled	Short	Bent	Doubled	
													
GP1: Control	2.46 ±0.57	2.21± 0.43	1.63 ±0.73	0.10 ±0.31	0.52 ±0.42	0.87 ±0.38	0.44 ±0.45	0.51 ±0.6	1.60 ±0.51	1.61 ±0.70	1.60 ±0.55	3.18 ±1.09	16.73 ±6.74
GP2:Ascorbic acid 100mg/kg/day	2.92 ±0.51	1.89 ±0.41	1.74 ±0.73	0.10 ±0.30	0.50 ±0.38	0.65 ±0.45	0.51 ±0.42	0.42 ±0.65	1.65 ±0.41	1.39± 0.75	2.10 ±0.50	2.20 ±0.88	16.07 ± 6.39
GP3: $NaNo_2$ low dose 16 mg/kg/day	20.46 ±0.8**	16.63 ±0.7**	14.63 ±0.7**	3.18 ±0.33**	0.79 ±0.29	1.40 ±0.59*	0.68 ±0.43	1.10 ±0.39*	1.75 ±0.71	1.77 ±0.69	3.80 ±0.74*	3.45 ±1.12	69.64 ±7.49**
GP 4: $NaNo_2$ high dose 32 mg/kg/day	23.44 ±0.9**	15.49 ±0.7**	16.49± 0.7**	4.08 ±0.42**	0.82 ±0.46	2.55 ±1.4**	0.72 ±0.33	1.59 ±0.33*	2.5 ±0.56	2.10 ±0.68	4.60 ±0.74**	3.9 ±1.15	78.28 ±8.37**
GP 5: $NaNo_2$ low dose +Ascorbic acid	3.42 ±0.56	3.17 ±0.12	2.64± 0.64	0.45± 0.16	0.58 ±0.23	0.89 ±0.42	0.41 ±0.73	0.60 ±0.43	1.63 ±0.45	1.68 ±0.69	1.90 ±0.74	2.26 ±1.10	19.63 ±6.27
GP6: $NaNo_2$ high dose +Ascorbic acid	3.50 ±0.73	3.77 ±0.15	2.87 ±0.73*	0.52 ±0.14*	0.63 ±0.45	1.02 ±0.45	0.59 ±0.75	0.76 ±0.85	1.74 ±0.51	1.73 ±0.69	2.10 ±0.61	2.34 ±1.12	21.57 ±7.12

Data are represented as the mean of 10 samples ±SE. The means with the same letter for each parameter in the same column are not significantly different, otherwise, they do (Duncan multiple range test).
*P<0.05 Significant. **P<0.01Highly significant.

Morphometric Analysis:

Data concerning the morphometric parameters were shown in Table 5. A significant reduction in the longitudinal diameter of the seminiferous tubules in both studied doses of sodium nitrite treated groups (104.90±4.56 and 94.96±9.60µm) in comparison with the control (177.74±5.89µm) and ascorbic acid (179.90±5.87µm) groups. A significant betterment has been shown in these parameters (175.60±5.12µm and 173.58±4.10 µm), after treatment with ascorbic acid concurrently with low and high doses of sodium nitrite. In the same manner, the transverse tubular diameter of the seminiferous tubules in the case of sodium nitrite treated groups showed a significant reduction with (72.57±6.72 and 61.32±9.45µm) in comparison with control (82.24±4.68µm) and ascorbic acid (84±4.68µm) groups but treatment with ascorbic acid concurrently with low (83.21±5.38) and high (81.57±4.94 µm) doses sodium nitrite was showed significantly improve. The height of the spermatogenic cells lining the seminiferous tubules showed a marked reduction in both low and high doses of sodium nitrite treated groups (23.58±4.0µm and 20.60±3.25µm) relative to control and ascorbic acid groups (37.29±2.13µm and 39.11±2.88µm) whereas treatment with ascorbic acid concurrently with low and high doses sodium nitrite showed definite significant improve by 37.23±2.82µm and36.4±2.94µm. So, the lumen of the seminiferous tubules was dilated in sodium nitrite treated groups with low (21.27±4.65µm) and high (23.12±4.91µm) sodium nitrite doses and in comparison with control (7.67±3.91µm) and ascorbic acid (8.76±3.41µm) treated groups and returned back within normal levels in 16 and 32mg/kg doses of sodium nitrite concurrently treated with ascorbic acid groups to 10.54±2.34µm and 10.72±3.38 for both doses, respectively. In addition, the thickness of the tunica albugina was decreased in both doses of sodium nitrite treated groups (21.01±5.99 µm and18.45±3.25 µm) in comparison with the control (29.79±5.26 µm) and ascorbic acid (30.72±4.12 µm) groups. In groups treated with low and high doses of sodium nitrite concurrently treated with ascorbic acid, the thickness of the tunica albuginea was found within the normal level of the control for both treatments (29.27±3.73µm and 28.22±2.51µm) (Table 5.). In addition, the diameter of blood vessels of the testicular tissue was

increased in sodium nitrite low and high doses treated groups (75.67±5.44µm and 77.92±4.33µm) in comparison with the control (64.08±3.23µm) and ascorbic acid (65.24±4.14µm) groups. After treatment with ascorbic acid, it became within the normal diameter values in both treatments (64.96±3.32µm and 68.09±3.31µm).

Table 5: Morphometric analysis of testes of male albino mice treated with sublethal doses of sodium nitrite individually or co-administered with ascorbic acid as an antioxidant for 35 days.

Parameters / Groups	Tubular diameter(µm)		Epithelial height (µm)	Tubular lumen (µm)	The thickness of tunica albugina (µm)	Diameter of blood vessels (µm)
	L.S	T. S				
Control	177.74 ±5.89	82.24 ±4.68	37.29 ±2.13	7.67 ±3.91	29.79 ±5.26	64.08 ±3.23
Ascorbic acid 100mg/kg/day	179.90 ±5.87	84.00 ±4.68	39.11 ±2.88	8.76 ±3.41	30.72 ±4.21	65.24 ±4.14
$NaNo_2$ low dose (16 mg/kg/day)	104.90 ±4.56**	72.57 ±6.72**	23.58 ±4.0**	21.27 ±4.65**	21.01 ±5.99**	75.67 ±5.44**
$NaNo_2$ high dose (32 mg/kg/day)	94.96 ±9.60**	61.32 ±9.45**	20.60 ±3.25**	23.12 ±4.91**	18.45 ±3.25**	77.92 ± 4.33**
$NaNo_2$ low dose + Ascorbic acid	175.60 ±5.12	83.21 ±5.38	37.23 ±2.82	10.54 ±2.34*	29.27 ±3.73	64.96 ± 3.32
$NaNo_2$ high dose + Ascorbic acid	173.58 ±4.10	81.57 ±4.94	36.40 ±2.94	10.72 ±3.38*	28.22 ±2.51	68.09 ±3.31

Histological Examination of The Testis Control and Ascorbic Acid Groups:

The testis of the untreated control mouse revealed that it is made up of many seminiferous tubules, each of which is a round, oval, or elongated compartment containing several layers of spermatocytes, secondary spermatocytes, spermatids, and spermatozoa. It also has an outer serous membrane made of simple squamous epithelium, a tunica albuginea composed of dense connective tissue containing blood vessels, Interstitial cells (Leydig cells), which are clusters of tiny, darkly coloured polygonal cells that constitute the endocrine part of the testis, are found in the intertubular connective tissue (Fig. 4).

The outermost layers of cells in seminiferous tubules are called spermatogonia, which are tiny, spherical cells. Between these cells are sertoli cells, which have oval nuclei and slender, pyramidal forms. The secondary spermatocytes are smaller cells that are located closer to the tubule lumen than the primary spermatocytes, which are larger cells with conspicuous nuclei and characteristic chromatin threads. The tubule lumen contains the spermatids, tiny spherical cells with darkly pigmented nuclei. Spermatozoa aggregate toward the sertoli cells (Fig. 4.) As demonstrated in Figure (4), a histological study of transverse sections from the testes of male mice treated with ascorbic acid for 35 days revealed similar histological architecture.

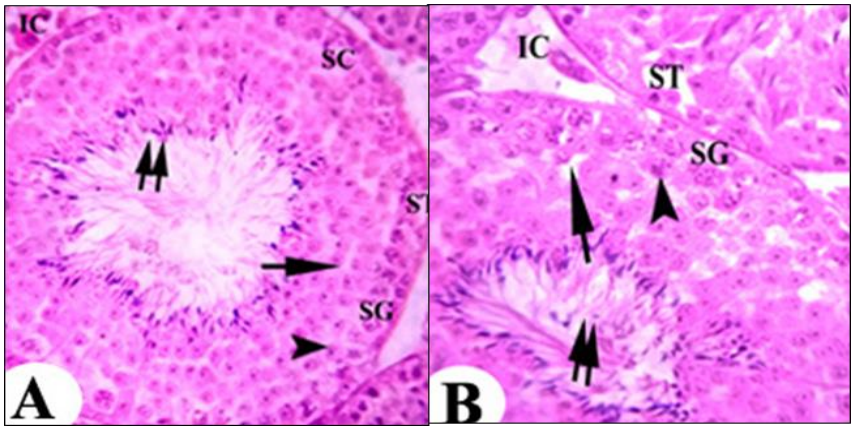

Fig. 4: Photomicrographs of transverse sections in the testes of control (A) and ascorbic acid (B) treated mice showing a normal histological structure of the testes. Note complete seminiferous tubule (ST), spermatogonia (SG), primary spermatocytes (arrow), secondary spermatocytes (arrowhead), spermatozoa (double arrows in A and B) and Sertoli cell in between (SC). Interstitial connective tissue (IC) with Leydig cells (Lc) (HE, X 400).

Sodium Nitrite-Treated Groups:

Microscopic analysis of the examined paraffin transverse sections from the testes of mice given 16 and 32 mg/kg of nitrite revealed numerous seminiferous tubules filled with primary spermatogonia but with no sperm production as a result of the sloughing of spermatogenic cells with pyknotic nuclei. The lumen of the seminiferous tubules showed accumulation of the shed cells. The lumina of numerous seminiferous tubules were found to contain enormous multinuclear spermatocytes. The germinal epithelium, which has numerous vacuoles, was also disrupted and irregularly positioned on the uneven basement membrane in some areas. The basement membrane and the germinal epithelium may separate along the direction of the vacuoles of the seminiferous tubules. There were spermatogenic arrest phenomena in several tubules and testicular tissues. (Fig.5.).

In contrast to the mice in the normal control group, the interstitial tissue displayed edematous stroma including tiny clusters of Leydig cells. Additionally, significant hemorrhages as well as dilated and congested blood vessels were seen in the interstitial tissue. Each of these lesions is dose-dependent, and the incidence of each is shown in Figure (Fig. 5.).

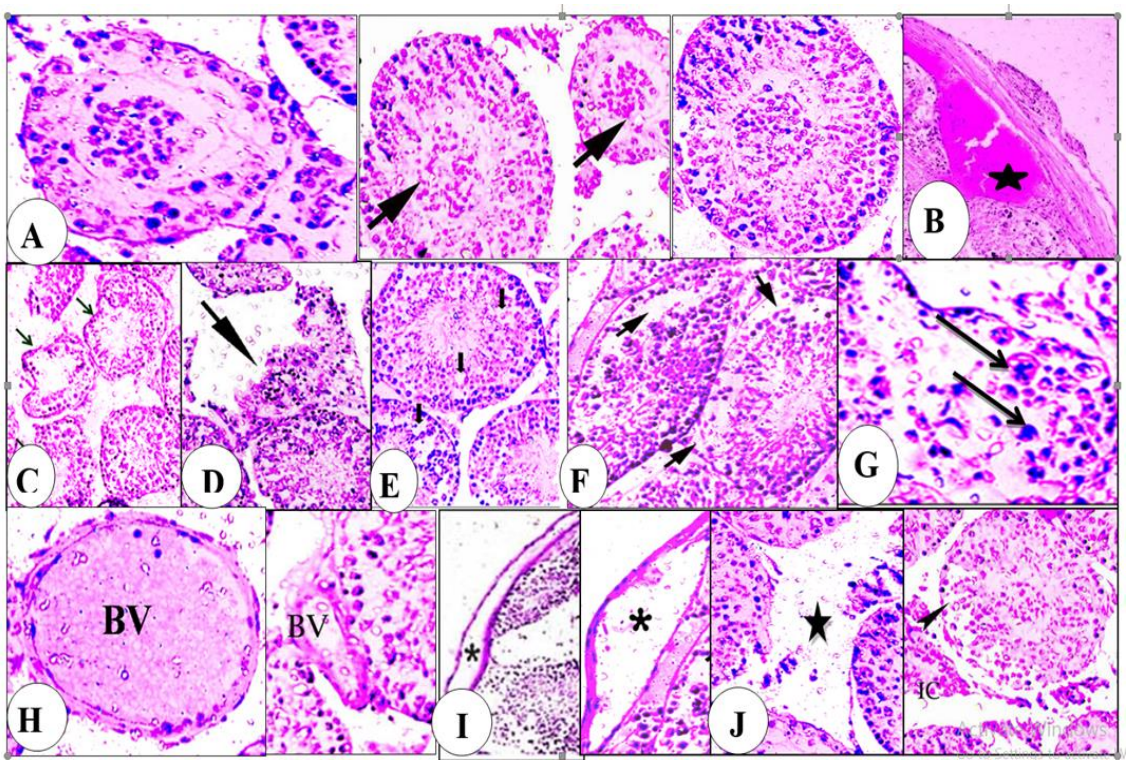

Fig. 5: Photomicrographs of transverse sections in the testes of mice treated with sodium nitrite low dose 16 mg/kg and high 32 mg/g showing: A) Spermatogenic arrest (arrow). B) Interstitial bleeding (star). C) Shrinkage & Irregular basement membrane (arrow). D) Rupture of basement membrane (arrow). E) Vacuolation (arrow). F) Detachment of spermatogenic cells (arrow). G) Gian multinuclear spermatocyte (arrow). H) Dilatation and congestion of blood vessels (BV). I) Edema (*). J) Degenerated interstitial cell (IC) (star) and detachment of spermatogenic cells (arrow) HE, X 400.

Sodium Nitrite with Ascorbic Acid Treated Groups:

Microscopical analysis of paraffin-sections from mice testis in both doses of sodium nitrite along with ascorbic acid (100 mg/kg) for 35 days revealed that most of the lesions previously mentioned, such as the germinal epithelium of the seminiferous tubules and interstitial tissue, had repaired themselves. The majorities of the seminiferous tubules displayed nearly typical histological architecture and were lined with primary and secondary spermatocytes, spermatogonia, and other spermatogenic cells arranged normally in between Sertoli cells. Furthermore, mature spermatozoa are present in their lumina. Be aware that the low dose (16 mg/kg) treated group showed better ascorbic acid repair against sodium nitrite repro-toxicity than the higher dose (32 mg/kg), where some cells remained vacuolated and separated from the basement membrane (Fig. 6).

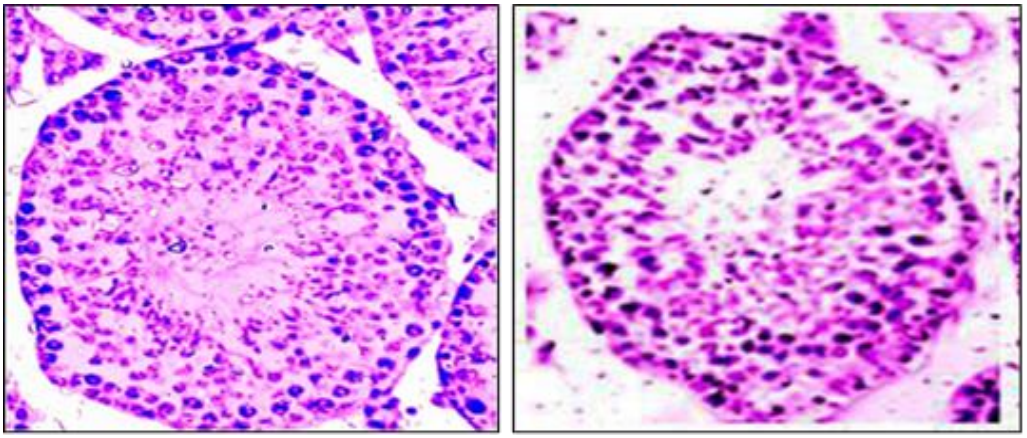

Fig. 6: Photomicrographs of transverse sections in the testes of mice co-administered ascorbic acid with a low dose of sodium nitrite and ascorbic acid with a high dose of sodium nitrite.

Immunohistological Studies:

Proliferating Cell Nuclear Antigen (PCNA) Expression:

PCNA is a deoxyribonucleic protein marker that is required for mammalian cells to synthesise deoxyribonucleic acid (DNA). In the nuclei of spermatogonia and a portion of spermatocytes, brown granules indicative of a positive PCNA reaction were visible. The primary spermatocytes and spermatogonial cells in the control and ascorbic acid-treated animals had robust immunopositive PCNA responses. In the spermatogonial cells' nuclei, the seminiferous tubule epithelium displayed a positive immune response reaction for PCNA (brown colour) (Fig 7. A & B).

In contrast to the control and ascorbic acid treated groups, other prarffen sections from testes of mice given low and high doses of sodium nitrite displayed decreased PCNA positive expression in the seminiferous epithelium cells' nucleus as violet colour (Fig. 7 C & D). However, in contrast to other groups treated with sodium nitrite, mouse testes from the two examined groups that also received ascorbic acid and sodium nitrite demonstrated increased PCNA immune expression in spermatogenic cells (Fig 7. E& F). In all groups analysed, Sertoli and Leydig cells displayed a negative PCNA reactivity.

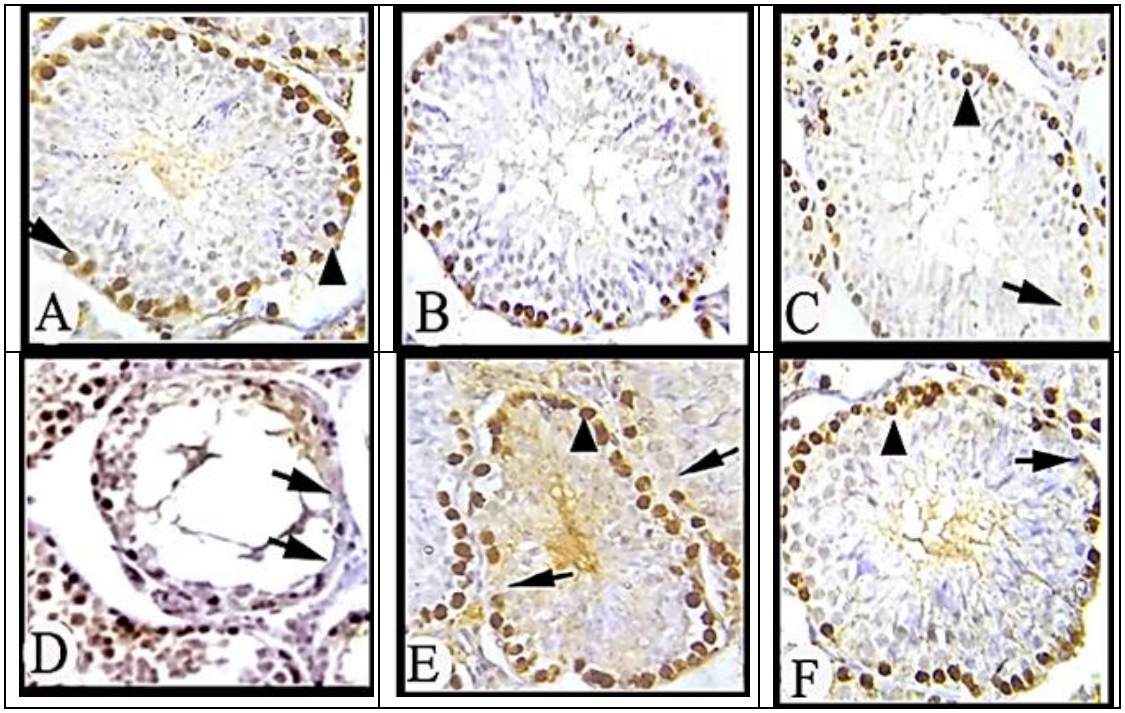

Fig. 7: Photomicrographs showing immunohistochemical demonstration of PCNA in transverse sections of mice testes of different studied groups**. (A)** Control group. **(B)** Ascorbic acid (100 mg/kg ascorbic acid). **(C)** Sodium nitrite low dose (16 mg/kg). **(D)** Sodium nitrite high dose (32 mg/kg). **(E)** Sodium nitrite low dose (16 mg/kg) co-administered 100 mg/kg ascorbic acid. **(F)** Sodium nitrite high dose (32 mg/kg) co-administered 100 mg/kg ascorbic acid. Note: nuclei of spermatogonic cell exhibited brown colour indicate positive reactivity (arrowhead) others give violet colour indicating weak or negative reaction (arrow). X400.

DISCUSSION

Body Weight Change:

Here, the body weight of mice administered 16 or 32mg/kg/d of sodium nitrite revealed a significant decrease in a dose-dependent manner comparing with the control and ascorbic acid groups. Our results are in consent with Helal *et al*. (2008) study who revealed that the body weight of male albino rats was significantly reduced after treated with 30 mg/kg BW sodium nitrite alone. Also, similar results in male rats after 30 days of daily oral 0.04mg/kgbw, 0.06mg/kg bw and 0.08mg/kg bw of nitrite respectively via oral route (Akintunde *et al*., 2014). Similarly, Adelakun *et al* (2019) found that nitrite injection (0.08 mg/kg) for 4 weeks days via gastric gavage reduced the body weight of the rats. This decrease could be attributed to an increase in nitrite levels in the body, which led to an increase in the rate of catabolic processes due to the reaction of $NaNO_2$ with food amines in the stomach, which produced free radicals and nitrosamines, possibly causing lipid peroxidation and oxidative stress (Choi *et al*., 2002). On the other side, ascorbic acid treatment in our investigation resulted in an increase in the body weight gain of the experimental mice. The overall results of Qasim, (2020) manifested that 0.8% ascorbic acid was the best antagonism effect of 0.4% sodium nitrate in addition, to a rise in body weights through all periods of the experiment due to giving all groups good concentrated forage.

Testes Weight:

Here, significant reduction in the testis's weights and GSI values of $NaNO_2$ administered mice compared with the control and other studied groups. This is in concurrence with the results of Aly *et al*. (2010), who found a significant reduction in rat testis weight, after treatment with 100 to 200 mg/kg $NaNO_2$

for 60 days and Adelakun *et al*. (2019), who found that giving rat's 0.08 mg/kg /d of nitrite for 28 days reduced the relative weights of their testes.

Reproductive Performance:

Zero reproductive index in case of sodium nitrite-treated mice in our study. Similarly, rats given 0.08 mg/kg body weight of nitrite had lower fertility potential, where more than 90% of the female rats they mated with failed to conceive (Adelakun *et al*., 2019). The tests indicate statistical differences between the control and nitrite exposure groups. The high dose (120 mg/kg nitrite in saline) shows a statistically significant difference in the rate of infertility after one against two rounds of nitrite exposure, indicating an greater effect upon recurrent exposure (Wu, *et al*., 2022).

Sperm Parameters:

Our results showed a significant decrease in the motility of the sperms, count and viability and significant increase in the abnormal sperm morphology in sodium nitrite-treated groups comparing with the control and ascorbic acid treated groups. In the same respect, Aly *et al*. (2010) recorded that sperm motility and count, as well as daily sperm production and testis weight, were all decreased dramatically at orally 100, or 200 mg/kg/day doses for 60 days of $NaNO_2$. Also, Adelakun *et al*. (2019) reported that, nitrite administration had significantly reduced sperm viability, motility, and count by subjecting that spermatozoa damage may increase by the induced oxidative stress after 0.08 mg/kg bwt of nitrite for 4 weeks via gastric gavage. Pavlova *et al*. (2013) mentioned that hypobaric hypoxia combined with high altitude 50 mg/kg/d of sodium nitrite exposure for 30 days results in oligoasthenospermia, which is characterized by decreased motility, a decrease in the total quantity of motile sperm, and an increase in defective or immature spermatozoa in male adult rats. Also, the same authors (2017) mentioned that the weight of the epididymis and the ratio of epididymis weight to body weight of mice were both decreased by 30-50 % and the mouse sperm count was reduced by 20-60% compared to the control value by the fifth hour following treatment with 50 mg/kg/day for month. Additionally, Pavlova *et al*. (2012) observed that Four-month-old male rats were intraperitoneally injected with sodium nitrite at a dose of 50 mg/kg, which resulted in a decrease in sperm count. The present results showed that animals treated with the studied two doses of sodium nitrite and 100 mg/kg of ascorbic acid showed great improvement in all sperm parameters.

Testicular Morphometric Change:

When compared to control and ascorbic acid-treated mice in the current investigation, mice treated with sodium nitrite had a significant number of alterations in the testicular tissues' evaluated morphometric parameters. The diameter of the tubules, the height of the germinal epithelium, the tunica albuginea, and the diameter of the blood vessels and the lumen of the seminiferous tubules were all altered.

In this regard, Adelakun et al. (2019) discovered that after 28 days, the volume of germinal epithelium in controls was (64.900.89) and significantly different from that in rats fed nitrite (58.080.33). The lumen density of the nitrite group was significantly lower (9.600.23) than that of the control group (15.420.20). The interstitium significantly increased (26.730.21) when compared to the controls (21.060.26) in the nitrite group.

Histopathological Findings:

Microscopical analysis of the testes tissues from mice treated with sodium nitrite revealed that the treatment resulted in testicular damage in the form of atrophied seminiferous tubules, focal seminiferous tubule disarray, and substantial depletion of the spermatogenic cell populations. Several of the seminiferous tubules' tubular lumina showed exfoliation of the injured spermatocytes and spermatids. Moreover, the intertubular connective

tissue became hyalinized, Sertoli cells shrank in size and number, and there was a comparable decrease in interstitial cells (Leydig cells). These observations were conceding with the measured morphometric parameters such as increased seminiferous tubule diameter, increased luminal tubular diameter and decreased height of the spermatogenic cell. Also, these results agree with the results of Akintunde *et al*. (2014) who reported that at 0.06 mg/kg sodium nitrite, histological analysis reveals seminiferous tubules with focal regions of lost germ cells, hyperplasia of Leydig cells, and inadequate maturation of spermatocytes. In addition, Adelakun *et al* (2019) found that the spermatogenic series cells were severely reduced in the rats' seminiferous tubules, hypocellularity in the interstitium, tubular lumen widening, tubular atrophy, and fewer spermatozoa in the tubular lumen. Also, Hammoud *et al*. (2014) recorded that degeneration of spermatogoneal cells lining seminiferous tubules and necrosis Leydig cells was found in the testes of rats treated with 1g/L $NaNO_2$. Interstitial edoema and degradation of spermatogoneal cells lining seminiferous tubules were also found in the testes of rats given 2g/L $NaNO_2$. Pavlova *et al*. (2013) reported that male Wistar rats were given $NaNO_2$ (50 $mg.kg^{-1}$ B.W) intraperitoneally and then sacrificed at various time intervals (1 h, 5 h, 24 h, and 48 h) after the injection, resulting in disruption of the seminiferous epithelium and assembling of undifferentiated germ cells in the luminal portion of the tubules. Also, incomplete spermatogenesis and the absence of spermatozoa in the lumen of some seminiferous tubules, however, complete degeneration and necrosis of the lining epithelial cells of some seminiferous tubules were also identified.

Immunohistochemical Findings:

Spermatogenesis is a complicated process in which the testis' spermatogonia undergo continual mitosis, meiosis, and cell differentiation in order to mature into spermatozoa (Yao et al., 2015; Griswold, 2016). Spermatogenic cells regularly divide, develop, and differentiate in a physiologically-regulated manner. Spermatogenesis is a complicated process in which the testis' spermatogonia undergo continual mitosis, meiosis, and cell differentiation in order to mature into spermatozoa (Yao et al., 2015; Griswold, 2016).

During spermatogenesis, spermatogenic cells commonly multiply, grow, and differentiate in ways that are physiologically significant. Proliferating cell nuclear antigen (PCNA) and Ki-67 are two proliferation factors that are involved in the growth and differentiation of spermatogonia in the testis (Wrobel et al., 1996; Angelopoulou et al., 2008). Thus, Zhao *et al.* (2018) revealed that the positive PCNA protein expression levels of in the testis cells are the typical marker of spermatogenic cells proliferation and assess the state of spermatogenic cell proliferation (smooth spermatogenesis).

PCNA:

A co-factor of DNA polymerase-d, proliferating cell nuclear antigen (PCNA) is an intranuclear polypeptide involved in DNA duplication and repair (Shivji et al., 1992; Georgescu et al., 2015). Being a necessary molecule for the beginning of DNA duplication, PCNA is crucial for the beginning of cell proliferation (Jaskulski et al. 1988; Xue et al., 2007). In a previous study, the presence and distribution of PCNA immunoreactivity in the cells of many species' testes were described (Schlatt and Weinbauer, 1994; Wrobel et al., 1996). In cases of male infertility, its staining intensity was also employed to assess the cell proliferation and spermatogenic activity of the testes (Tousson et al., 2012).

Based on the analysis of PCNA immunohistochemistry in the testes tissue, the goal of our study was to investigate the potential of ascorbic acid in preventing or reducing sodium nitrite-induced reprotoxicity. In both the control

group and the ascorbic acid-treated group, numerous positive nuclear responses were seen in the spermatogonia and proliferating spermatocytes of the testes, but in all sodium nitrite-treated groups with the exception of groups 5 and 6 the reactions were weak or negative. Also, the sodium nitrite-treated group had fewer PCNA-positive germinal cells and a reduced signal density of these cells. These findings are consistent with the sodium nitrite-treated groups' decreased sperm counts.

In conclusion, the results of the current investigation redirect clearly that sodium nitrite induced reproductive toxicity in male mice. These symptoms were decreased by ascorbic acid treatment suggesting that ascorbic acid is a protective antioxidant against male reproductive toxicity induced by sodium nitrite. So, intensive research is required to explore sodium nitrite-related molecular and metabolic mechanisms. It is recommended to perform further studies using other experimental tools, measurements, immunohistochemical markers and antioxidant to evaluate its protective or preventive effects against the reprotoxicity of other doses of sodium nitrite. The use of sodium nitrite as a food preservative must be limited for damage caused by it. We must eat food rich in ascorbic acid to reduce the harmful effect of sodium nitrite.

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