



## **Histological and Biochemical Studies of Germ Cell Toxicity in Male Rats Exposed to Sodium Benzoate**

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### **Authors' contributions**

*This work was carried out in collaboration among all authors. Author ODA designed the study and performed the statistical analysis. Author JPI wrote the protocol and wrote the first draft of the manuscript. Authors ODA and OAB managed the analyses of the study. Author OJA managed the literature searches. All authors read and approved the final manuscript.*

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### **ABSTRACT**

**Background:** One of the consequences of increasing urbanization is that many people eat processed foods that contain various chemical substances applied as additives. Some of them may have the ability to suppress male fertility.

**Aims:** To determine the effects of sodium benzoate (NaB) on the histology of the testis, and biochemical and semen parameters in rats. The potential of Vitamin E to protect the testis was also studied.

**Methodology:** Six groups of 8 rats each were treated with these substances: Group A (Control) had olive oil. Groups B and C had 200 mg/kg of NaB with Vitamin E added to group C. Groups D and E had 400 mg/kg of NaB with Vitamin E added to group E. Groups F and G were given NaB only at 200 mg/kg and 400 mg/kg respectively but were left for an extra 4 weeks after the last treatment dose. All groups were treated daily for 8 weeks. Outcome measures were testosterone assays and cell counts and morphometry in the testis. We also examined biochemical parameters such as catalase, glutathione and malondialdehyde levels.

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**Results:** This study showed that NaB impaired reproduction. Total sperm count in some treated groups were reduced to half (50%) of the count in control animals. There was reduction in anti-oxidant levels and elevation in markers of lipid peroxidation suggesting oxidative stress. There was histologic evidence of impaired spermatogenesis and considerable testicular damage with micrographs showing widespread germ cell loss and sloughing of germinal epithelium in many places. Vitamin E offered significant protection from testicular damage in the groups given the vitamin.

**Conclusion:** We conclude that NaB has the potential to impair fertility in rats and more studies are needed to determine its safety level in the male reproductive system.

*Keywords: Sodium benzoate; testis; fertility; histomorphometry.*

## 1. INTRODUCTION

Mass urbanization is a social phenomenon in the last several decades in many parts of the world. Rural-urban drift intensified in many developing countries as the rural population migrated in droves to cities in search of a better livelihood. Many of these people spend so much time at work and on the road daily, they have to depend on food from restaurants or fast food outlets where the food is made with processed and semi-processed materials. It has been estimated that up to 75% of the diet consumed today in the western world is factory processed with each person ingesting 8 to 10 pounds of additives each year [1]. Through these foods and drinks, people are exposed daily to a large number of chemical and sometimes hormonal substances collectively called endocrine disrupters. Many of these compounds have now been shown to have harmful effects on male reproduction [2,3]. It is believed that these substances are a major factor in the relentless fall in average sperm counts that has been observed in many parts of the world over the last several decades [4,5]. These observations were first made in the developed countries but perhaps because of westernization of lifestyles, a similar decline has begun to occur in the less developed nations.

Man has practiced food preservation for thousands of years. Excess food is preserved by a variety of methods which use either natural or chemical agents [6]. A food additive is any substance that is added to food to perform functions such as enhancing colour, texture, flavor, taste and prolonging shelf life. Preservatives are substances added to food to protect it from microbial damage [7,8]. Many of these agents however have selective affinity for specific organs in the body, where they bio-accumulate and eventually cause tissue damage.

Many chemicals from food and drugs including hormonally active substances used in animal

husbandry have been found to have a negative effect on the testis [2,3]. Sodium benzoate (NaB) is a white crystalline powder with the formula:  $C_7H_5NaO_2$ . It is widely used as a food preservative. It is given an E number of E211 in the food industry. Its major use is as a preservative in food and beverages (60%) and also as a cooling liquid (10%). It is used typically in salads, fruit juices, jam and carbonated drinks [9]. There is research evidence that exposure to sodium benzoate can harm fetal development and cause degenerative changes in the liver and renal tissue [10,11]. While NaB has been shown to reduce the weight of ovaries and serum levels of FSH and LH in mice [12], very little has been reported concerning the effects of this ubiquitous food additive on male reproduction. This study was therefore carried out to investigate its effects on the reproductive system in male rats. We carried out a detailed and quantitative evaluation of key germ cell populations in the seminiferous epithelium. We also evaluated histological changes in the light of alterations in the levels of markers of tissue oxidation status and serum testosterone levels.

## 2. MATERIALS AND METHODS

### 2.1 Chemicals and Reagents

Food grade NaB, was purchased from a local store in Lagos, Nigeria. Vitamin E ( $\alpha$ T) made by Biopharma Nigeria Limited, was purchased from a local Pharmacy in Lagos as well. Absolute alcohol, Xylene, Hydrochloric acid, and all other chemicals used in this study were products of Sigma-Aldrich, Ontario, Canada. They were all of analytical grade.

### 2.2 Animals

Fifty-six (56) male Wistar rats, 12 weeks old and weighing between 180 and 200 g were obtained from an animal breeding stock at the University

of Ibadan. They were allowed to acclimatize for 2 weeks and were fed freely on standard commercial rat food. Relatively constant environmental conditions were maintained and there was 12 hours' light and darkness rhythm and average humidity of 70% in the lab during the experiment. Experimental procedures involving animals and their care were approved by our Faculty's Ethical Committee (Approval Number: AEC/2019/12) and were conducted in conformity with guidelines for the care of laboratory animals in Biomedical Research and in accordance with the Helsinki Declaration and the Guiding Principles in the Care and Use of animals (American Physiological Society, 2002).

### 2.3 Methods

Two doses of NaB dissolved in distilled water were used in this study: 200 and 400 mg/kg body weight (kbw). A single dose of  $\alpha$ T, 100 mg/kbw dissolved in Olive oil was used in this study. One set of the animals was killed after being treated with these doses for 8 weeks. We treated for 8 weeks because that is the duration of spermatogenesis in rats. [13] In order to study the likelihood of spontaneous recovery from the effects of NaB, we treated another set of animals with the same doses and left them for 4 weeks after the last dose was given [14]. They were killed after this waiting period. All treatments were administered orally by gavage. The animals were randomly assigned into seven groups of eight animals each and treated as follows:

**Group A:** Control: Olive oil, 0.5ml and then 0.5 ml distilled water daily for 8 weeks.

**Group B:** 200 mg/kbw of NaB only, daily for 8 weeks.

**Group C:** 200 mg//kbw of NaB and 100mg/kbw  $\alpha$ T daily for 8 weeks

**Group D:** 400 mg/kbw of NaB only, daily for 8 weeks.

**Group E:** 400 mg/kbw of NaB and 100mg/kbw  $\alpha$ T daily for 8 weeks.

**Group F:** 200 mg/kbw of NaB only, daily for 8 weeks, left for 4weeks after last dose.

**Group G:** 400 mg/kbw of NaB only, daily for 8 weeks, left for 4weeks after last dose

The animals were weighed daily at the time of treatment to determine dose. At the end of the experiment the animals were weighed and killed using ketamine anaesthesia. Their testes, epididymis and other accessory sex organs were carefully dissected out, freed of fat and weighed. Blood was collected by cardiac puncture into

plain haematocrit bottles and centrifuged at 3000 rpm for 10 minutes to obtain serum. One testis was fixed in Boiun's fluid for histological examination. The other testis was homogenised in 10ml of distilled water, centrifuged at 5000 rpm for 5 minutes and the supernatant was stored at  $-40^{\circ}\text{C}$  and used for biochemical assays.

### 2.4 Epididymal Sperm Parameters

Progressive motility was estimated by modifying a method previously described [15]. Briefly, a segment of the cauda epididymis was removed and fluid from it aspirated by pipette into 1ml physiological saline solution. It was left to stand and rock for a few minutes to liberate its sperm cells. Two drops from this suspension were placed on a warm slide, two drops of 2.9% sodium citrate buffer was added and it was examined at a magnification of 400. The average from 5 fields was taken for each sample.

Total sperm count was determined according to a method described by the same authors. Briefly, epididymal sperm was diluted 1:20 with fresh physiologic saline. A drop of 35% formalin was added to immobilise sperm. The new Neubauer hemocytometer was charged with 10 $\mu$ L of this suspension. The count was made after allowing cells to settle over 5 minutes using a Ceti microscope [15].

Sperm morphology was evaluated from the preparation made for motility. One hundred sperm cells from each sample were scored for morphological abnormalities [16]. Briefly, in wet preparations using phase-contrast optics, spermatozoa were categorized into two classes. In this study, spermatozoa were considered abnormal morphologically if they had one or more of the following features: rudimentary tail, round or swollen head, broken neck, double head, double tail, or if they were headless or tailless.

### 2.5 Determination of Biochemical Parameters

Catalase (CAT) activity was measured according to the method described by Rukmini et al. In this method, buffer was added to testicular homogenate and reacted with hydrogen peroxide ( $\text{H}_2\text{O}_2$ ). The rate of decomposition of  $\text{H}_2\text{O}_2$  was measured by absorbance at 240 nm. The activity of the enzyme was expressed as  $\mu\text{mol/ml}$  protein [17].

Superoxide dismutase (SOD) activity was measured according to a method described by Winterbourn et al, [18]. It is based on the ability of SOD to inhibit the reduction of nitro-blue tetrazolium (NBT). The absorbance was measured at 560nm and enzyme activity was expressed as  $\mu\text{mol/ml/mg}$  protein.

Glutathione peroxidase (GPX) activity was measured by the method described by Rotruck et al, [19]. The reaction mixture contained Tris- HCl buffer, sodium azide, glutathione and  $\text{H}_2\text{O}_2$ . The absorbance of the product was read at 430 nm and the enzyme activity was expressed as  $\mu\text{mol/ml/mg}$  protein.

Reduced glutathione (GSH) level was determined by the method of Elman [20]. The supernatant from testicular homogenate was treated with Elman's reagent. Water and phosphate buffer were added and the absorbance was read at 412 nm. Reduced glutathione concentration was expressed as  $\mu\text{mol/ml/mg}$  protein.

Lipid peroxidation in testicular tissue was estimated colorimetrically by method of Buege & Aust, [21]. This method employs thiobarbituric acid reactive substances (TBARS) and includes HCl, tricarboxylic acid and water. The absorbance of clear supernatant was measured against reference blank at 535 nm. Concentration was calculated using the molar absorptivity of malondialdehyde (MDA) and expressed as  $\mu\text{mol/ml}$ .

Serum testosterone level was estimated using the Enzyme Immunology Assay (EIA) kits, (Immunometrics, London U.K) according to manufacturer's protocol. Plasma samples were collected and stored at  $-20^\circ\text{C}$  until assayed. The kits contained controlled testosterone substrate reagents and quality control samples. A quality control sample was run for the hormone at the beginning and at the end of the assay for variation. The EIA kit used had a sensitivity level of  $0.3\text{nmol/L}$  ( $0.1\text{ ng/mL}$ ). The intra and inter assay variations were 11.00% and 10.10%, respectively [22].

## 2.6 Histology

For histological examination, the testes were dehydrated in graded ethanol and cleared in xylene. They were then infiltrated in molten paraffin at  $58^\circ\text{C}$ , and embedded in wax with the long axis of the testis perpendicular to the

section plane in order to generate spherical sections. They were later blocked out and serial sections,  $5\mu$  thick were cut out of them. They were then rehydrated and stained with haematoxylin and eosin [23].

To determine morphometric parameters, four sections from each testis were made from polar and equatorial regions of the organ. In each section, 25 tubules were randomly selected and assessed. In all, 100 tubules profiles were assessed per testis following previously described techniques [24,25]. Cell types were characterized throughout by nuclear features. Morphometry and cell counts were performed on a Ceti research microscope (Ceti, UK) fitted with an XLI, Still Image Capture Software Version 12.

## 2.7 Evaluation of Indices of Spermatogenesis and Spermiogenesis

Tubular differentiation index (TDI) was estimated as the percentage of seminiferous tubules that had more than three layers of differentiated germinal cells beginning from the layer of spermatogonia. These samples were considered as having positive TDI.

Repopulation index (RI) was calculated as the ratio of inactive spermatogonia (spermatogonia with darkly stained nucleus) to active spermatogonia, (spermatogonia with lightly stained nucleus) in each seminiferous tubule [26]. Positive spermiogenesis index (PSI) was estimated as the percentage of seminiferous tubules that had spermatozooids: late spermatids, elongated spermatids, and luminal spermatozoa [27].

## 2.8 Histopathologic Evaluation

To obtain a semi-quantitative evaluation of pathological changes in the testis 100 tubules were examined in each testis and classified as intact, atrophic/depleted, sloughed, degenerate (with or without giant cells) depending on the predominant histological feature seen in each tubule. Values obtained were expressed in percentage [28].

## 2.9 Statistical Analysis

All data were expressed as mean  $\pm$  SD. The level of homogeneity among the groups was tested using one-way Analysis of Variance (ANOVA). A value of  $P < 0.05$  was considered to

be significant. Data Analysis was carried out with the SPSS software version 16.0 (SPSS Inc. Chicago, Illinois).

### 3. RESULTS

#### 3.1 Weight of Animals, Testes and Accessory Sex Organs

The animals tolerated the experiment well though hyperactivity was generally seen among animals treated with Nab alone. The results of weight changes in the animals and sex organs are presented in Table 1. The animals gained weight in all groups during the study period. The weight of testes and prostate were significantly reduced in groups B, D and G respectively. These groups were treated with only NaB.

#### 3.2 Changes in Biochemical Parameters

The results for biochemical anti-oxidant parameters as well as malondialdehyde levels are presented in Table 2. This additive caused a general reduction in testicular levels of the enzymatic anti-oxidants. NaB caused a significant reduction in levels of SOD and reduced GSH in all unprotected treatment groups (B, D and G). Lipid peroxidation levels indicated from MDA were correspondingly high in these same groups B, D and G. Co-treatment with  $\alpha$ -T provided considerable protection from the effects of NaB, as significant changes in the levels of these biomarkers did not occur in groups C and D.

Also, CAT and GPX levels were only reduced significantly in group D which had the highest dose of NaB.

#### 3.3 Alterations in Levels of Serum Testosterone

Serum testosterone levels were lower in the unprotected groups, B, D and G but the difference was not statistically significant (Table 3).

#### 3.4 Alterations in Sperm Parameters

The results for sperm parameters in this study are presented in Figs. 1 to 3. NaB caused a significant reduction in sperm motility only in group D which had 400mg of NaB only for 8 weeks. Motility was about 45 % compared to over 70% in control (Fig. 1). However, sperm concentration was significantly reduced by about

50% in all unprotected groups (B, D and G) exposed to NaB (Fig. 2). The effect was largely independent of dose and considerable recovery had occurred when the animals were left for 4 weeks after the last treatment before they were sacrificed (groups F and G). Changes in sperm morphology followed closely after those in concentration. Significant reduction was seen in groups B and D which were exposed to NaB without any protection (Fig. 3).

#### 3.5 Germ Cell Alterations and Indices of Spermatogenesis

Exposure to NaB caused impaired spermatogenesis as changes in indices of spermatogenesis occurred along the same lines as in the other parameters evaluated in this study. Tubular differentiation index (TDI), repopulation index (RI) and positive spermiogenesis index (PSI) were all significantly lower in the unprotected study groups—B, D and G (Figs. 4 to 6). Again, animals that were co-treated with  $\alpha$ -T were considerably protected from these effects.

#### 3.6 Changes in Histomorphometry and Quantitative Histopathology

Alterations in histo-morphometric parameters are presented in Table 4 and a semi-quantitative evaluation of changes in histopathology is presented in Table 5. Qualitative histological changes are shown in micrographs numbered as Figs. 7 to 15.

Sections of the testis from control group showed normal histo-architecture. All morphometric parameters assessed in this study: tubular diameter (*D*), cross sectional area (*Ac*) and height of germinal epithelium were significantly reduced in unprotected groups B, D and G with maximum effect in group D, which was exposed to the higher dose. In group D, cross sectional area of tubules (*Ac*) was reduced by up to 50% (Table 4).

Massive germ cell loss manifesting as tubules with cell atrophy and the presence of vacuoles were the predominant histopathological findings in unprotected study groups: B (Figs. 8 & 9), D (Figs. 11 & 12) and G (Fig 15). This was especially prominent in groups B and D where, in quantitative terms, 46% and 61% respectively, of all tubule profiles examined showed clear evidence of germ cell loss (Table 5).

**Table 1. Effect of sodium benzoate (NaB) and  $\alpha$ -tocopherol ( $\alpha$ T) on body and organ weight**

Parameter	Group A (Control)	Group B (200mg/kg NaB)	Group C (200mg/kg NaB + $\alpha$ T)	Group D (400mg/kg NaB)	Group E (400mg/kg NaB + $\alpha$ T)	Group F (200mg/kg NaB- W)	Group G (400mg/kg NaB- W)
Initial body weight (g)	184.60±20.67	191.40±21.59	189.00±17.20	194.80±3.96	192.20±12.19	189.80±18.55	196.40±3.58
Final body weight (g)	238.40±20.58	223.00±10.56	226.80±9.09	232.40±20.03	244.50±14.80	235.80±15.79	232.00±8.46
Testis weight (g)	1.00±0.19	0.64±0.23*	0.94±0.15	0.74±0.28	0.65±0.17	0.74±0.21	0.80±0.16
Epididymis weight (g)	0.32±0.08	0.26±0.05	0.26±0.55	0.28±0.08	0.18±0.10*	0.23±0.05	0.24±0.05
Seminal vesicle weight (g)	1.12±0.31	0.96±0.25	0.94±0.27	0.98±0.29	0.95±0.33	0.68±0.18	0.72±0.08
Prostate weight (g)	0.28±0.08	0.18±0.05	0.18±0.04	0.20±0.10	0.15±0.06*	0.17±0.05*	0.18±0.04

\*  $P < 0.05$ , when compared to control. Values are expressed as mean  $\pm$  SD, N= 8 per group. NaB-W are withdrawal groups

**Table 2. Effect of sodium benzoate (NaB) and  $\alpha$ -tocopherol ( $\alpha$ T) on biochemical parameters**

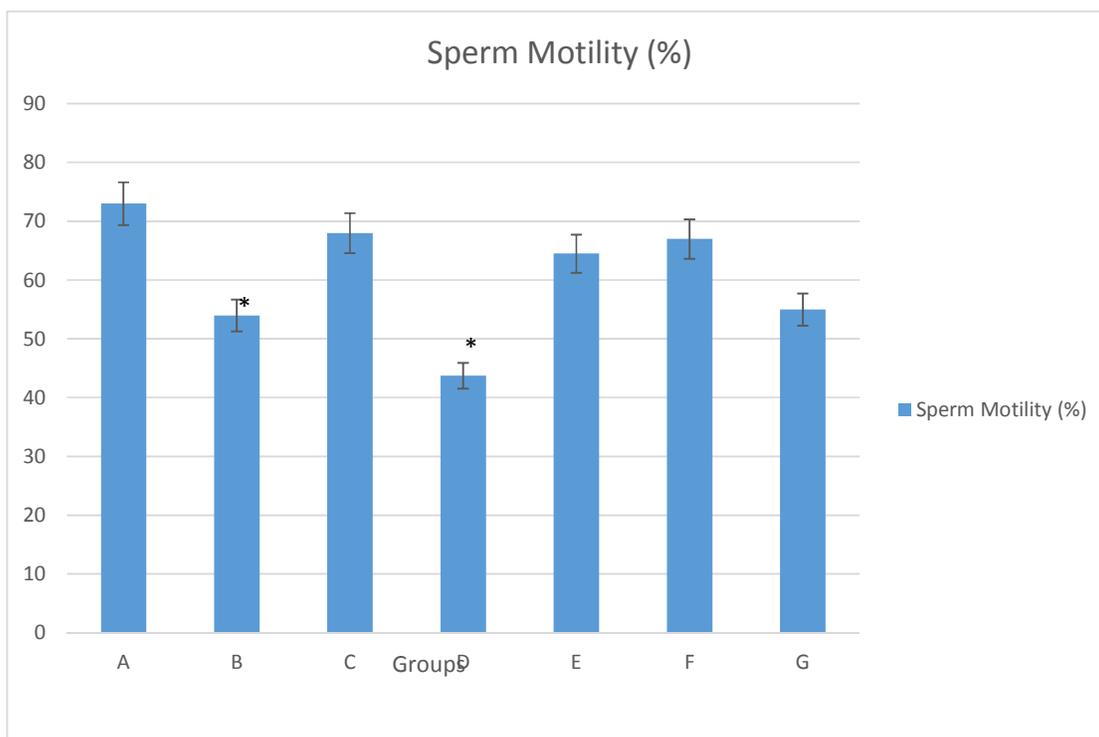
Parameter	Group A (Control)	Group B (200mg/kg NaB)	Group C (200mg/kg NaB + $\alpha$ T)	Group D (400mg/kg NaB)	Group E (200mg/kg NaB $\alpha$ T)	Group F (200mg/kg NaB- W)	Group G (400mg/kg NaB- W)
(SOD)	6.1±0.25	3.82±0.19*	6.56±1.03	2.8±0.17**	5.18±0.28	5.08±1.0	2.91±0.4**
(CAT)	12.34±1.15	9.23±1.09	10.96±1.01	7.3±1.12*	10.21±0.88	10.03±1.42	9.78±1.48
(GPX)	0.62±0.02	0.4±0.09	0.57±0.03	0.29±0.02*	0.55±0.08	0.53±0.07	0.49±0.01
(MDA)	1.57±0.26	2.82±0.31*	1.7±0.17	3.97±0.19**	2.22±0.28	2.5±0.47	3.17±0.28*
(GSH)	0.8±0.04	0.39±0.08**	0.69±0.02	0.24±0.07**	0.47±0.03**	0.37±0.06**	0.3±0.02**

All measurements are in  $\mu$ mol/ml/mg protein. \*  $P < 0.05$ , when compared to control. \*\*  $P < 0.001$ , when compared to control. Values are expressed as mean  $\pm$  SD

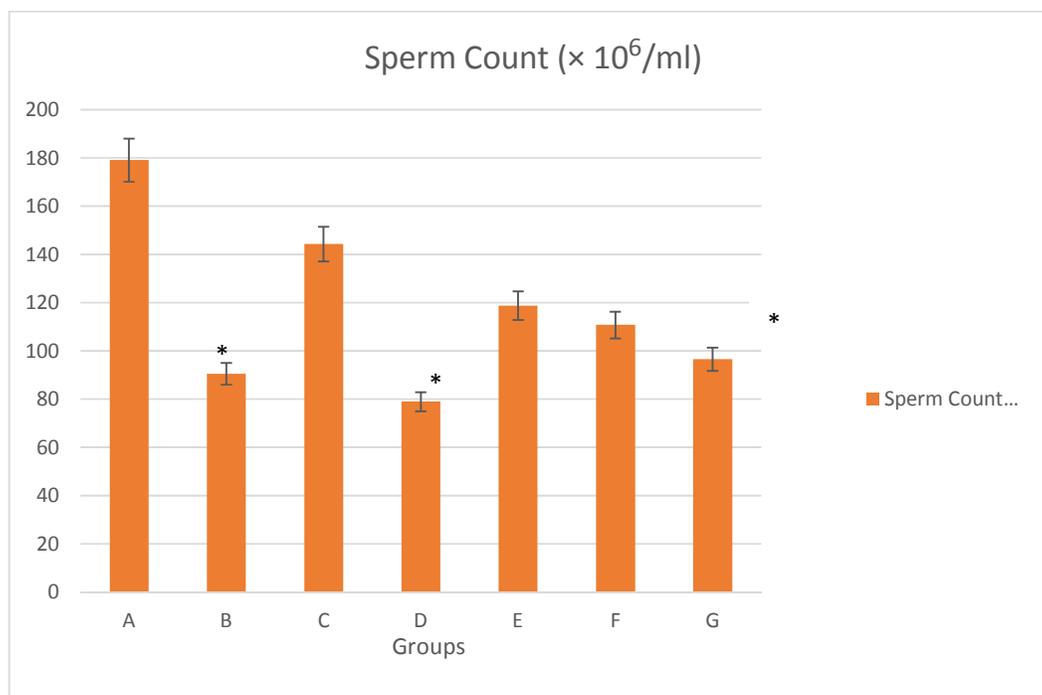
**Table 3. Effect of sodium benzoate (NaB) and  $\alpha$ -tocopherol ( $\alpha$ T) on plasma testosterone**

	Group A	Group B	Group C	Group D	Group E	Group F	Group G
Testosterone (ng/ml)	3.77±0.75	2.62±0.59	3.66±1.01	2.59±0.68	3.36±0.41	3.24±0.53	2.60±1.12

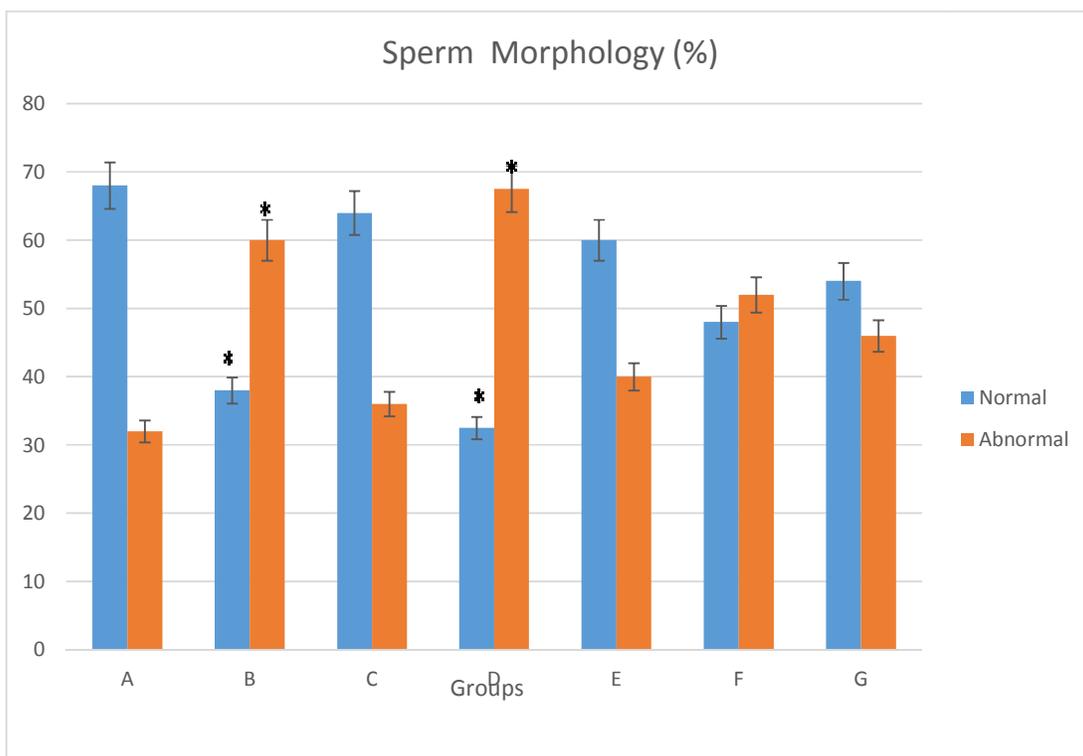
\*  $P < 0.05$ , when compared to control. Values are expressed as mean  $\pm$  SD



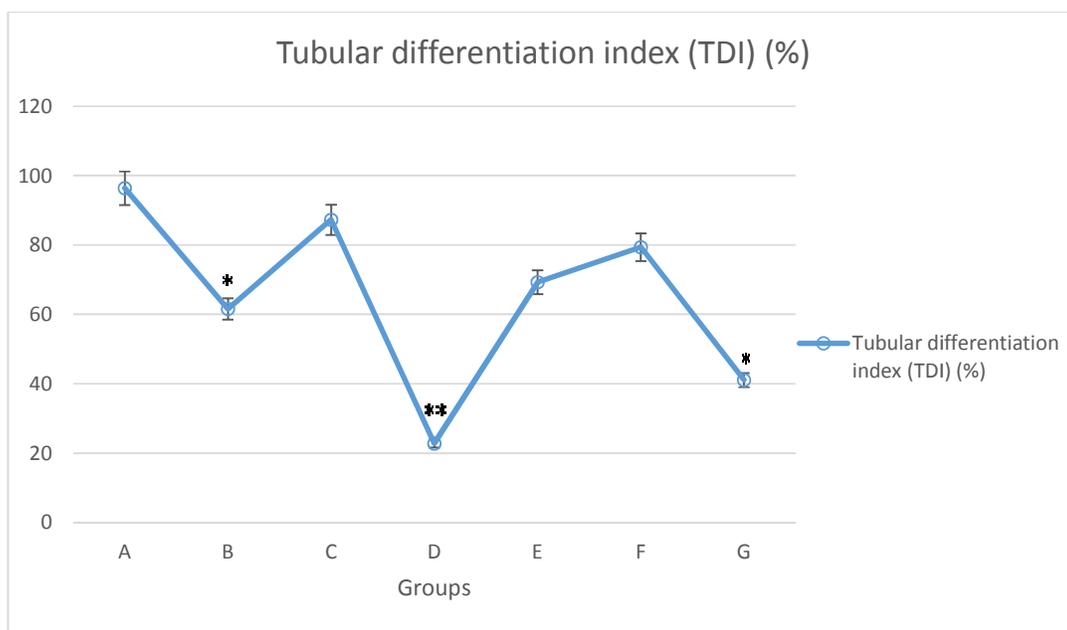
**Fig. 1. Effect of sodium benzoate (NaB) and  $\alpha$ -tocopherol ( $\alpha$ T) on sperm motility (%)**  
 \*  $P < 0.05$ ; significantly different from control. Values are expressed as Mean  $\pm$  SD



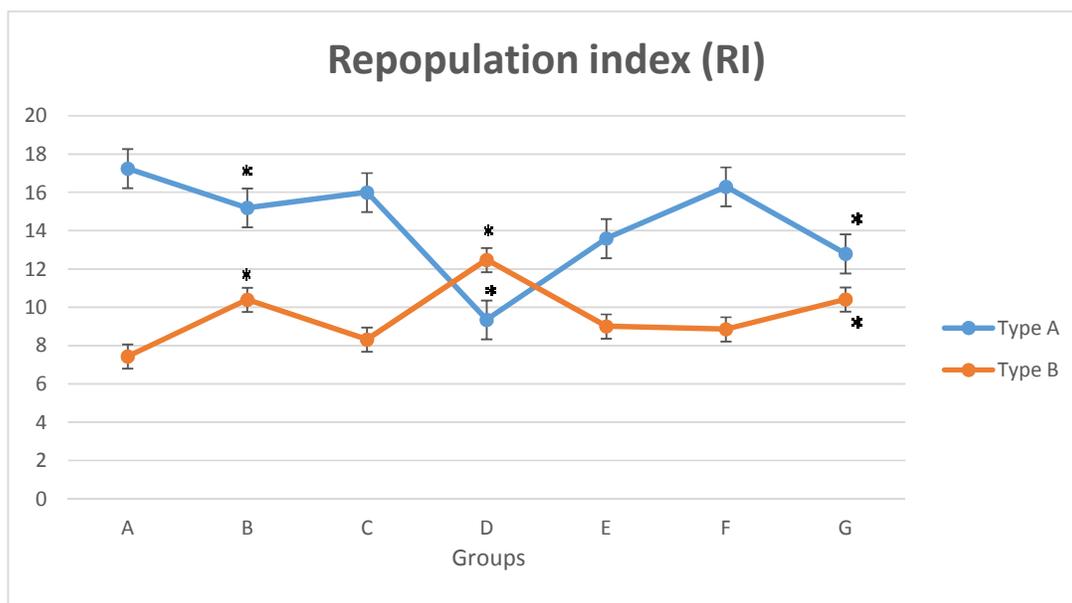
**Fig. 2. Effect of sodium benzoate (NaB) and  $\alpha$ -tocopherol ( $\alpha$ T) on sperm count ( $\times 10^6$ /ml)**  
 \*  $P < 0.05$ ; significantly different from control. Values are expressed as Mean  $\pm$  SD



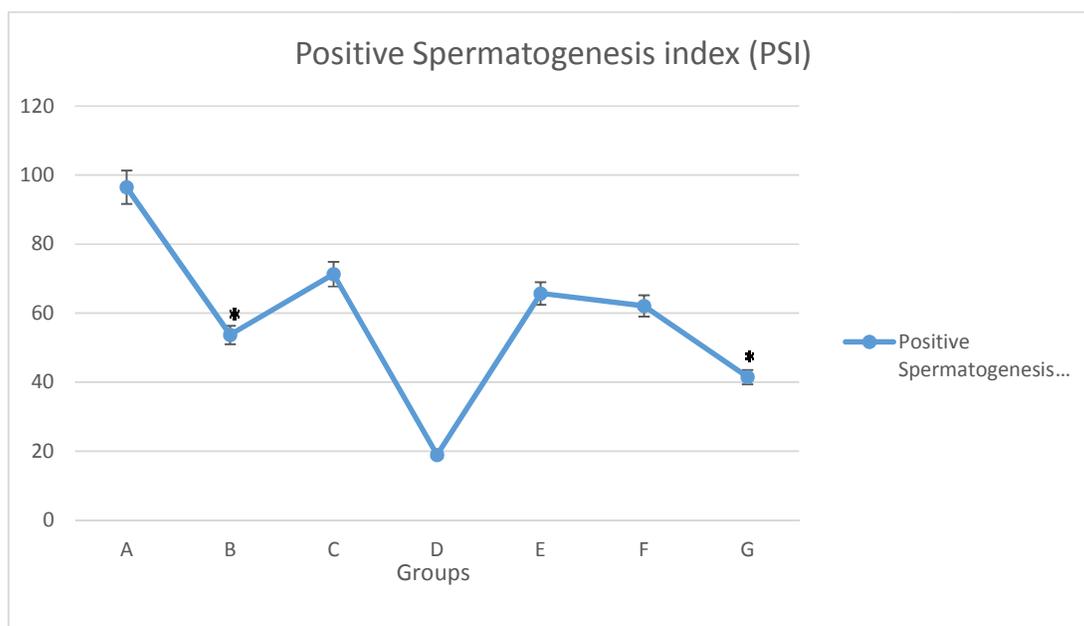
**Fig. 3. Effect of sodium benzoate (NaB) and  $\alpha$ -tocopherol ( $\alpha$ T) on sperm morphology (%)**  
 \*  $P < 0.05$ ; significantly different from control. Values are expressed as Mean  $\pm$  SD



**Fig. 4. Effect of Sodium Benzoate (NaB) and  $\alpha$ -Tocopherol ( $\alpha$ T) on Tubular differentiation index (TDI)**  
 \*  $P < 0.05$ ; \*\*  $P < 0.001$ , significantly different from control. Values are expressed as Mean  $\pm$  SD



**Fig. 5. Effect of sodium benzoate (NaB) and  $\alpha$ -tocopherol ( $\alpha$ T) on repopulation index (RI). A - blue chart is type a spermatogonia; B-light brown chart is Type B spermatogonia**  
 \*  $P < 0.05$ ; \*\*  $P < 0.001$  significantly different from control. Values are expressed as mean  $\pm$  SD

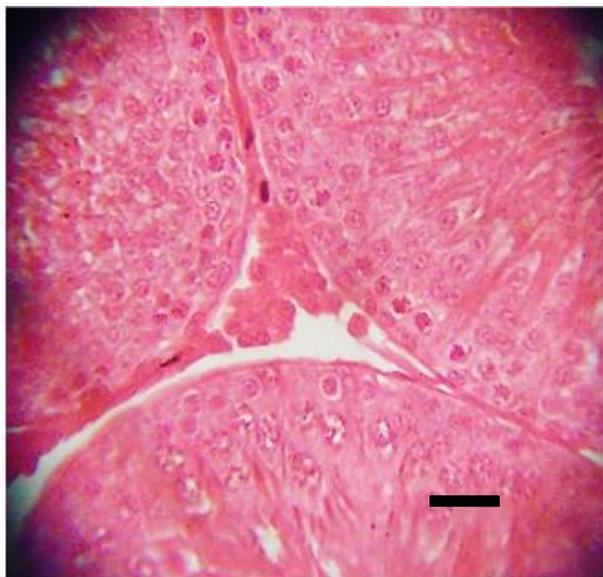


**Fig. 6. Effect of Sodium Benzoate (NaB) and  $\alpha$ -Tocopherol ( $\alpha$ T) on Positive Spermatogenesis index (PSI)**  
 \*  $P < 0.05$ ; \*\*  $P < 0.001$  significantly different from control. Values are expressed as mean  $\pm$  SD

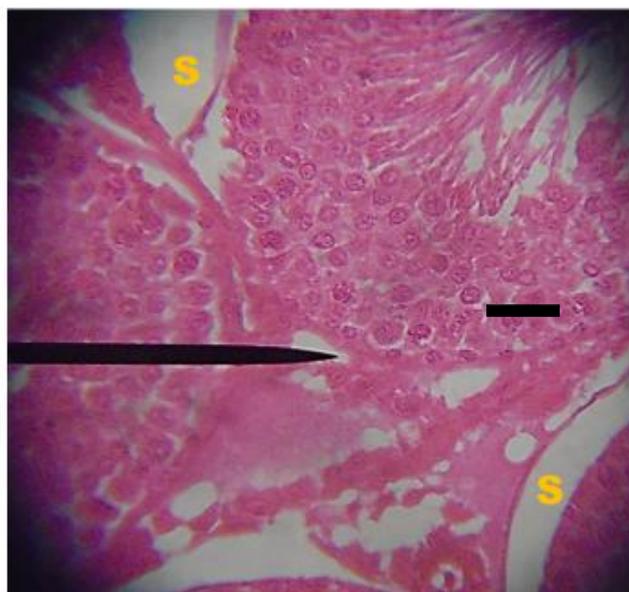
Sloughing from the basement membrane and vacuoles or open spaces between germ cells were the next most common histologic alteration observed in treated groups. In many cases, the interstitium was widened and infiltrated with fluid

as well (Figs 8-10 and 12). Semi-quantitative estimates of histopathological changes are captured in Table 5. Micrographs from groups C and E show that  $\alpha$ -T conferred significant protection from these effects as histological

features of tissue damage in these sections were less severe (Figs 10 and 13). In the groups where spontaneous recovery was studied after withdrawal of the chemical, significant recovery occurred, especially in group F which had the lower dose (Fig. 14).



**Fig. 7.** Micrograph from group A (control) animal showing parts of three seminiferous tubules with normal germ cell complement. Tissue architecture is normal. Scale bar = 35µm. H & E, ×400



**Fig. 8.** Micrograph from group B animal (200mg/kg NaB) showing parts of three seminiferous tubules with germ cell layers ripped clean of the basement in several tubules (S). Black arrow: interstitium with vacuoles and mild fluid infiltration. Scale bar = 35µm. H & E, × 400

**Table 4. Effect of sodium benzoate (NaB) and  $\alpha$ -tocopherol ( $\alpha$ T) on histo-morphometric parameters in seminiferous tubules**

Parameter	Group A (Control)	Group B (200mg/kg NaB)	Group C (200mg/kg NaB + $\alpha$ T)	Group D (400mg/kg NaB)	Group E (200mg/kg NaB $\alpha$ T)	Group F (200mg/kg NaB- W)	Group G (400mg/kg NaB-W)
Diameter (D) ( $\mu$ m)	251.4 $\pm$ 2.13	213.2 $\pm$ 1.70*	241.8 $\pm$ 7.25	196.5 $\pm$ 5.13**	224.5 $\pm$ 15.00	226.3 $\pm$ 2.26	204.0 $\pm$ 3.62*
Sectional area ( $A_c$ ). $\times 10^{-3}$ mm <sup>2</sup>	65.2 $\pm$ 0.96	41.6 $\pm$ 0.35*	60.4 $\pm$ 1.80	28.1 $\pm$ 0.90**	50.8 $\pm$ 1.30	52.4 $\pm$ 0.20	37.6 $\pm$ 0.70*
Height of Germ layers ( $\mu$ m)	87.5 $\pm$ 3.70	68.4 $\pm$ 1.30*	80.9 $\pm$ 1.40	56.5 $\pm$ 1.90**	78.7 $\pm$ 5.10	76.6 $\pm$ 2.40	64.5 $\pm$ 6.10*
Width of interstitial space ( $\mu$ m)	92.4 $\pm$ 1.20	261.3 $\pm$ 4.10*	102.0 $\pm$ 1.00	482.5 $\pm$ 8.30*	121.3 $\pm$ 4.20	152.2 $\pm$ 1.30*	362.0 $\pm$ 5.10*

\*  $P < 0.05$ , when compared to control. \*\*  $P < 0.001$ , when compared to control. Values are expressed as mean  $\pm$  SD

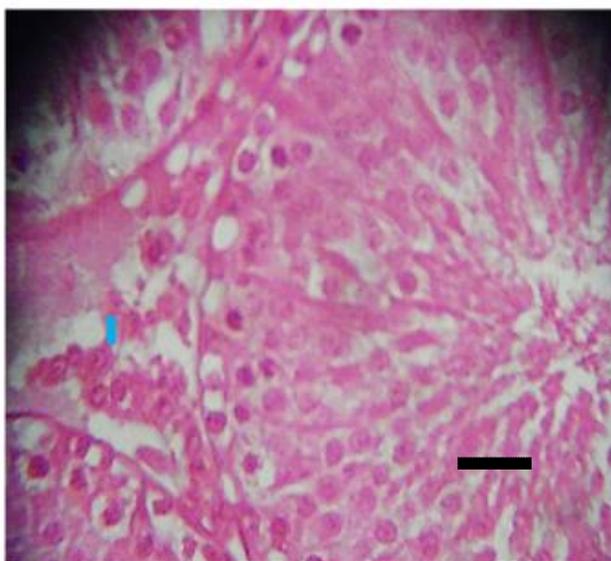
**Table 5. Effect of sodium benzoate (NaB) and  $\alpha$ -tocopherol ( $\alpha$ T) on quantitative histopathologic score**

Parameter	Group A (control)	Group B (200mg/kg NaB)	Group C (200mg/kg NaB + $\alpha$ T)	Group D (400mg/kg NaB)	Group E (200mg/kg NaB $\alpha$ T)	Group F (200mg/kg NaB-W)	Group G (400mg/kg NaB- W)
Intact (%)	96.5 $\pm$ 2.76	20.3 $\pm$ 6.14*	78.52 $\pm$ 7.4	11.85 $\pm$ 3.48**	66.5 $\pm$ 2.12	58.6 $\pm$ 3.83*	47.24 $\pm$ 3.74*
Atrophic/ Depleted (%)	0.0	46.3 $\pm$ 3.67*	5.22 $\pm$ 4.63	61.20 $\pm$ 1.76*	11.1 $\pm$ 5.32	10.3 $\pm$ 7.2	23.9 $\pm$ 6.12*
Sloughing (%)	0.0	15.9 $\pm$ 3.72*	0.0	11.3 $\pm$ 4.93*	5.83 $\pm$ 6.32	5.87 $\pm$ 1.96	12.84 $\pm$ 8.12
Degenerate (%)	0.0	2.4 $\pm$ 3.51	0.0	5.25 $\pm$ 6.21	7.37 $\pm$ 4.11	14.5 $\pm$ 3.83*	10.35 $\pm$ 2.65*
Vacuolised (%)	3.5 $\pm$ 3.6	15.1 $\pm$ 3.8*	16.26 $\pm$ 3.1*	10.4 $\pm$ 4.1*	9.2 $\pm$ 4.01*	10.7 $\pm$ 6.1*	5.67 $\pm$ 2.5

\*  $P < 0.05$ , when compared to control. Values are expressed as mean  $\pm$  SD



**Fig. 9.** Micrograph from group B animal (200mg/kg NaB) showing parts of three seminiferous tubules with massive germ cell loss in the tubule on the right, with an empty lumen L, and large vacuoles V. There are no visible germ cells in this tubule. The interstitium also has vacuoles and moderate fluid infiltration. H & E, × 400



**Fig. 10.** Micrograph from group C (200mg/kg NaB +  $\alpha$ -T) animal showing parts of three seminiferous tubules with better preservation. Germ cell layers are complete in the tubule on the right, though empty spaces are visible on its basement with nuclei that appear to be shrinking. The interstitium is infiltrated with cells and fluid. H & E, × 400

#### 4. DISCUSSION

The food industry makes use of a wide range of chemical substances of which NaB and MSG are typical examples. These are agents that have

been connected with toxicity in a number of organs at specific doses [11,29]. As we have already noted, male fertility has been declining and WHO cut off levels for diagnosing sub-fertility has been reduced from 40million to

20million per ml in the latest guidelines [30]. Research evidence has incriminated endocrine disruptors as the chief culprits in this process [31]. In this study, NaB at the doses used suppressed all sperm parameters and caused extensive alterations in histology of the testis in

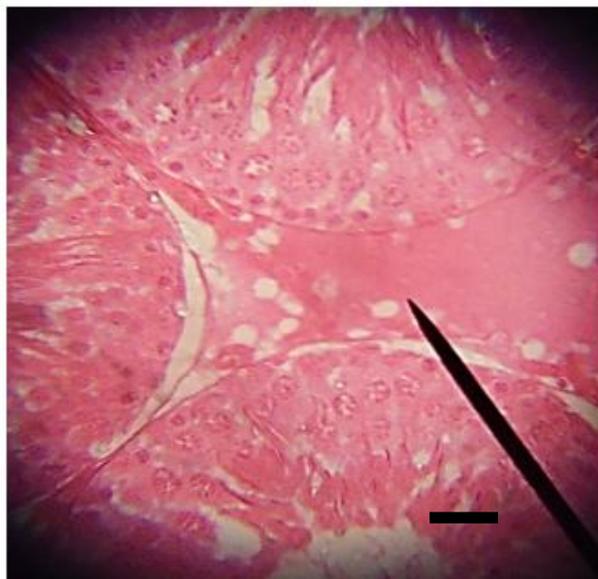
the unprotected groups. These results support the findings of some previous researchers [32,33]. The anti-fertility effects were moderated by the presence of  $\alpha$ T. This study therefore adds to published evidence of the toxicity of this agent in other organs such as liver and kidneys [11].



**Fig. 11. Micrograph from group D (400mg/kg NaB) animal showing parts of three seminiferous tubules. There is massive germ cell loss in the tubule on the top, with an empty widened luminal space and a large vacuole at the arrow point. H & E,  $\times$  400**



**Fig. 12. Micrograph from group D (400mg/kg NaB) animal showing parts of four seminiferous tubules. The two tubules in the top have germ layers ripped off the basement membrane leaving the empty space (S) above the arrow point. The interstitium is widened and infiltrated below the arrow. H & E,  $\times$  400**



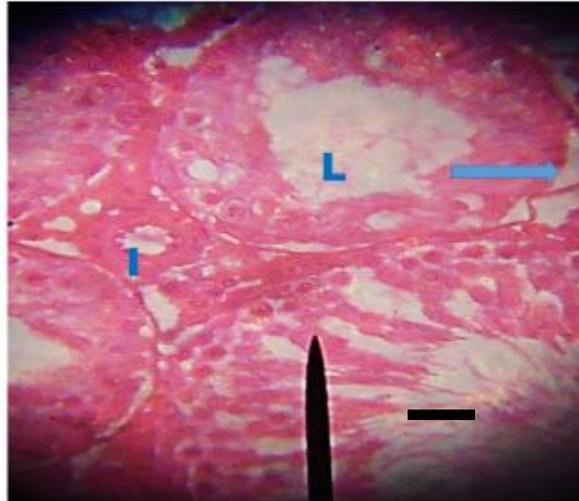
**Fig. 13.** Micrograph from group E (200mg/kg NaB +  $\alpha$ -T) animal showing parts of three seminiferous tubules with better preservation of cells and there is normal tissue architecture. The basement has been ripped off in parts of the tubule to the left of the micrograph. Black arrow: shows a widened interstitium with only empty vacuoles and fluid. H & E,  $\times$  400



**Fig. 14.** Micrograph from group F (200mg/kg NaB, withdrawal) animal showing parts of three seminiferous tubules. There is sloughing of the germ cell layer from the basement membrane at the arrow point and in the middle of germ cell layers (S). H & E,  $\times$  400

The ability of NaB to affect the morphology of spermatozoa, the final product of spermatogenesis suggests that it crosses the blood-testis barrier in quantities sufficient to

impair that process [31]. Many testiculotoxic agents act in a dose-dependent manner. The effect of NaB on sperm concentration in this study was largely independent of dose,



**Fig. 15. Micrograph from group G (400mg/kg NaB withdrawal) animal showing parts of four seminiferous tubules. There is massive germ cell loss in the tubule on the top right with open luminal area (L) and sloughed germ cells from the basement which takes a wavy outline indicated by the blue arrow. The interstitium (I) also has some vacuoles in it. H & E, × 400**

suggesting that beyond a critical threshold dose, at least concerning this parameter, the all or none law might apply to the effects of this substance.

A careful examination of germ cell populations in this study suggests that NaB acts at virtually all levels of the seminiferous epithelium. There was considerable germ cell atrophy and reduction in both tubular differentiation and positive spermiogenesis indices. Studies have reported similar reduction in indices of spermatogenesis and tubular morphology when diabetic rats were exposed to high doses of MSG [34]. Both MSG and NaB are ubiquitous food additives, and it is quite significant that they reduce both spermiation and the process of spermatogonial repopulation. Al-Ani et al. [35], had reported the reduction of spermatocytes and spermatids with NaB but this study shows that in addition, there is a significant reduction in the production of spermatogonia to replace those being recruited into the spermatogenic process. To sustain sperm production, the pool of spermatogonia must be replenished by the production of new cells from mitotic division as older ones are utilized in spermatogenesis. NaB was shown in this study to interfere with this process.

Testicular levels of the key anti-oxidants were reduced in unprotected groups in this study. This suggests that the treatment caused a down-regulation in their production. It is noteworthy that

lipid peroxidation level was elevated and almost all deleterious effects of NaB seen in this study were significantly attenuated by co-administration of  $\alpha$ T. This suggests that oxidative stress may have been a major contributor to the adverse effects of NaB. Studies have implicated oxidative stress in testicular damage induced by substances such as Adriamycin [14], artesunate [36] and green tea [37].

In all complex organisms, normal cellular metabolism, especially energy generating processes, require oxygen. Unfortunately, these metabolic processes also generate free oxygen radicals. These molecules are so reactive and unstable that if not removed quickly enough, they destroy tissues by oxidizing critical components of cells such as DNA, proteins and membrane lipids. Mature sperm membrane has a high content of phospholipids. Spermatogenesis also involves a high level of mitotic activity at least in its early stages. The later stages involve major morphologic transformation. The entire process is now known to require the expression of a large number of genes [38]. The testis is therefore highly vulnerable to any substance that damages DNA.

Living tissues such as the testis prevent oxidative stress and remain healthy by maintaining a delicate balance between the production of pro-oxidants and anti-oxidants. Studies of men with varicoceles show that high seminal reactive

oxygen species (ROS) is associated with reduced fertility. It has been suggested that a useful oxidative stress score can be calculated from the ratio of ROS to total anti-oxidant capacity (TAC) in these cases [39-41].

The testis is equipped with a wide array of natural anti-oxidants and free radical scavengers such as ascorbic acid, alpha-tocopherols and GSH. A number of studies, including those in which researchers used ascorbic acid [42]) and  $\alpha$ -tocopherol [43], show that testicular damage can be mitigated if exogenous anti-oxidants are administered along with the toxic agents. The findings of the present study agree with these previous ones. It is significant that  $\alpha$ -tocopherol mitigated testicular damage in this study because there is a study which reported that when NaB was administered with ascorbic acid, rather than protecting the testis, the two substances combined to damage the organ in a synergistic manner [32]. This emphasizes the need for more studies on the effects of these substances on male reproduction.

## 5. CONCLUSION

In this study we have characterized in some detail, germ cell perturbations and fertility suppression induced by NaB in male rats. We have demonstrated that this food additive causes considerable impairment of fertility indices in rats. We have also shown from the changes in the oxidative status of testicular tissue and the effects of  $\alpha$ -tocopherol, that oxidative stress is likely to be a key pathway for the deleterious effects that were observed in this study. In view of the widespread use of NaB, which is commonly called E112 in the food industry, more studies are needed to increase our knowledge of the effects of this agent on male reproduction.

## DISCLAIMER

The products used for this research are commonly used in our area of research and country. There is absolutely no conflict of interest between the authors and producers of the products because we do not intend to use these products as an avenue for any litigation but for the advancement of knowledge. Also, the research was not funded by the producing company rather it was funded by personal efforts of the authors.

## CONSENT

It is not applicable.

## ETHICAL APPROVAL

The experiments were conducted according to guiding principles in the care and use of animals of the American Physiological Society, 2002. Reference number for approval of Ethics Committee is: AEC/2019/12.

## COMPETING INTERESTS

Authors have declared that no competing interests exist.

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