Histo-morphometric Evidences for Testicular Derangement in animal models submitted to chronic and Sub-chronic Inhalation of Fragrance

[1*]Akunna G.G.; [1]Saalu L.C. [2]Ogunlade B.; [1]Akingbade A.M.; [3]Anderson L.E.; [4]Olusolade F.S

[1] Department of Anatomy, College of Medicine, Lagos State University, Ikeja Lagos, Nigeria
[2] Department of Anatomy, College of Medicine, University of Lagos, Idi Araba, Lagos, Nigeria
[3] Department of Anatomy, Afe Babalola University, Ado Ekiti, Nigeria
[4] Department of Parasitology, Afe Babalola University, Ado Ekiti, Nigeria
Corresponding Author: AKUNNA, Gabriel Godson
Department of Anatomy, College of Medicine, Lagos State University, Ikeja Lagos, Nigeria
+2348038619526, ggakunna@yahoo.com

ABSTRACT

Only few people are aware that in the manufacturing of a single bottle of perfume, about 600 individual chemical ingredients coined as fragrance are used. Exposure and a lingering culture of trade secrecy and undisclosed substances within the fragrance industry has remained an important factor when considering the hazardous and degenerative effect of these so called scents.

In this study, we evaluated the histopathological effect of two popular perfumes used in Nigeria on the testis of rat. Sixty adult male Wistar rats were randomly divided into six groups of ten rats each. Group A and B rats (Controls) rats were exposed (6hrs day^{-1}) to 5 ml kg^{-1} body weight of normal saline for 56 days and 112 days via whole body inhalation respectively, Group C and D rats were exposed (6hrs day^{-1}) to 5 ml kg^{-1} body weight of one of the perfume designated as F^1 for a period of 56 days and 112 days via whole body inhalation respectively while Group E and Group F rats were exposed (6hrs day^{-1}) to 5 ml kg^{-1} body weight of one of the perfume designated as F^2 for a period of 56 days and 112 days via whole body inhalation respectively.

Result indicated that the testes of exposed groups of rat had worst geometric values and histological profiles compared to the control group of rat. These results indicated and validated the histopathological role of fragrance components in rat.

Keywords: Fragrance, Testes, Stereology, Infertility, Oxidative Stress

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INTRODUCTION

A sturdy debate over whether male reproductive ability is influenced by environmental factors has long persisted. In 1977, there was a remarkable and sensational report by Whorton *et al.* (1977) in which 14 out of 25 male workers involved in producing dibromo-3-chloropropane (DBCP), were diagnosed as azoospermic or oligospermic. In 1992, Carlsen *et al.* (1992) reported a marked decrease in sperm count in previous 50 years. While some researchers continue registering their doubts over any correlation between environmental factors and male infertility (Chapin *et al.*, 1994), many researchers have asserted that societal progress in advanced countries and worsening of the natural environment have an imperative negative bearing on male fertility. Long-reported risk factors include exposure to radiation, electromagnetic waves, and a variety of chemical substances including fragrance.

Fragrance refers to a combination of substances that gives perfume or cologne a unique scent. Copious documentations have indicated that 82 percent of perfumes labeled "natural ingredients" actually contain synthetic fragrances (Rastogi *et al.,* 1996).

The consumers are kept back about the actual chemicals in fragrance that poses a potential health risks. Such chemicals that affect male reproductive hormones may be a factor in infertility and has been known as endocrine disruptors (Giudice, 2006, Saalu *et al.,* 2010, Akunna *et al.,* 2013).

It has been reported that perfumes, colognes, body sprays and care products contained an average of four potential hormone-disrupting chemicals.

In male reproductive anatomy, endocrine disruptors have severally been implicated as teratogens, resulting in cryptorchidism, hypospadias and impairment of body function normally regulated by natural hormone signaling (Wang and Baskin, 2008, Akunna *et al.,* 2011, Akunna *et al.,* 2013). Studies have shown that these chemicals causes damage by mimicking or disrupting natural estrogen, testosterone and thyroid pathways (Soto et al., 2009).

Although the implication of subsequent exposure to these chemicals have not been critically understood, recent findings has clearly demonstrated disruption in spermatogenesis (Akunna et al., 2014), liver damage (Akunna *et al.,* 2011) and other tissue toxicity in animals exposed to fragrance components (Johansen et al., 2003, Elberling *et al.,* 2004, Breast Cancer Fund, 2008, Schnuch *et al.,* 2010). In animal model studies, fragrance exposure has lead to spermatotoxicity and infertility, congenital malformation in penises and abnormal testes (Akunna *et al.,* 2014**).**

According to published scientific studies, diethyl phthalate and octinoxate which are major components of perfume and sunscreen respectively has been implicated in sperm damage, apoptosis and interference with estrogen and androgens in human respectively (Giudice,

2006, Wang and Baskin, 2008, Silva *et al.,* 2004 ,Schreurs *et al.,* 2005, Swan, 2008, CDC, 2009). Sperm DNA damage was reported in a study of 168 men and 379 men exposed to diethyl phthalate and DEHP respectively (Duty *et al.,* 2003, Hauser *et al.,* 2007, López-Carrillo *et al.,* 2010).

A strong relationship between diethyl phthalate exposure during pregnancy and changes in male genital development and alterations in levels of male sex hormones in the baby boys has been established (Swan *et al.,* 2005, Main *et al.,* 2006)**.**

However, some animal model studies of fragrance components could not report any alteration in the reproductive anatomy (Howdeshell *et al.,* 2008). However, at the highest levels of exposure, DEP has been linked to liver abnormalities, elevated cholesterol (Sonde et al., 2000) and birth defects (ATSDR, 1995). In our laboratory in 2013, we reported a decrease in sperm count, motility and an increase in abnormal sperm morphology following exposure to fragrance components (Akunna *et al.,* 2011, Kwack *et al.,* 2009). In this study, we demonstrated the testicular histopathological effect of these components in rat using scientifically proven histo-morphometric principles.

MATERIALS AND METHODS

In this study, two (2) commonly used perfumes in Nigeria designated as F1 and F2 were obtained from Bayous Cosmetics in Lagos, Nigeria and were kept under standard temperature. As described by Akunna *et al.* (2011), sixty adult male Wistar rat (12-13 weeks old) weighing 190-220 g were used for the study. The rats were randomly divided into six groups (**A**-**F**) of ten rats and the average weight difference between and within groups did not exceed ± 20% of the average weight of the sample population. **Group A** rats served as the first control (Control I) and were exposed (6hrs day^{-1}) to 5 ml kg^{-1} body weight of normal

saline for 56 days. **Group B** rats served as the second control (Control II) group and were exposed (6hrs day^{-1}) to 5 ml kg^{-1} body weight of normal saline for a period of 112 days**. Group C (**Sub-chronic I**)** and **Group D (**Chronic I**)** rats were exposed (6hrs day^{-1}) to 5 ml kg^{-1} body weight of Fragrance I **(F^1)** for a period of 56 days and 112 days respectively. **Group E** (Sub-chronic II) and **Group F (**Chronic II**)** animals were exposed (6hrs day^{-1}) to 5 ml kg^{-1} body weight of Fragrance II (**F^2**) for a period of 56 days and 112 days respectively. The study is consistent with the standard of the use of laboratory animals. Small balls of cotton wool were soaked with the fragrance (Experimental Groups) and normal saline (Control Groups) respectively. The wools were then placed in a Petri dish inside the cages and covered with perforated plastic to prevent direct contact for an exposed duration of 6hrs day^{-1} throughout the period of study (Akunna *et al.*, 2011).

Animal sacrifice and sample collection

The rats were first weighed and then were sacrificed by cervical dislocation. The abdominal cavity was opened up through a midline abdominal incision to expose the reproductive organs. The testes were excised and trimmed of all fat. The testicular weights of each animal were evaluated with an electronic analytical and precision balance (BA 210S, d=0.0001-Sartoriusen GA, Goettingen, Germany). The testes volumes were measured by water displacement method. The two testes of each rat were measured and the average value obtained for each of the two parameters was regarded as one observation. One of the testes of each animal was fixed in 10% formol-saline for histological examination (Akunna *et al.*, 2013).

Histo-morphometric evaluation

The testes after whole body perfusions were transferred to a graded series of ethanol. On day 1, they were placed in 70% alcohol for 7 hours, then transferred to 90% alcohol and left in the

latter overnight. On day 2, the tissues were passed through three changes of absolute alcohol for an hour each and then cleared in xylene. Once cleared, the tissues were infiltrated in molten paraffin wax in the oven at 58ºC.

Three changes of molten paraffin wax at one-hour interval were made, after which the tissues were embedded in wax and blocked out. Prior to embedding, it was ensured that the mounted sections to be cut by the rotary microtome were orientated perpendicular to the long axes of the testes. These sections were designated “vertical sections”. Serial sections of 5 μm thick were obtained from a solid block of tissue, fixed on clean slides to which Mayer’s egg albumin had been coated to cement the sections to the slides properly and were stained with haematoxylin and eosin stains, after which they were passed through a mixture of equal concentration of xylene and alcohol. Following clearance in xylene the sections were oven-dried between 35°C and 40°C (Sheehan and Hrapchak, 1987).

The slides were viewed under a research microscope connected to a computer monitor for qualitative and quantitative evaluation. For each testis, seven “vertical sections” from the polar and the equatorial regions were sampled (Gundersen and Jenson, 1987) and an unbiased numerical estimation of the following morphometric parameters was determined using a systematic random scheme: testicular volume and weight; diameter (D) and cross-sectional area of the seminiferous tubules (A_C); number of profiles of seminiferous tubules per unit area of testis (N_A); and numerical density of the seminiferous tubules (N_V). Seven “vertical sections” per testis were selected by a systematic sampling method that ensured fair distribution between the polar and equatorial regions of each testis. Briefly, a section was taken at the equator of each testis; one on each side of the equator, three-quarters of the distance between the pole and the equator; another half-way between each pole and the equator; and one on each side of the equator, a quarter of the distance from each of the pole. For each stereological parameter (D, A_C, N_A and N_V), five randomly selected fields from all

the seven sections of a single testis were viewed, and estimation on each carried out. The average from a total of seventy readings from five fields in seven sections of the two testes of one rat was obtained and this was recorded as one observation. The evaluation of the diameter was done with calibrated eyepiece and stage grids mounted on a light research microscope at X 100 magnification. Estimation of volume density of testicular components and number of seminiferous tubules were done on a computer monitor onto which a graph sheet was superimposed and on which slides were projected from a research light microscope.

Diameter (D) **of seminiferous tubules**: The diameter of seminiferous tubules with profiles that were round or nearly round were measured for each animal and a mean, D, was determined by taking the average of two diameters, $D_1$and D_2 (Perpendicular to one another). $D_1$and D_2were taken only when $D_1/D_2 \geq 0.85$.

Cross-sectional area (A_C) **of the seminiferous tubules**: The cross-sectional areas of the seminiferous tubules were determined from the formula $A_C = \pi D^2/4$, (where π is equivalent to 3.142 and D the mean diameter of the seminiferous tubules).

Number of profiles of seminiferous tubules in a unit areaof testis (N_A): The Number of profiles of seminiferous tubules per unit area was determined by using the unbiased counting frame proposed by Gundersen and Jenson (1987). Using this frame, in addition to counting profiles completely inside the frame we counted all profiles with any part inside the frame provided they did not touch or intersect the forbidden line (full-drawn line) or exclusion edges or their extension.

Numerical Density (N_V) **of seminiferous tubules**: This is the number of profiles per unit

volume and was determined by using the modified Floderus equation:

$N_V = N_A / (D + T)$ (Gundersen and Jenson, 1987) where, N_A is the number of profiles per unit area, D is the diameter and T the average thickness of the section.

Statistical Analysis

All data were expressed as mean ± SD of number of experiments (n = 10). The level of homogeneity among the groups was tested using Analysis of Variance (ANOVA) as done by (Snedecor and Cochran, 1980). Where heterogeneity occurred, the groups were separated using Duncan Multiple Range Test (DMRT). Values of $p < 0.05$ and $P < 0.005$ were considered to indicate a significant difference between groups (Duncan, 1957). Analysis of data was done using both electronic calculator and Statistical Package for Social Sciences (SPSS)/ PC computer program (version 11.0 SPSS, Cary, NC, USA).

RESULT AND DISCUSSION

Changes in gross anatomical parameters

There was a significant decrease ($p<0.05$ and $p<0.005$ at 56days and 112days respectively) in body weight, testicular weight, testicular volume and testis weight/body weight ratio of exposed rats which was time dependent**.** This could be due to reduction in food intake by the exposed animals and the level of stress which could have been caused in part, by the inhaled substances (Akunna *et al.*, 2011). The result in this study conform to previous reports of significant reduction in gross anatomical parameters due loss in testicular integrity as a result of various oxidative derangements (Akunna *et al.*, 2011, 2014). Carlssen *et al.* (1992) reported a decrease in body weight and gonad somatic index following exposure of animal models to musk ketone.

The significant decrease in testicular weight in our study could have been as a result of Sertoli cell apoptosis due to fragrance toxicity. A direct association between testis size and spermatogenesis has been documented. Testicular size which is said to be determined at perinatal and prepubertal period when the sertoli cell mass is established. More Sertoli cells means more germ cells per testis, and the number of sertoli cells per gram of tissue combined with the number of spermatids per sertoli cells is associated with sperm production per gram of testis (Saalu *et al.,* 2008). This report is in accordance with our previous report on decreased sperm concentration, sperm motility and increase in abnormal sperm morphology of rat exposed to fragrance. As shown in Table [**1**], the control group of rats had a significant (*p<0.005*) increase in body weight when compared to fragrance-exposed rats that loss significant amount of weight. Again the increase in body weight of the control rats could mean that they were still in their active growth phase during the study (Saalu *et al.,* 2008, 2011).

Table 1: Body weight changes in experimental animal exposed (6hrs day^{-1}) to fragrance components (5ml/kg body weight for 56 days and 112 days). *Values are expressed* as Mean ± SD for n=10; *P**<0.05, *P***<0.005 significantly different from control.

Gross Anatomical Parameters	*Initial Body Weight (g)*	*Final Body Weight (g)*	*Body Weight. Differences*
Group A	220.3±0.1	273.1±3.1	52.8
Group B	210.2±1.4	281.1±1.4	70.9
Group C	205.6±0.1	180±1.3	25.6*
Group D	198±1.1	140±0.4	58*
Group E	210.1±2.2	180±3.2	30.1**
Group F	203.1±5.0	150.3±1.0	52.8**

Where:

A: 5 ml kg^{-1} body weight normal saline (6hrs day^{-1}) for 56 days (Control I)
B: 5 ml kg^{-1} body weight of normal saline (6hrs day^{-1}) for 112 days (Control II)
C: 5 ml kg^{-1} body weight of F^1 (6hrs day^{-1}) for 56 days (Sub-chronic I)
D: 5 ml kg^{-1} body weight of F^1 (6hrs day^{-1}) for 112 days (chronic I)
E: 5 ml kg^{-1} body weight of F^2 (6hrs day^{-1}) for 56 days (Sub-chronic II)
F: 5 ml kg^{-1} body weight of F^2 (6hrs day^{-1}) for 112 days (Chronic II)

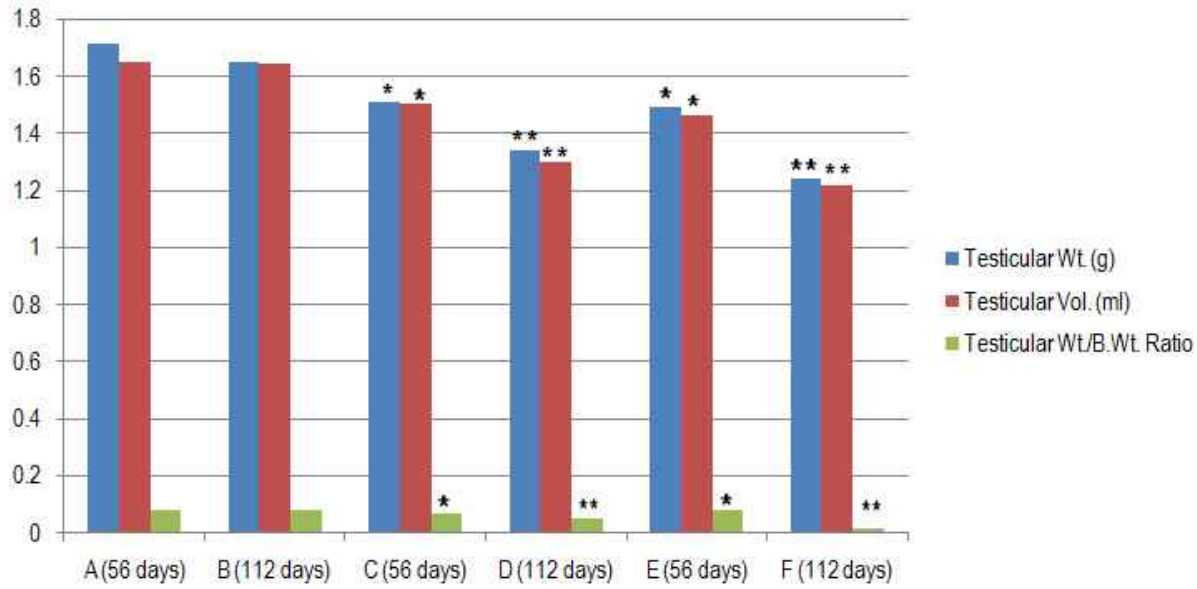

Figure 1: The effect of Fragrance exposure (5ml kg^{-1} body weight for 56 days and 112 days) on testicular weight, testicular volume and testicular weight/body weight ratio of experimental rat. *Values are expressed* as Mean ± SD for n=10; *P**<0.05, *P***<0.005 significantly different from control.

Where:

A: 5 ml kg^{-1} body weight normal saline (6hrs day^{-1}) for 56 days (Control I)
B: 5 ml kg^{-1} body weight of normal saline (6hrs day^{-1}) for 112 days (Control II)
C: 5 ml kg^{-1} body weight of F^1 (6hrs day^{-1}) for 56 days (Sub-chronic I)
D: 5 ml kg^{-1} body weight of F^1 (6hrs day^{-1}) for 112 days (chronic I)
E: 5 ml kg^{-1} body weight of F^2 (6hrs day^{-1}) for 56 days (Sub-chronic II)
F: 5 ml kg^{-1} body weight of F^2 (6hrs day^{-1}) for 112 days (Chronic II)

Testicular morphometry

In other to quantify the histological changes in this study scientifically, stereological evaluation of the tissues was employed. It has been shown that the level of testicular

androgen is directly proportional to the size of the testicular interstitium and the number of Leydig cell (Yama *et al.,* 2011, Akunna *et al.,* 2014).

In this study, there was a significant ($p<0.05$ at 56days and 112days respectively) reduction in the mean seminiferous tubular diameters, cross sectional area, number of profiles per unit area and the mean numerical density of seminiferous tubules of rat exposed to fragrance components (5 ml kg^{-1} b. wt for 56 days and 112 days) when compared to the control groups that had significant increase in stereological parameters [Table **2**].

In experimental animals such as rats, the interstitial and tubular compartment comprises about 2.6% and about 60–80% of the total testicular volume. In the human testis, the interstitial compartment represents about 12–15% of the total testicular volume, 10–20% of which is occupied by Leydig cells. The Leydig cells being the most important cell of the interstitium and the source of testicular testosterone and of insulin-like factor 3 (INSL3).

Deducing from previous documentation, our result herein could only suggest a significant decrease in interstitium which could be due to reduction in the number of androgen producing cells (Interstitial cells of Leydig) (Saalu *et al.,* 2006, Yama *et al.,* 2011). However, the stereological changes evidenced in our study were not time-dependent.

Although the stereological values obtained from our study are actually sound evidence of the three-dimensional characteristics of the rat testis exposed to fragrance components, it will be scientifically incorrect to predict the expected consequence of these degenerative changes on entire spermatogenic process because the present morphometric data only forms part of the entire delineation. Other factors are clearly important. These include number of spermatogenic cells in the basal compartment and the Sertoli-Sertoli cell barrier which determines the number of cells in the adluminal compartment (Yama *et al.,* 2011).

Table 2: The effect of Fragrance components (5 ml kg^{-1} b. wt for 56 days and 112 days) on seminiferous tubular diameter (µm), cross sectional area Ac (×103µm2), numerical densities of seminiferous tubules NA (×10-8µm-2) and number of profiles per unit area Nv (×10-10µm-3). *Values are expressed* as Mean ± SD for n=10; *P**<0.05, *P***<0.005 significantly different from control.

Treatment Groups	*D(µm)*	*A_c (×10^3µm^2)*	*N_A (×10^{-8}µm^{-2})*	*N_v (×10^{-10}µm^{-3})*
Group **A**	161.3±2.3	33±1.1	29.1±1.8	21.3±2.2
Group **B**	152±8.1	34±1.2	30.2±1.1	20.1±2.1
Group **C**	110±2.2*	26.1±0.1*	20.2±3.3*	15±1.3*
Group **D**	107.3±0.3*	20±1.1*	14.1±3.1*	12.3±1.6*
Group **E**	125.9±0.3*	24.8±3.3*	20.2±2.1*	17.1±2.1*
Group **F**	96.3±2.0*	22±0.4*	17.3±9.0*	13.1±4.0*

Where:

A: 5 ml kg^{-1} body weight normal saline (6hrs day^{-1}) for 56 days (Control I)
B: 5 ml kg^{-1} body weight of normal saline (6hrs day^{-1}) for 112 days (Control II)
C: 5 ml kg^{-1} body weight of F^1 (6hrs day^{-1}) for 56 days (Sub-chronic I)
D: 5 ml kg^{-1} body weight of F^1 (6hrs day^{-1}) for 112 days (chronic I)
E: 5 ml kg^{-1} body weight of F^2 (6hrs day^{-1}) for 56 days (Sub-chronic II)
F: 5 ml kg^{-1} body weight of F^2 (6hrs day^{-1}) for 112 days (Chronic II)

Histological profile of the testicular tissue

Increased spermatogenic efficiency has been implicated with higher seminiferous epithelium, increased number of spermatogonia production, and decreased germ cell loss (Saalu *et al.*, 2007).

About 35–40% of the volume of the germinal epithelium is represented by Sertoli cells. The intact testis with complete spermatogenesis contains 800–1200 × 10^6 Sertoli cells (Zhengwei *et al.*, 1998) or approximately 25 × 10^6 Sertoli cells per gram testis (Raleigh *et al.*, 2004).

In this study, rats that were exposed to fragrance showed destructive changes in their seminiferous tubular epithelium and interstitial tissues evidenced by an uneven arrangement

at the basal portion of the germinal epithelium Fig [**4-7**]. There profile was characterized by hypo spermatozoa formation in the tubules when compared to the control group of rat Fig. [**2-3**]. The tubular epithelium of groups of rat treated for 54 days (Sub-chronic exposure) exhibited lesser pathological alterations to the when compared to the group treated for 112 days (Chronic exposure). This was a sound conclusion of the time dependent manner of fragrance toxicity.

From our studies on fragrance, we can conclude herein that fragrance components are testiculotoxic in rat. Although numerous reports on the effect of fragrance on human health have been documented, there is a need for further investigation on whether fragrance acts directly as spermatotoxins or through a steroidal pathway. This is more pertinent when one consider that in species such as rat with lower proportion of Sertoli cells in the seminiferous epithelium, there is higher Sertoli cell and greater spermatogenic efficiencies when compared to humans.

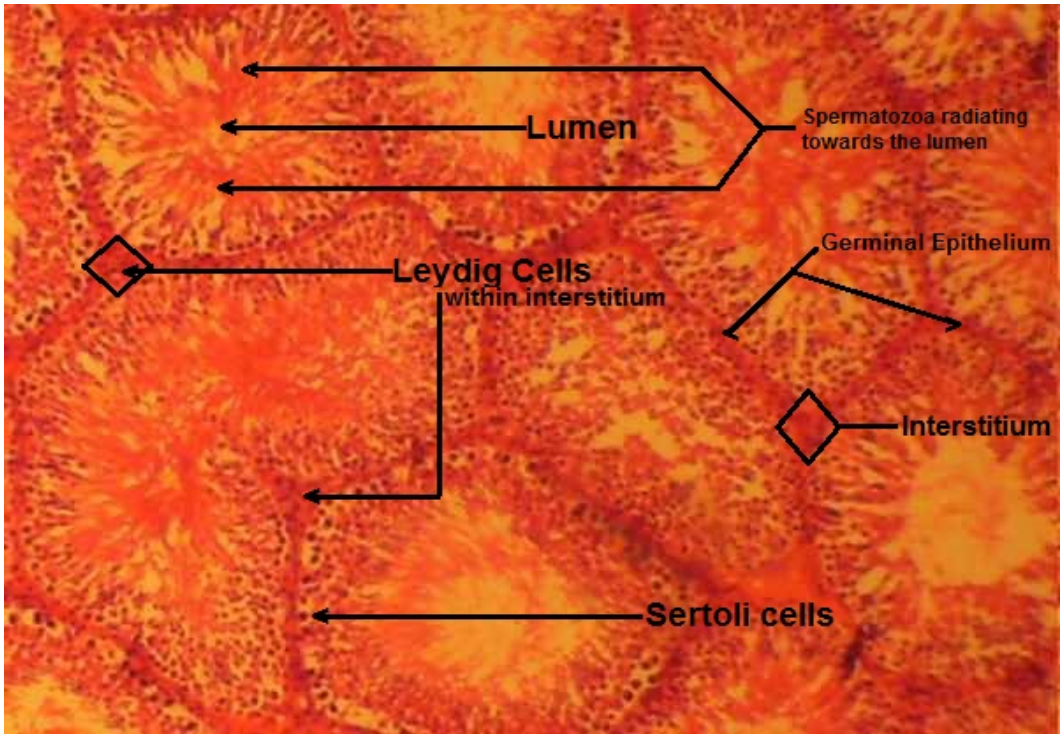

Figure 2: Testicular histological profile of Group A rats (Control I) (5 ml kg^{-1} body weight of normal saline for 56 days).

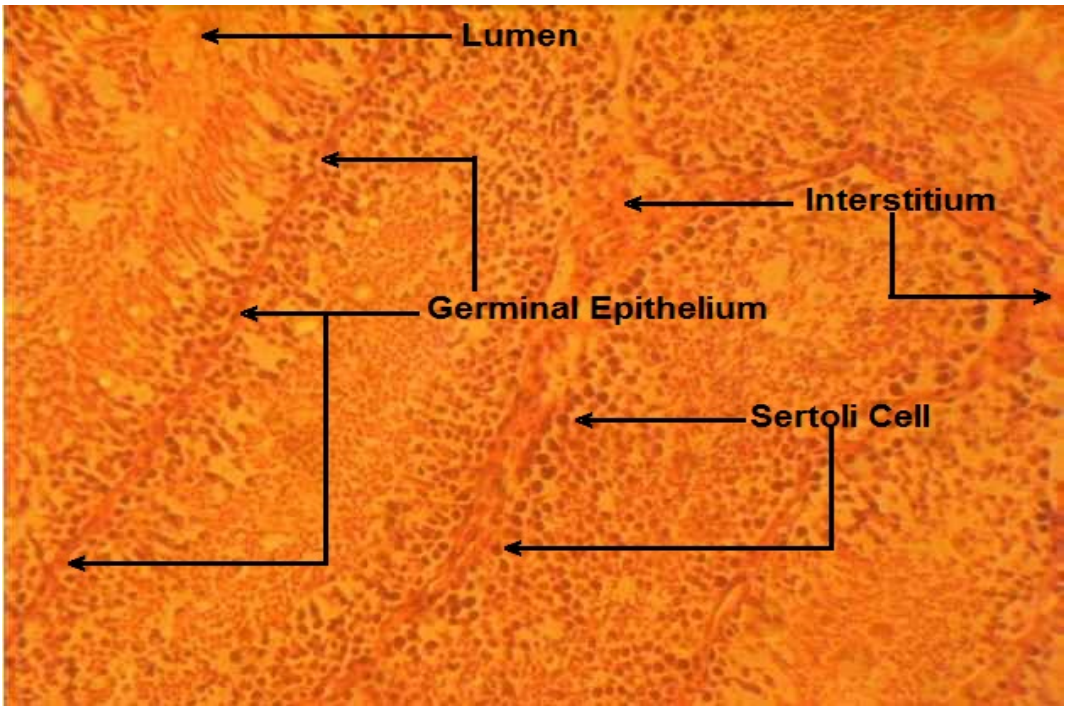

Figure 3: Testicular histological profile of Group B rats (Control II) 5 ml kg^{-1} body weight of normal saline for 112 days).

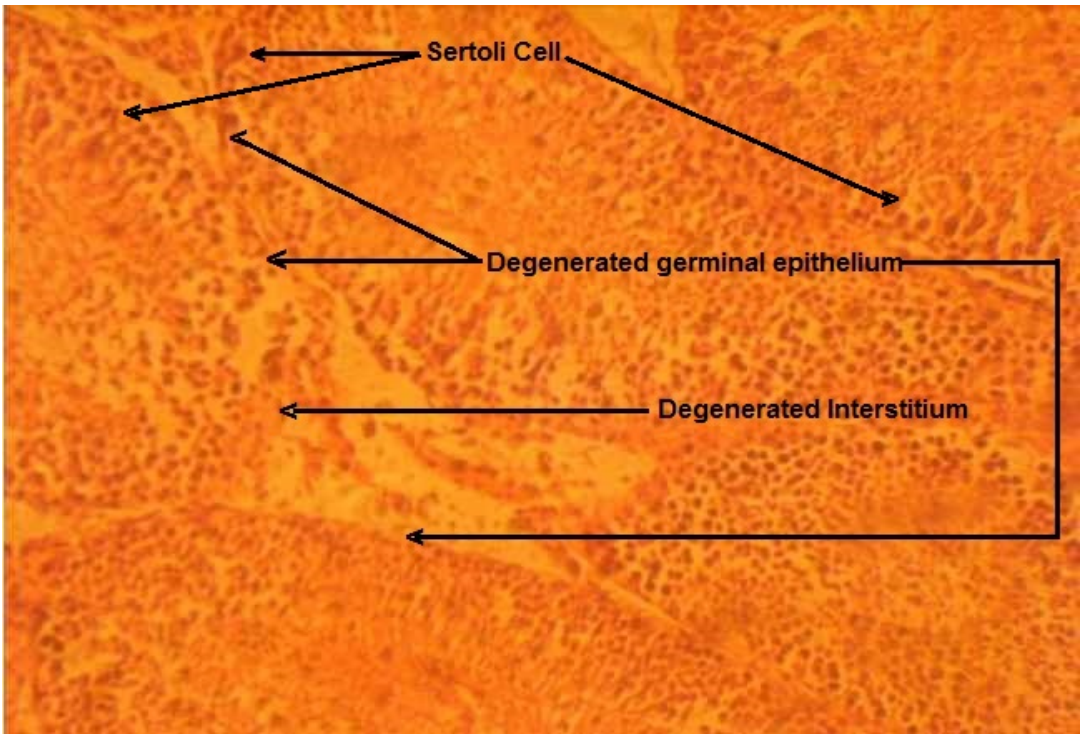

Figure 4: Testicular histological profile of Group C rats (5 ml kg^{-1} body weight of F^1 for 56 days).

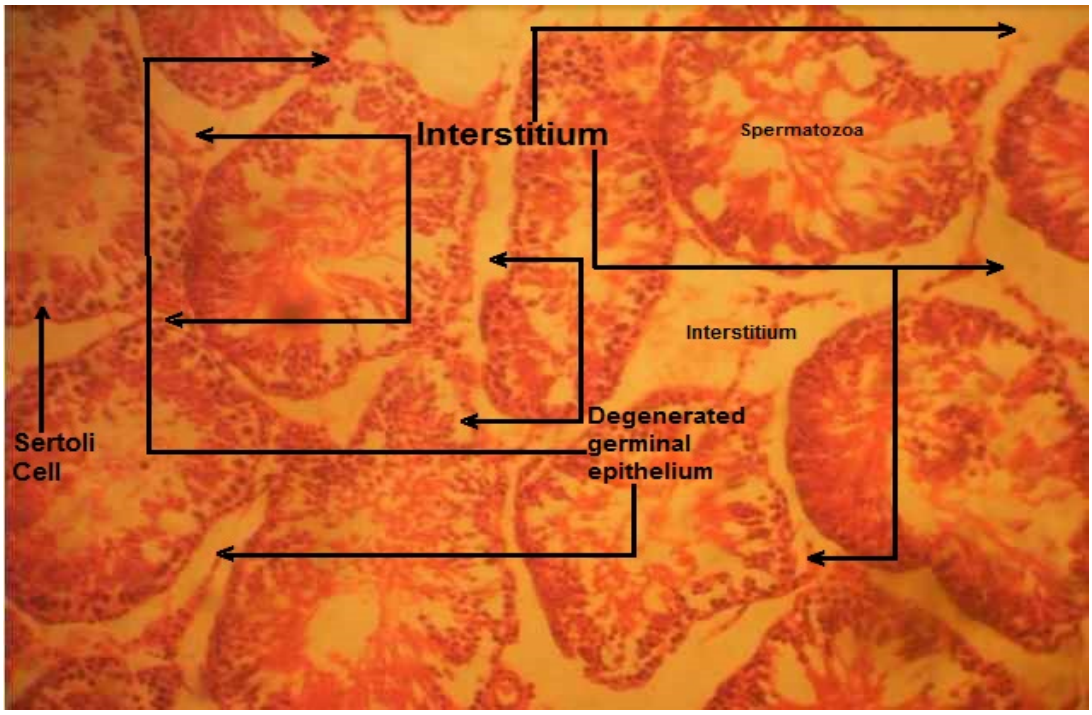

Figure 5: Testicular histological profile of Group D rats (5 ml kg^{-1} body weight of F^2 for 112 days).

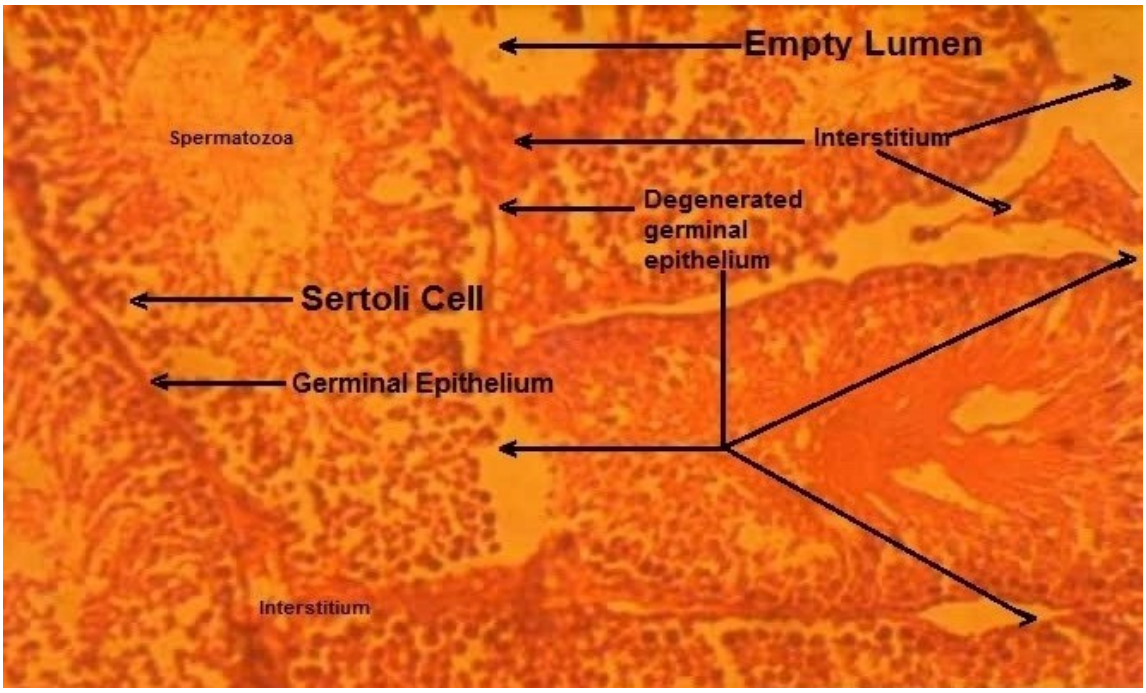

Figure 6: Testicular histological profile of Group E rats (5 ml kg^{-1} body weight of F^2) for 56 days).

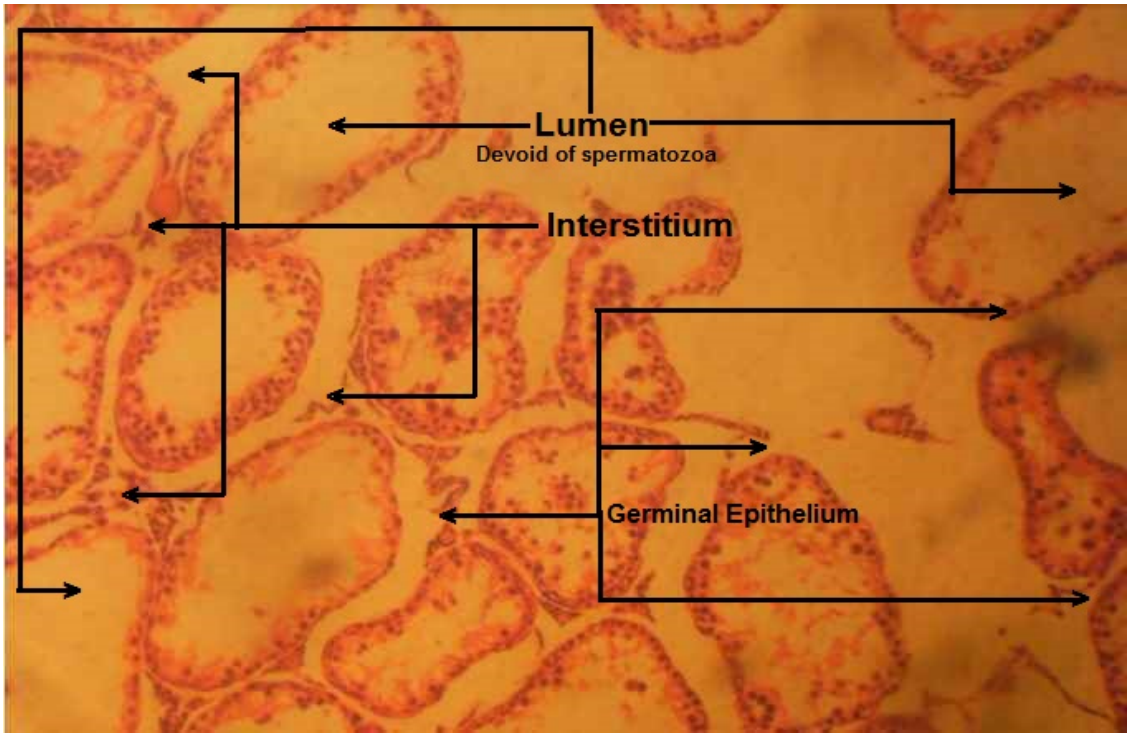

Figure 7: Testicular histological profile of Group E rats (5 ml kg^{-1} body weight of F^2 for 112 days).

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