



## Commercial Artificial Sweeteners Affect Spermatogenesis in Mice

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**Abstract:** The use of artificial sweeteners has increased in recent years. More young people are using them as a non- caloric sweetener in coffee or tea. They are also present in many manufactured food products such as biscuits and cakes. Many studies explored the potential biological effects of artificial sweeteners. However, most studies explored single types of artificial sweeteners, while very few were done on the actual commercial artificial sweeteners compounds present in the market and used the normal permitted daily dose. Spermatogenesis is one of the important biological functions that leads to the production of the male germ cells, the sperms. Studies showed that male infertility has increased in recent years. - The aim of this research was to study the effect of commercial artificial sweeteners on mouse spermatogenesis. Male albino mice were divided in two main groups: 1. Control group and 2. Treated group. Each group was then divided into 4 groups (C1, C2, C3 and C4) and (T1, T2, T3 and T4). Treated Swiss albino male mice were orally given 50mg/kg commercial artificial sweetener solution for four weeks. While the controls were given distilled water. Artificial sweeteners caused multiple malformations in spermatozoa such as a ring shape at the end of the sperm tail, coiled sperm, short tail and cytoplasmic droplet seen in the mid-piece compared to the controls. Alteration of spermatogenesis process was seen in treated testis histological sections compared to the controls, by the increased number of deformed seminiferous tubules, the shrinkage in their size and the disappearance of the membrane surrounding some of the seminiferous tubules causing their fusion. Our results showed that daily consumption of 50mg/kg of commercial artificial sweeteners affects the spermatogenesis process in mice and produces malformed sperms.

**Keywords:** Artificial sweeteners, spermatogenesis, spermatocytes, spermatozoa, morphometry, sperm malformation.

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## I. INTRODUCTION

Testis is an important part in the male reproductive system. It is where spermatogenesis takes place and goes under regulation of hormones to give mature sperm. Each testis is composed of coiled tubules called seminiferous tubules<sup>1</sup>. The seminiferous tubules are enclosed by a thick basal lamina and few layers of smooth muscles. The seminiferous epithelium is divided into basal and luminal. In the base of the seminiferous epithelium two types of spermatogonia are present: type A spermatogonia which is attached to the basal membrane of the seminiferous epithelium and is considered to be spermatogonial stem cell (SSC). Subtypes of spermatogonia that are given by type A and Type B cells that are committed to go under different divisions to give mature spermatozoa that are able to fertilize<sup>2,3</sup>. Spermatogenesis is the process of production of sperms from spermatogonia. It takes place in the seminiferous tubules in male testes. Meiosis is responsible for producing gametes with haploid number (1n) of the original cell.<sup>4-7</sup> The sperm consists of head and tail which include (mid piece, principle piece and end piece). Head consists of the nucleus, acrosome, perinuclear theca and plasma membrane. The nucleus takes up most of the head size and contains the chromosomes of haploid numbers tightly packed to protamine. The head takes different shapes, this depends on the species of the organism. Acrosome consists of two regions: acrosome cap (anterior acrosome) positioned on top of the nucleus in the anterior dorsal part of the head and containing digestive enzymes that have a role in penetration of zona pellucida during fertilization. Second region is the head cap that covers the lateral surface of the head and it is a thin layer. The midpiece consists of a part that connects the head with the tail. Midpiece has a mitochondrial sheath that is needed for energy supply for sperm. The tail segment is attached to the basal plate of the nucleus and to the nine fibers of the flagellum posteriorly.<sup>8-11</sup>. Artificial sweeteners are sweetening agents that include many compounds with different chemical structures and sizes. They are considered as calorie sweeteners.<sup>12</sup> Artificial sweeteners are used in the industry of different kinds of food, such as soft drinks, dessert mix, yoghurt, cereal, Strepsils and gum<sup>13</sup>. Sucralose is considered to be a non-nutritive sweetener that is used as a low calorie product. It is used in baking and cooking. It is 600 times sweeter than sucrose<sup>14</sup>. In a study on the effect of daily consumption of saccharin and sucrose on the testicular biologic function in mice. The result showed that high doses of saccharin affected the mouse testicular function and decreased the male reproductive performance. While in the high and middle concentration of sucrose disturbed spermatogenesis and sperm motility was seen<sup>15</sup>. In another study where mice were treated with water containing saccharin (2%) for 35 days. The results showed a decrease in sperm motility in the treated group. Also the rate of DNA fragmentation and apoptosis in the spermatozoa was higher in saccharin treated mice. The authors concluded that consumption of saccharin affects sperm parameters<sup>16</sup>. As many products contain artificial sweeteners and many people consume artificial sweeteners in hot and cold drinks. Moreover given the increased infertility seen in young males in the last ten years. It was important to investigate the effect of normal doses of commercial artificial sweeteners present in the market on spermatogenesis. The mouse was chosen as a model for this study for its suitability as a mammalian model. Therefore the aim of this study was to investigate the effect of artificial sweeteners on mouse sperm formation, morphology and

histological and histometrical changes in seminiferous tubules....

## 2. MATERIALS AND METHODS

All experimental procedures were approved by the Biology Department at King Abdulaziz University. Swiss, albino adult males were purchased from Pharmacy College at King Saud University in Riyadh, mean weight 23.42 g. They had normal animal feed, 12:12-light: dark and temperature at 22 ± 2C. A commercial artificial sweetener containing: Sorbitol 1.98 g, sucralose 9.8 mg, Acesulfame potassium 8 mg/ 2gm, Corn powder and Chromium picolinate, was purchased from a local supermarket. In this research, a dose of 50 mg/Kg body weight was used. The dose was determined according to the study of<sup>18</sup>.

### 2.1 Study design

Male albino mice were divided into two main groups: Control group (C) (n = 8 mice) and Treated group (T) (n = 8 mice). Each group was then divided into 4 groups (C1,C2,C3 and C4) and (T1,T2,T3 and T4). The control groups were given 0.5ml of distilled water and the treated groups were given 0.5 ml of the experimental solution. All groups were given the dose orally by a gavage needle for five days each week.

### 2.2 Sample collection

Each week one group of each control and treated was weighed then scarified and the testis were collected, weighed then fixed in 5% formalin for histological studies. The epididymis was dissected and fixed in 5% of formalin.

### 2.3 Sperm collection and examination

Epididymis were collected and dissected into small pieces. Then each epididymis was fixed in 5% formalin in a separate Eppendorf tube for around 6 months. A smear was taken from the fluid for the preparation of semen smear slides. Sperm smears were repeated three times from each sample tube. Slides were then examined and photographed. Each slide photo was then studied to count normal and abnormal spermatozoa and to perform morphometric studies on sperms. Sperm abnormality was detected according to<sup>19</sup>. No examination was done to C1 sperms as the samples were lost.

### 2.4 Histological techniques

Extracted testes were fixed in 5% formalin solution. They were then prepared and stained with Ehrlich's Hematoxylin and eosin according to the method of<sup>20</sup>.

### 2.5 Photography

Sperms smears and testis sections photos were taken by Olympus BX51 microscope connected to Olympus camera DP72 in King Fahd Center for Medical Research, KAU.

### 2.6 Morphometric and Histometric studies

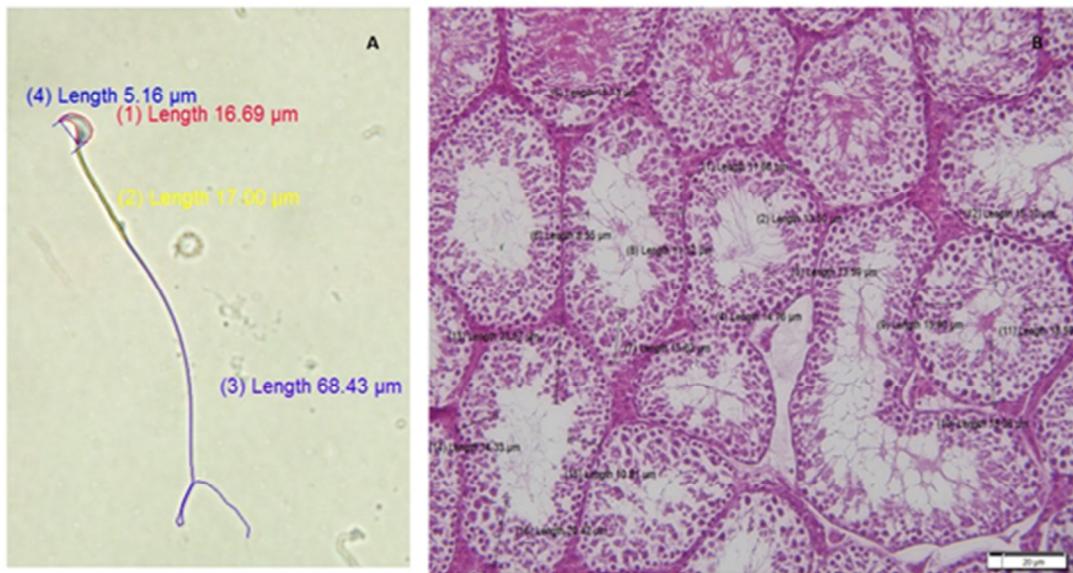
A cellSense Standard program was used to do all morphometric and histometric measurement. In the sperm, the head circumference, mid piece and tail length were

measured see fig 1 A . In the testis the thickness of the spermatogenesis area in the seminiferous tubules was taken see Fig 1B. For counting the seminiferous tubules, the paint program was used by marking each tubule with a dot. Two colors has been chosen as marker the yellow color was used for normal seminiferous tubule that has almost all stages of the spermatogonia and sperm seemed to have a normal distribution while the green color was used for abnormal seminiferous tubules for several reason such as it either doesn't have all stages of spermatogonia or it was filled with too much seminal fluid or it doesn't have sperms in it.

Raghuvanshi et al. study <sup>19</sup> was used as a reference to detect sperm abnormality.

### 3. STATISTICAL ANALYSIS

Data was collected into excel, then transferred into (SPSS). The tests used with the normal distribution were ANOVA and student-Newman Keuls test .In case of abnormal distribution Mann-Whitney U test was used from the non-parametric test. Significance (\*) was at  $P \leq 0.05$ .



**Fig1. Methodology of histometric measurements for (A) sperm and (B) C. S. of seminiferous tubules in testis.**

## 4. RESULTS

### 4.1 Sperm morphology

#### 4.1.1 The effect of artificial sweeteners on sperm morphology

The sperms of the control groups C2, C3 and C4 had a very clear hook shaped head followed by neck and the mid-piece that seemed darker in color than the rest of the tail which was long and thin. Most sperm of treated animals groups T2, T3 & T4 seemed similar to the controls. However many deformations were seen such as (a ring shape at the end of the sperm tail, coiled sperm, short tail and cytoplasmic droplet seen in the mid-piece). Oval heads appeared in T2 treated animal sperms. While T3 and T4 treated animal sperms had different head shapes and free heads (see figures 2,3 and 4).

#### 4.1.2 Effect of artificial sweeteners on sperm head circumference

In this study the mean of control sperm head circumference was (17.64  $\mu$ m) for C2, (17.90  $\mu$ m) for C3 and ( 17.41  $\mu$ m ) for C4. Artificial sweeteners caused a significant increase in Head circumference in T2 ( $P=0.016$ ) compared to the control .While in T3 and T4 no significant change in sperm head circumference was seen compared to the controls see Fig 5 .

#### 4.1.3 Effect of artificial sweeteners on the distance between the tip and the back of the sperm head

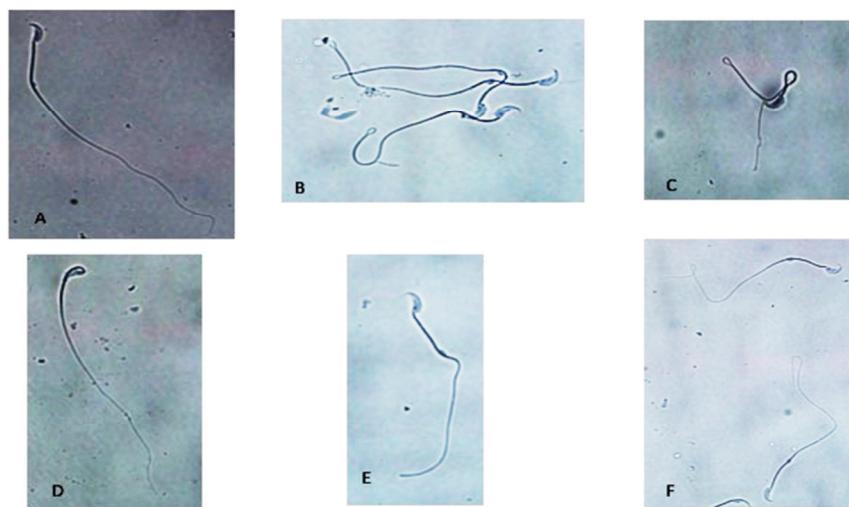
In this study the mean of the distance between the tip and the back of the sperm head in controls was (8.85  $\mu$ m) for C2, (6.35  $\mu$ m) for C3 and (6.54  $\mu$ m) for C4. Group T2 showed a significant decrease in the distance between the tip and the back of the head compared to the control ( $P=0.003$ ). In addition, the T3 group showed a significant decrease compared to the controls ( $P=.000$ ).While no significant change in group T4 was seen (see Fig 5).

#### 4.1.4 Effect of artificial sweeteners on the middle piece length

In this study the mean of the middle piece length in controls were (16.45  $\mu$ m) for C2, (15.61  $\mu$ m) for C3 and (15.28  $\mu$ m) for C4. Group T4 showed a significant increase in the middle piece length compared to the controls ( $P=.001$ ). While no significant change in the middle piece length was seen in groups T2 and T3 compared to the controls. (see Fig 5).

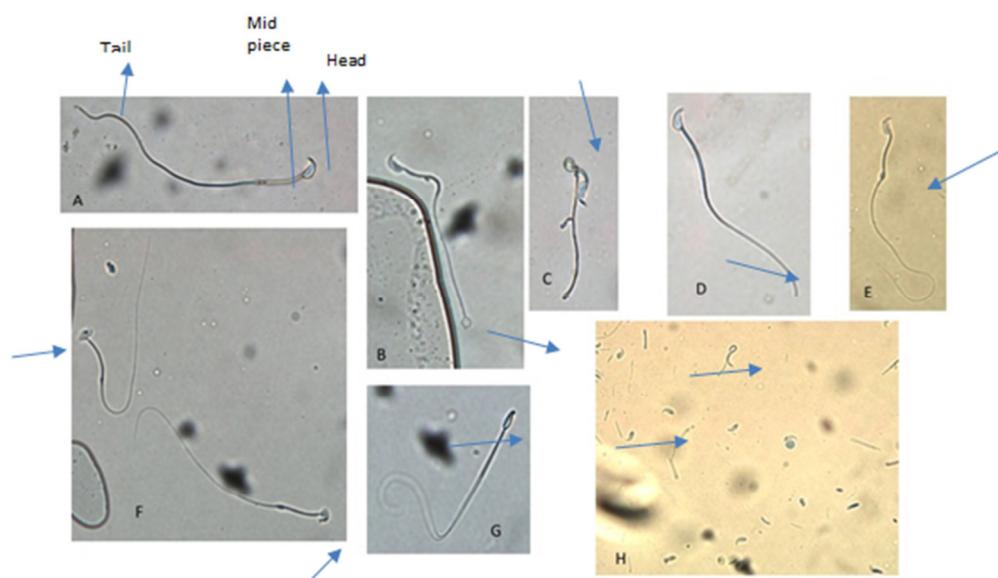
#### 4.1.5 Effect of artificial sweeteners on sperm tail length

In this study the mean of the tail length in controls was (70.66  $\mu$ m) for C2, (69.44  $\mu$ m) for C3 and (72.54  $\mu$ m) for C4. There was no significant change in tail length in T2 and T3 groups compared to the controls. However a slight decrease in tail length of T4 group was seen compared to the controls. (see Fig 5).



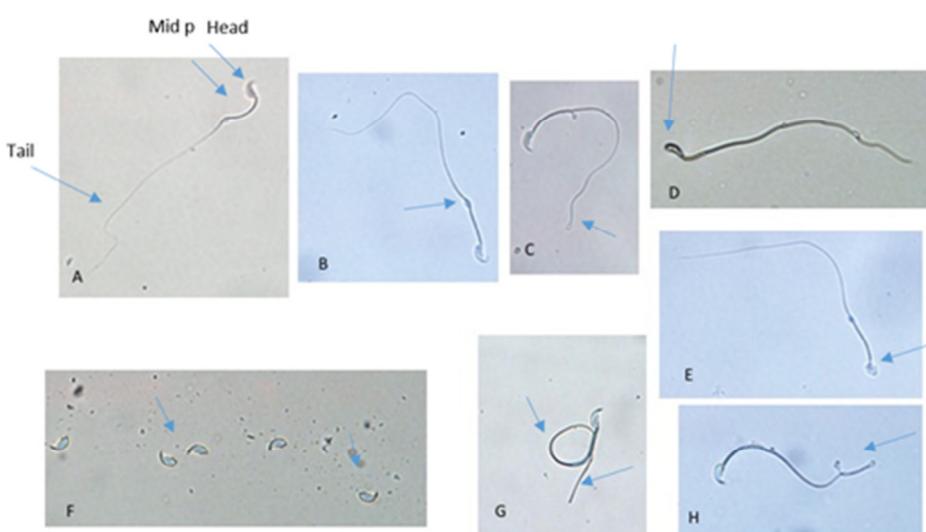
**Fig 2. Sperm malformations seen after 2 weeks of treatment**

(A) Control, (B-F) Treated. Note: the ring shape at the end of the tail (B). Coiled sperm (C). Oval head (D). Shorter tail (E) and Cytoplasmic droplet (F) under 600X magnification.



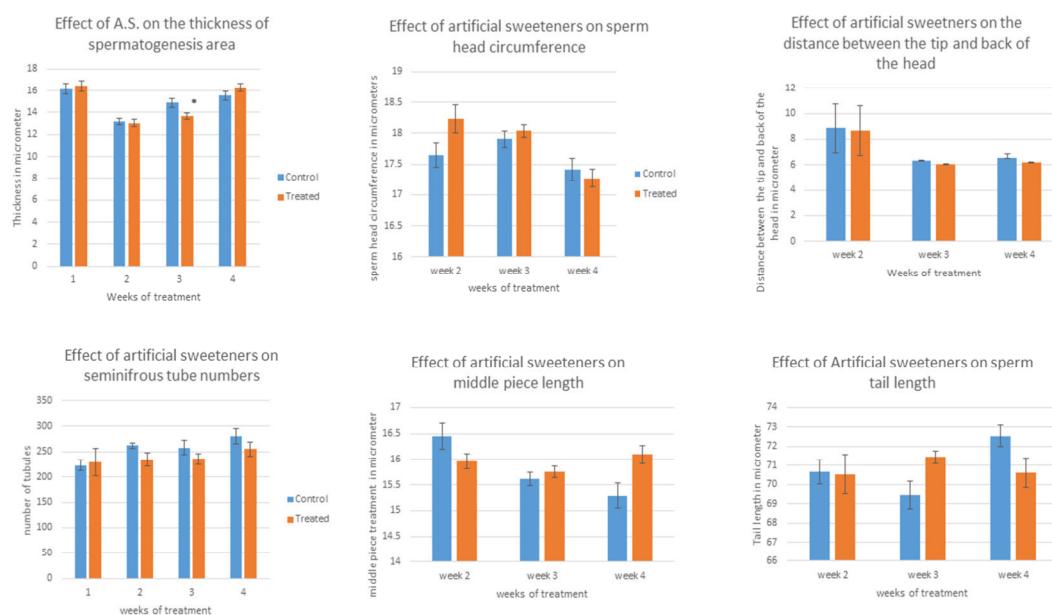
**Fig 3. Sperm malformations seen after 3 weeks of treatment .**

(A) Control, (B-H) Treated. Note: The ring shape at the end of the tail (B). Coiled sperm in (C). Short sperm- (D). Cytoplasmic droplet (E) Abnormal sperm head in (F-G). Free heads and tails (H) under 600X magnification .



**Fig 4. Sperm malformations seen after 4 weeks of treatment**

(A) Control , (B-H) Treated. Note : Cytoplasmic droplet (B) .the ring shape at the end of the tail (C) .Oval head in (D) . Abnormal sperm head(E) . Free heads (F ) Shorter tail and coil (G) Coiled sperm (H) under 600X magnification.



**Fig 5. Effects of artificial sweeteners on sperm morphometry**

Graphs showing the effect of artificial sweeteners on C.S. morphometry of sperm and testis. Values are Mean  $\pm$  Standard error ( $M \pm SE$ ) of 8 samples (\*).  $P < 0.05$ . Mean Measurements of thickness of spermatogenesis area were taken from 135 readings where 3 readings were taken from 5 tubes from 3 different sections from 3 different testis for each treatment group. The number of tubes were counted in these sections. Mean Sperm measurements were taken from 180 measurements from 30 sperm from 3 different smears from each mouse in each treatment group except week 1 group.

#### 4.2 Testis histology

The cross section of the testis in all control groups showed tunica albuginea lining the testis. The seminiferous tubules appeared having a circular or oval shape and they were surrounded by interstitial tissues containing blood vessels, and Leydig cells. The wall of the seminiferous tubules consisted of lamina propria that consisted of fibroblasts and the inner layer that formed the basement membrane. The seminiferous tubules contained the different cell stages of spermatogenesis. These were as follows; spermatogonia that were seen to perform cell division in several sections and were found on the basement membrane. Primary spermatocytes, that were seen as large cells with round or spherical shape nuclei, secondary spermatocytes that appeared smaller than the primary spermatocyte, spermatids were the smallest in size and they appeared gathered in groups attached to Sertoli cells. Sertoli cells were seen extending from the basement membrane to the lumen. Spermatozoa were seen in the lumen of the seminiferous tubules (see figure 6 and 7). Testis cross sections of all treated groups seemed similar to the control groups with some changes that were seen in treated testis sections, such as; tunica albuginea which seemed to be thinner compared to the control groups, spaces between seminiferous tubules seemed enlarged compared to the controls. On the cellular level within the seminiferous tubule primary spermatocyte seemed bigger in size with absence of secondary

spermatocytes in some tubules. In other tubules no sperm was seen in the lumen. Disruption of spermatogenesis stages and disappearance of the membrane surrounding some of the seminiferous tubules was also seen. This caused the tubules to open and connect with the neighboring tubules and for the spermatogenesis stages to spill out in the inter tubular space (see figure 6 and 7).

#### 4.3 Testis Histometry

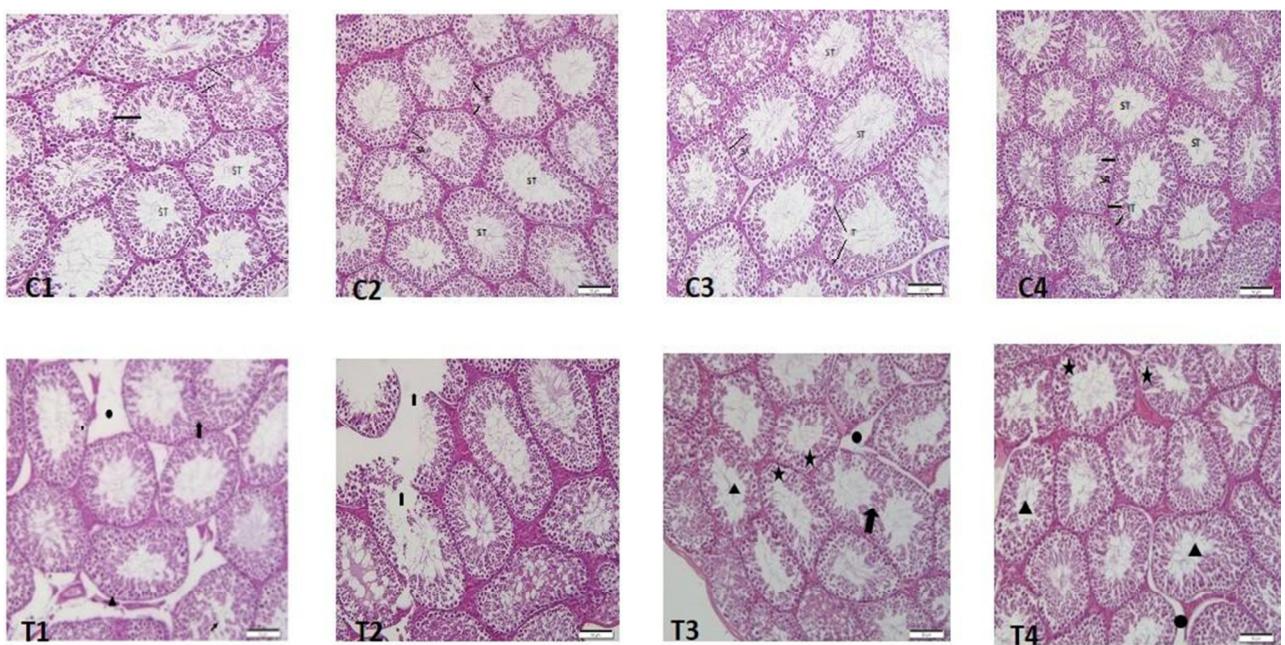
In this study the mean thickness of spermatogenesis area in control groups was (16.16  $\mu$ m) for C1, (13.189  $\mu$ m) for C2, (14.86  $\mu$ m) for C3 and (15.52  $\mu$ m) for C4. Artificial sweeteners caused a significant decrease in T3 group ( $p=0.00$ ) compared to the control group. While there were no significant changes in T1, T2 and T4 groups compared to the controls. (see Fig 5)

##### 4.3.1 Effect of artificial sweeteners on the Seminiferous tubules numbers

In this study the mean of seminiferous tubules numbers in control groups was (233) for C1, (262.33) for C2, (258.33) for C3 and (280) for C4. Artificial sweeteners caused a non-significant decrease in T2, T3 and T4 Seminiferous tubules numbers compared to the control groups. While there was a slight non-significant increase in T1 group compared to the controls. (see Fig 5).

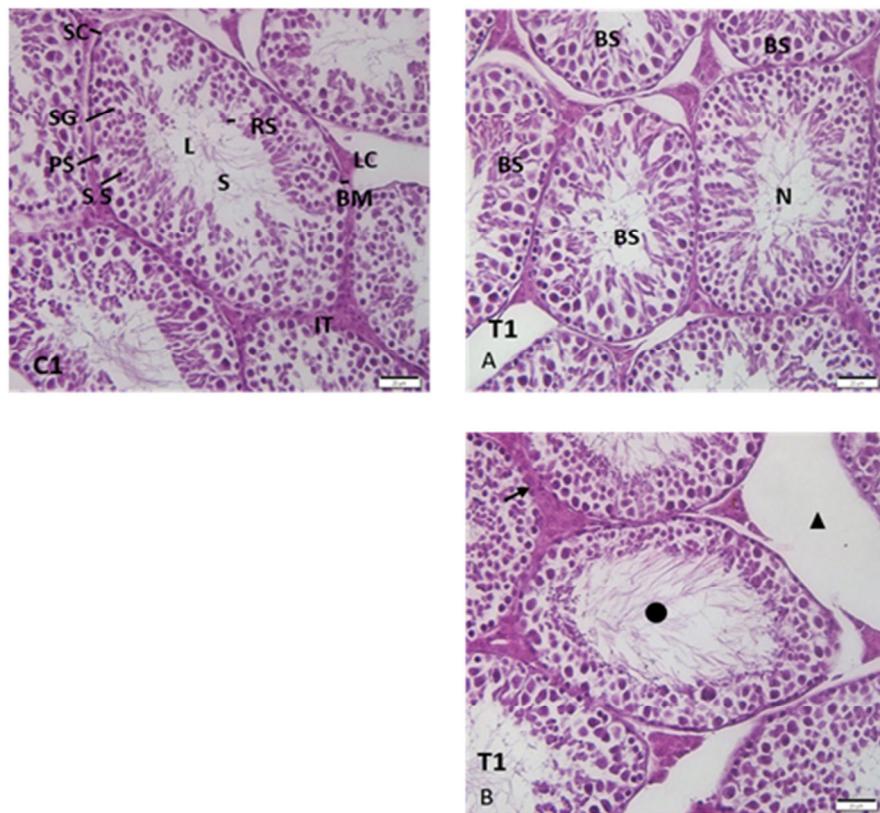
##### 4.3.2 Effect of artificial sweeteners on the percentage of abnormal seminiferous tubules

In this study the percentage of abnormal seminiferous tubules in control groups was (29.75) for C1, (25.31) for C2, (24.793) for C3 and (24.794) for C4. Artificial sweeteners caused a non-significant increase in the percentage of abnormal seminiferous tubules in T3 and T4 groups compared to the control groups. While no change was seen in T1 and T2 groups compared to the controls (see Fig 5).



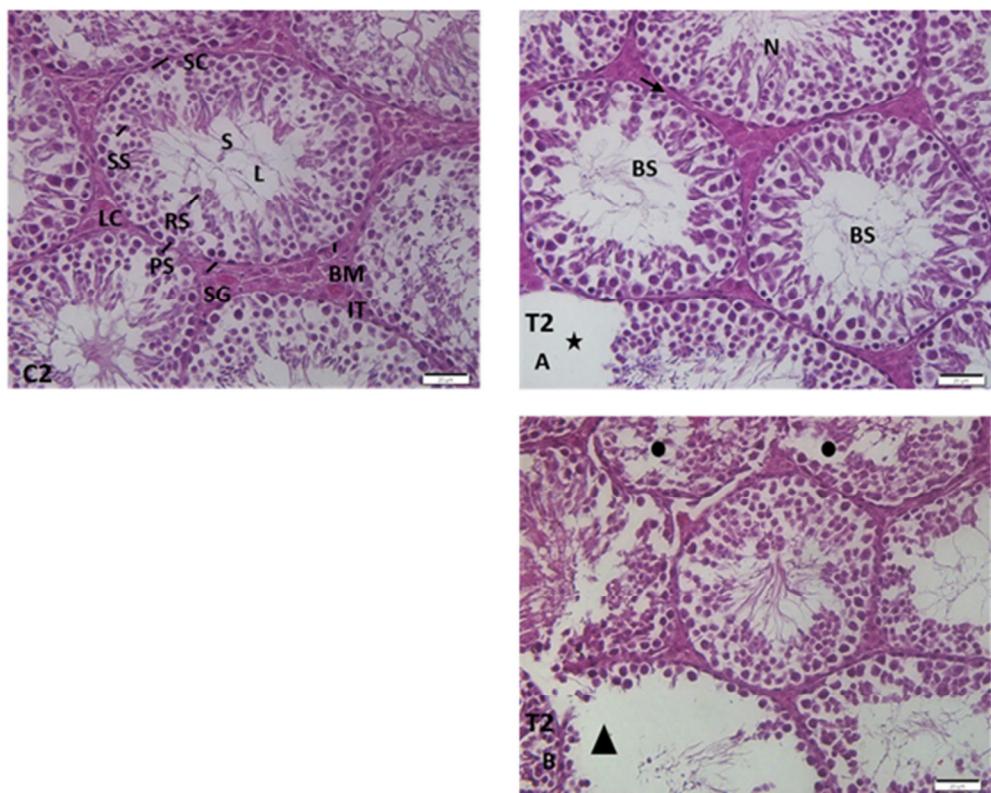
**Fig 6. Light micrographs of transverse section of testis of swiss albino mice of control groups.**

showing spermatogonia area (SA), basal membrane (BM), spermatogonia (SG), Sertoli cell (SC), primary spermatocyte (PS), secondary spermatocyte (SS), round spermatid (RS), sperm (S), lumen of the seminiferous tubule (L), Leydig cell (LC) and Interstitial tissue (IT). Second row for Treated group showing (black dot) increased of space between (ST), fusion of spermatocyte between two of the ST (arrow in T1 ), absence of some spermatocyte stages and unorganized spermatocyte (arrow in T2 ), bigger size of the spermatocyte (stars and arrow heads in T3 and T4 ) (H&E stain, magnification 200x



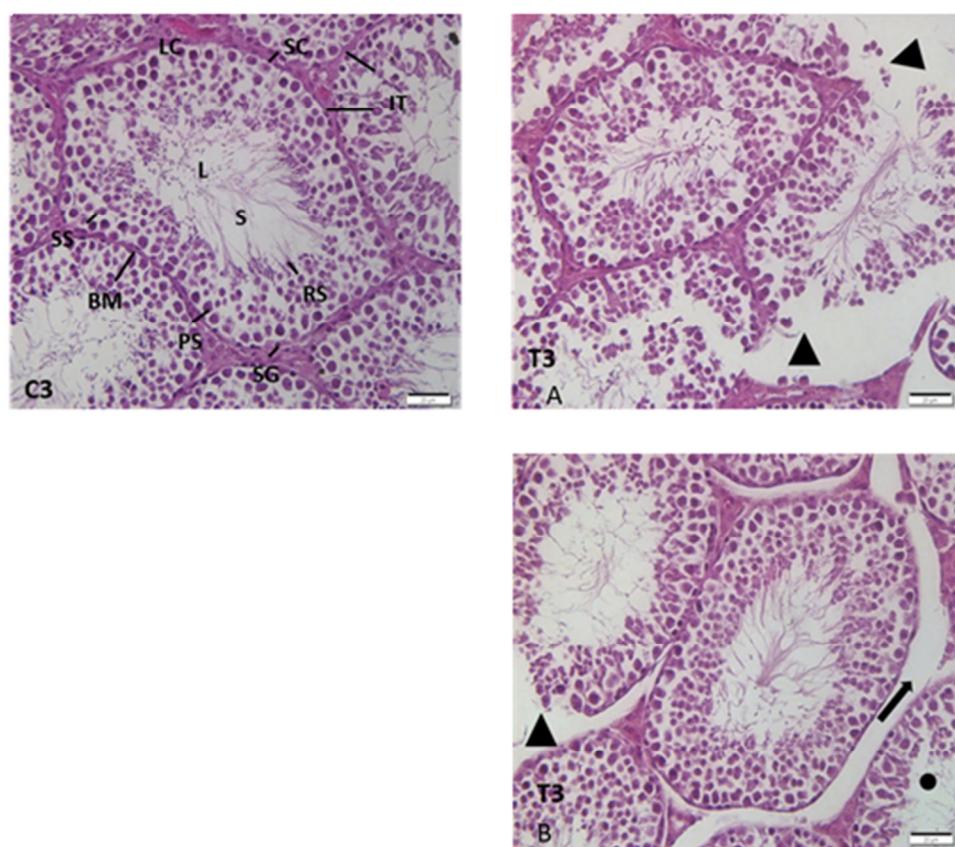
**Fig 7 : Light micrographs of transverse section of testis of swiss albino mice of control group C1,**

showing basal membrane (BM), spermatogonia (SG), Sertoli cell (SC), primary spermatocyte (PS), secondary spermatocyte (SS), round spermatid (RS), sperm (S), lumen of the seminiferous tubule (L), Leydig cell (LC) and interstitial tissue (IT). Transverse section of testis of Treated group T1 showing: (A) normal seminiferous tubules (N), bigger size of the spermatocyte (BS) while (B) showing increment of space between seminiferous tubules (arrow head), increased of connective tissues thickness between ST (black arrow) and unorganized spermatocyte (black dot). (H&E stain, magnification 400 x



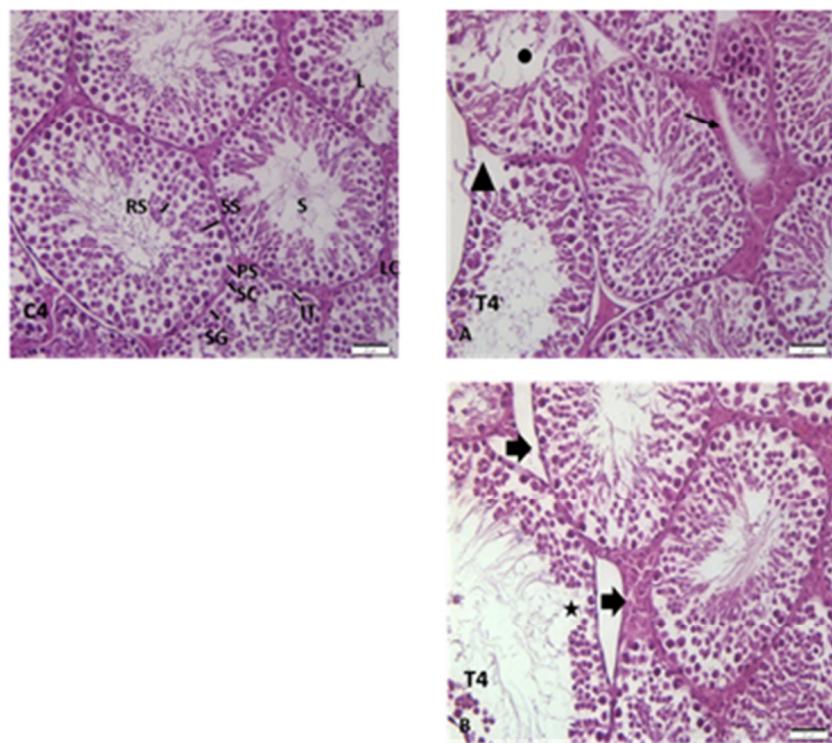
**Fig 8 : Photomicrographs of transverse section of testis of Swiss albino mice control group C2,**

showing basal membrane (BM), spermatogonia (SG), sertoli cell (SC), primary spermatocyte (PS), secondary spermatocyte (SS), round spermatid (RS), sperm (S), lumen of the seminiferous tubule (L), Leydig cell (LC) and interstitial tissue (IT). Transverse section of testis of Treated group T2 showing: (A) normal ST (N), bigger size of the spermatocyte (BS), absence of Spermatocyte in the seminiferous tubules (star) and increased of connective tissues thickness between ST (black arrow) while (B) showing fusion of the seminiferous tubules (arrow head), and unorganized spermatocyte (black dot). (H&E stain, under the magnification of 400 X



**Fig 9 : Photomicrographs of transverse section of testis of Swiss albino mice control group C3,**

showing basal membrane (BM), spermatogonia (SG), sertoli cell (SC), primary spermatocyte (PS), secondary spermatocyte (SS), round spermatid (RS), sperm (S), lumen of the seminiferous tubule (L), Leydig cell (LC) and interstitial tissue (IT). Transverse section of testis of Swiss albino mice treated group T3, showing: (A) fusion of seminiferous tubules (arrow head). (B) showing space between seminiferous tubules (black arrow), primary spermatocyte bigger in size (arrow head) and fusion of seminiferous tubule (black dot). (H&E stain, under the magnification of 400 X.



**Fig 10 : Photomicrographs of transverse section of testis of Swiss albino mice control group C4,**

showing basal membrane (BM), spermatogonia (SG), sertoli cell (SC), primary spermatocyte (PS), secondary spermatocyte (SS), round spermatid (RS), sperm (S), leydig cell ( LC) and interstitial tissue (IT). Transverse section of testis of Treated group T2 showing: (A) unorganized spermatocyte (black dot), fusion of Spermatocyte in the seminiferous tubules (arrow head), bigger size of the spermatocyte (BS) (black arrow). (B) showing absence of Spermatocyte in the seminiferous (star) and space between seminiferous tubules (black arrow). (H&E stain, under the magnification of 400 X).

## 5. DISCUSSION

Artificial sweeteners are sweet substances that include a large number of compounds with different chemical structures and sizes.<sup>12</sup> Artificial sweeteners are available in the market these days and are of different types. They are almost used in every product with a different chemical formula.<sup>21</sup> There are two types of sweeteners available in the market: The natural sweeteners from natural sources from plants such as stevioside. Artificial sweeteners (synthetic) such as saccharin, aspartame, sucralose and cyclamate. Although these compounds serve as sweeteners with minimum or no calories some studies showed that they may induce the insulin secretion and have long term effect on weight gain.<sup>21, 22</sup> In this study Artificial sweeteners caused sperm morphologic and morphometric malformation such as (ring shape in the end of the sperm tail, coiled sperm, short tail and cytoplasmic droplet seen in the mid-piece). Oval head appeared in 2 weeks treated animal sperms. While in 3 and 4 weeks treated animal sperms different head shapes and free heads and tails were seen. Sperm morphometric results showed that artificial sweeteners caused a significant increase in Head circumference after two weeks of treatment compared to the controls. The groups T2 and T3 showed a significant decrease in the distance between the tip and the back of the head compared to the control C2 and C3 groups, which indicates increased curvature of sperm head. Also Group T4 showed a significant increase in the middle piece length compared to the controls. This was seen in a study of Swiss Albino Male Mice that were fed continuously with microwave exposed food for two, three and four weeks<sup>19</sup>, they concluded that microwave cooked food caused spermatotoxic effects. They also concluded that a coiled spermatozoa tail was a sign of biochemical changes of sperm

surface, and found that the presence of cytoplasmic droplets in the middle piece inhibited sperm motility. In another study, adult male rats were exposed to ethanol since puberty and had sperms abnormalities such as coiled tail, detached head and tails. They concluded that ethanol has spermatotoxic effects. While abnormalities in tail could affect sperm motility and fertility<sup>23</sup>. In a study of male sterility caused by sperm cell-specific structural abnormalities in ebouriff6 which is a new mutation of the house mouse done by Lalouette et al.<sup>24</sup>. Sperm abnormalities were seen such as coiled tail, short tail and abnormal head shape. The researchers concluded that the tail abnormalities were probably caused by mitochondria disorganization in the mid piece. They also concluded that the head abnormalities resulted from disruption of the cytoskeleton. As most of these sperm malformations were seen in this study on the morphological and morphometrical levels. It might be concluded that artificial sweeteners might be considered spermatotoxic for inducing sperm abnormalities, and changes in biochemical surface of sperm and inhibition of sperm motility. It seems also that the artificial sweeteners used in this study might have affected the sperm cell cytoskeleton. Changes in sperm morphology might affect the ability of sperm to perform fertilization, therefore reducing fertility. In this study, sections of treated mouse testis showed smaller seminiferous tubules with increased inter tubular spaces, also a disruption in cell order within the tubules was seen and giant spermatocyte cells were also seen. On the histometric side the thickness of the spermatogenesis area was significantly less than the controls and dilation of blood vessels was seen in some sections. Other studies had similar results caused by different factors. In a study<sup>25</sup> done on heavy human alcohol drinkers it was shown that alcohol caused morphological disorder of testis like partial spermatogenic arrest and changes on peritubular

fibrosis but researchers concluded that the spermatogenesis arrest could be reversible. Other studies showed effects of artificial sweeteners on spermatogenesis. Sodium saccharin caused a significant decrease in testosterone in treated rats<sup>26</sup>. Also, saccharin altered sperm quality in mice including sperm count, viability, motility, and augmented abnormalities.<sup>15</sup> In a study done by Lalouette et al.<sup>24</sup> on male sterility caused by sperm cell-specific structural abnormalities in Ebouriif6 which is a new mutation of the House Mouse the result was a reduction of spermatocyte population , decrease of round spermatid numbers , degeneration of germ cells that seemed like empty looking vacuoles , disorganization of spermatogenesis stages and the lumen seemed smaller in size in mutant mouse. They concluded that the ebo gene caused a defect in the testis and also hair. They also concluded that the sterility was caused from the abnormalities in cytoskeleton in spermatid maturation late stage such as the kinesin motor. Many abnormalities seen in<sup>24</sup> studies were seen in our study, which might indicate that the commercial artificial dose given to the mice in this study might have had an effect on gene expression needed to perform normal spermatogenesis.

## 6. CONCLUSION

In this study we used a commercial artificial sweetener

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present in the market and used the normal dose that is used by normal persons and yet there was a clear effect on the sperms and spermatogenesis process. The normal dose could be increased unintentionally through the everyday nutritious behavior as many snacks contain different types of artificial sweeteners. This puts an emphasis to do more intensive research to understand the mechanism of action of these artificial sweeteners, and whether they can cause epigenetic changes or not.

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## 8. AUTHORS CONTRIBUTION STATEMENT

F.Al-Qudsi and A. Aldossary designed experiments and reviewed the manuscript. Both authors read and approved the final version.

## 9. CONFLICT OF INTEREST

Conflict of interest declared none.

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