Niger. J. Physiol. Sci. 39 (December 2024): 215 – 221
www.njps.physiologicalsociety.com

Niger. J. Physiol. Sci.

Full length Research Article

Cross-Examination and Comparison of Effects of Nutritive and Non-Nutritive Sweeteners on Male Reproductive Health Using Wistar Rat Model

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Summary: Sucrose and saccharin are prevalent sweeteners in today's diet and play crucial roles in global food and beverage consumption. The intake of sucrose, a natural sweetener, alongside saccharin, an artificial alternative, has raised various health concerns related to its effects on reproductive health. This study specifically focuses on how these sweeteners influence male reproductive parameters, including sperm quality, hormonal levels (such as follicle stimulating hormone {FSH}, luteinizing hormone {LH}, and testosterone), reproductive gene expression, and the functionality of reproductive organs, in animal models (in vivo) and by also identifying the mechanisms through which these substances impact male reproductive health. Thirty male Wistar rats, each weighing 180–200 g, were randomly assigned to three groups: Group one served as the control, Group two received sucrose (100 mg/kg), and Group three was administered saccharin (5 mg/kg). The findings indicated that the levels of follicle stimulating hormone, luteinizing hormone, testosterone, and steroidogenic acute regulatory protein (StAR) significantly decreased in the sucrose and saccharin groups compared with those in the control group. Additionally, the testicular antioxidant malondialdehyde levels increased relative to those in the control, whereas superoxide dismutase and catalase levels were significantly decreased. Furthermore, the sperm profile—including sperm count, motility, and viability—was significantly altered compared with that of the control group. These results suggest that both sweeteners can negatively impact reproductive function by diminishing testicular antioxidant activity, altering sperm characteristics, and inhibiting StAR, ultimately leading to testicular damage, with the sucrose exhibiting a more pronounced negative effect on reproductive function.

Keywords*: Sucrose, Saccharin, Sweeteners, Hormone levels, Sperm quality. Wistar rats*

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Manuscript received- August 2024; Accepted: November 2024

DOI: https://doi.org/10.54548/njps.v39i2.7

INTRODUCTION

The evolution of the food industry has resulted in a diverse array of sweeteners available to consumers. These sweeteners offer a delightful taste, which can be either energy-providing (nutritive) or calorie-free (nonnutritive) (American Dietetic Association, 2004). In the early stages of human evolution, H. sapiens fulfilled its craving for sweetness by consuming naturally sweet foods such as fruits and vegetables (Reed and McDaniel, 2006). As the food industry has expanded, it has enabled the mass production of refined sugar derived from sugar beets and sugarcane (Arshad *et al*., 2022). Today, the market is flooded with a variety of sugar substitutes to meet the growing demand for sweeteners (ADA, 2004).

Sweeteners can be classified into two categories: nutritive and nonnutritive. Nutritive sweeteners supply energy, whereas nonnutritive sweeteners offer sweetness without contributing any calories (ADA, 2004; Fitch and Keim, 2012).

The male reproductive system comprises internal structures, such as the testes, epididymis, vas deferens, and prostate, as well as external structures, such as the scrotum and penis. These components are richly supplied with blood vessels and include various glands and ducts that facilitate reproduction, sperm storage, and ejaculation while also producing androgens essential for male development (Tiwana and Leslie, 2017). The key hormones involved in regulating these processes include follicle-stimulating hormone (FSH) and luteinizing hormone (LH), which are secreted by the anterior pituitary gland, along with gonadotropin-releasing hormone (GnRH), which is produced by the hypothalamus. Together, these hormones play crucial roles in promoting and sustaining sexual

development and function in males through the hypothalamic–pituitary–gonadal axis (Tiwana and Leslie, 2023).

In the mid-19th century, sugar was regarded as a "pure white and deadly" substance, largely because of its association with various harmful effects (Handelsman, 1989). While there is insufficient evidence directly linking sucrose intake to toxicity, numerous studies indicate that sugar consumption may be associated with a range of negative health outcomes (Howard and Wylie-Rosett, 2002).

Artificial and natural sweeteners have a long history of use and have become staple components of many diets. Anecdotal evidence suggests that these sweeteners may influence various organ systems. Increased dietary intake of sweeteners has been linked to the development of several health issues, including autoimmune and cardiovascular diseases, cancer, dental caries, gallstones, premature aging, appendicitis, depression, hyperactivity, and various metabolic disorders (Howard and Wylie-Rosett, 2002). Although these compounds are often marketed as low-calorie or calorie-free sweeteners, some studies indicate that they may lead to sperm abnormalities, alterations in sperm biochemical properties, and reduced sperm motility (Burke and Small, 2015). Changes in sperm morphology could hinder the ability of a sperm to fertilize an egg, thereby impacting fertility (Moretti *et al*., 2022). Additional research has shown that artificial sweeteners can affect spermatogenesis; for example, sodium saccharin significantly reduces testosterone levels in treated rats (Helal *et al*., 2019). This study aims to fill this knowledge gap by examining the differential impacts of sucrose and saccharin on sperm quality, hormone levels, testicular morphology, and reproductive organ function in male Wistar rats. By clarifying the specific effects of these two sweeteners on male reproductive health, this research will offer valuable insights into the potential risks associated with their consumption and aid in informed decision-making regarding dietary practices and public health policies.

MATERIALS AND METHODS

Duration and setting of the study: The study was conducted in the Department of Physiology Laboratory, Igbinedion University Okada, from May 2024 to July 2024. The animals were handled in accordance with the Guiding Principles for Research as recommended by the Declaration of Helsinki and the Guiding Principles for the Care and Use of Laboratory Animals.

Procurement and maintenance of animals: The experimental animals used in this study were thirty (30) healthy male Wistar rats weighing between 180 g and 200 g. The animals used were obtained from the central animal house of Igbinedion University Okada. The animals were provided with proper housing and were kept at a standard temperature. As a precaution against infection, bedding was changed daily, and the cages were washed every day. The animals were provided with water and feed on daily (Akhter *et al*., 2023).

Experimental design, animal treatment and collection of tissues: The 30 male Wistar rats were divided into three (3) equal groups at random, each containing ten rats, weighed on the day of arrival, before and after treatment, and properly labelled with markers for easy identification. The animals were treated daily for 64 days as follows: Group one: control (distilled water), Group two: sucrose (100 mg/kg), and Group three: saccharine (5 mg/kg) via oral gavage. The animals were anaesthetized with chloroform on the last day (Akpantah *et al*., 2003). Blood samples were obtained via cardiac puncture, and plasma was obtained for hormone (testosterone, FSH, LH) assays. The testis with the epididymis was dissected, and caudal epididymal sperm were analysed. The right testes were homogenized, and the resulting supernatant was used for superoxide dismutase (SOD), catalase activity, malondialdehyde (MDA) and StAR gene investigations. The left testes were fixed in Bouin's fluid for histological examination.

Hormonal profile investigations: Blood samples were obtained via cardiac puncture into plain bottles and spun at 3,500 rpm for 10 minutes in a cold centrifuge and samples were assayed for follicle-stimulating hormone, luteinizing hormone and testosterone using their respective Calbiotech ELISA kits as described by Okeke *et al*. (2023).

Gene expression Study

Isolation of Total RNA: Total RNA was isolated from tissue samples with Quick-RNA MiniPrep™ Kit (Zymo Research). The DNA contaminant was removed following DNAse I (NEB, Cat: M0303S) treatment. The RNA was quantified at 260 nm and the purity confirmed at 260 nm and 280 nm using A&E Spectrophotometer (A&E Lab. UK).

cDNA conversion

One (1 µg) of DNA-free RNA was converted to cDNA by reverse transcriptase reaction with the aid of cDNA synthesis kit based on ProtoScript II first-strand technology (New England BioLabs) in a condition of 3-step reaction: 65 °C for 5 min, 42 °C for 1 h, and 80 °C for 5 min (Elekofehinti *et al*., 2020).

PCR amplification and agarose gel electrophoresis:

Polymerase chain reaction (PCR) for the amplification of Steroidogenic Acute Regulatory Protein (StAR) was carried out with OneTaqR2X Master Mix (NEB) using its primer (Inqaba Biotec, Hatfield, South Africa). PCR amplification was performed in a total of 25 µl volume reaction mixture containing cDNA, primer and Ready Mix Taq PCR master mix. Under the following condition: Initial denaturation at 95 ◦C for 5 min, followed by 30 cycles of amplification (denaturation at 95 ◦C for 30 s, annealing for 30 s and extension at 72 ◦C for 60 s) and ending with final extension at 72 ◦C for 10 min. The amplicon was resolved on 1.0% agarose gel. The GAPDH gene was used to normalize the relative level of expression of the gene, and quantification of band intensity was done using "image J" software (Olumegbon *et al*., 2022).

Antioxidant activity: The obtained supernatant was used for the estimation of superoxide dismutase (SOD), catalase (CAT) and malondialdehyde (MDA) levels (Oyeyemi *et al*., 2020).

Sperm profile: The sperm count, motility, and viability assessment were performed immediately after the animals were anaesthetized. The right epididymis was excised immediately with care to minimize blood contamination and placed into a prewarmed (37°C) Petri dish containing two mL of a phosphate-buffered saline solution (pH 7.4). The spermatozoa were assessed for total sperm count and the percentages of motile, progressive, nonprogressive and immotile sperm cells (Tannenbaum *et al*., 2003).

Histological Investigations: The testes and epididymis sections were subjected to Harri's hematoxylin and eosin staining, and the nuclei were stained blue, while the cytoplasm was stained pink, following the method described by Akpantah *et al*. (2003).

Statistical analysis: GraphPad Prism version 8.0.1 was used for the data analysis. One-way analysis of variance (ANOVA) was used to determine the differences between means among the groups, followed by Tukey's post hoc test. The results are expressed as the means ± SEMs. Differences between means were considered statistically significant at $p<0.05$.

RESULTS

Hormones: Compared with the control, the oral administration of sucrose and saccharine significantly reduced the FSH, LH and testosterone levels ($P<0.05$) (Table 1).

Testicular oxidant and antioxidant enzymes: The testicular MDA concentration was significantly increased in the sucrose and saccharin groups compared with the control group ($p<0.05$). Testicular catalase and superoxide dismutase were significantly decrease in sucrose and saccharine group when compared to control group ($p<0.05$) (Figs. 1-3).

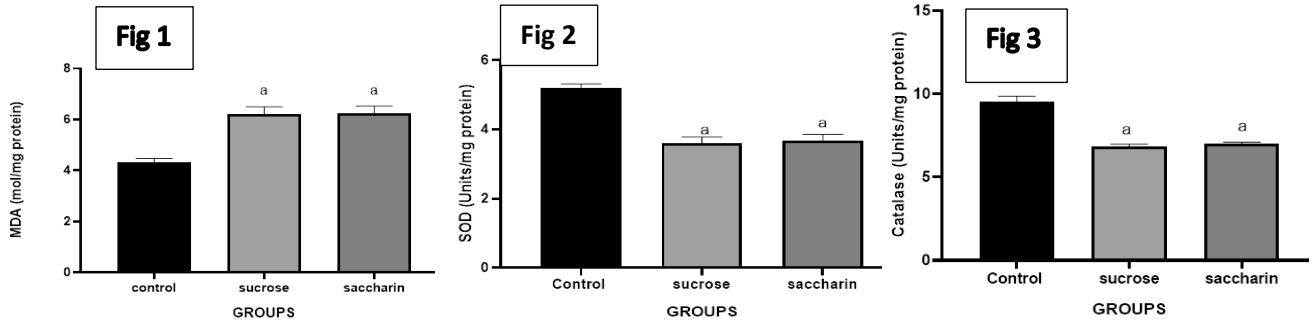
Sperm profile: Sperm count and viability (Figure 4 and 5) were significantly reduced in male Wistar rats treated with sucrose and saccharine compared with the control group ($P<0.05$).

Table 1:
Effects of natural and artificial sweeteners on reproductive hormone levels in male Wistar rats.

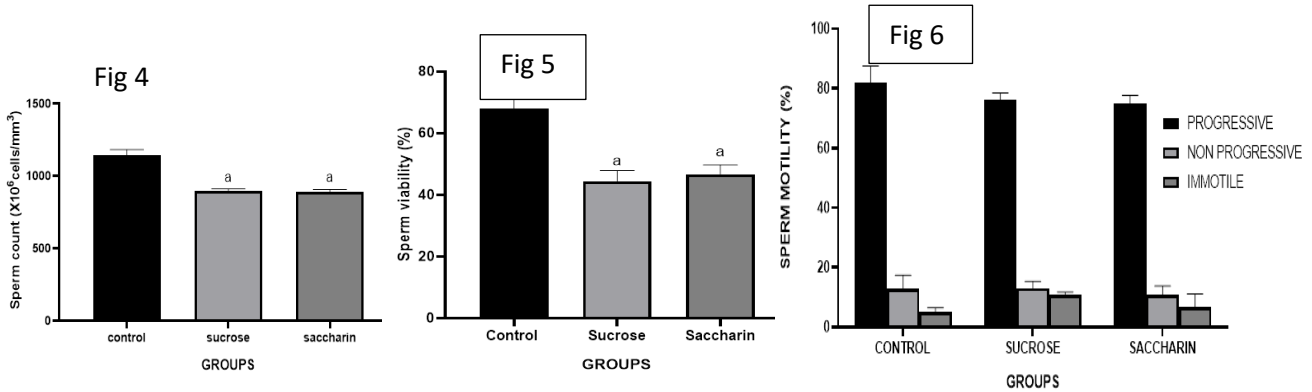
Group	FSH (U/L)	LH (U/L)	Testosterone (ng/ml)
Control	0.173±0.002	0.623±0.012	0.328±0.008
Sucrose	0.030±0.004[a]	0.120±0.015[a]	0.120±0.004[a]
Saccharin	0.110± 0.004[a,b]	0.180±0.001[a,b]	0.198±0.006[a,b]

(a, b) ($p < 0.05$) significantly different from the control group and sucrose group, respectively.

Testicular and epididymal histology: The control group (Plate 1A) showed tubules lined with normal spermatogenic series, Sertoli cells and interstitial Leydig cells, whereas the sucrose group (Plate 1B) showed Leydig cell degeneration, a tubular lumen packed with immature forms, and the saccharine group (Plate 1C) presented patchy spermatogenic arrest and Leydig cell hyperplasia. The epididymis of the rats in the control group presented normal ducts, dilated spermatozoa, and interstitial spaces. The epididymis of the rats in the sucrose group presented remarkable ductal depletion of spermatozoa, whereas the epididymis of the rats in the saccharine group presented patchy ductal depletion of spermatozoa.



Figures 1, 2, and 3: Effects of sucrose and saccharin on SOD, MDA and catalase in male Wistar rats.
(a, b) ($p < 0.05$) significantly different from the control group and sucrose group, respectively.



Figures 4-6: Effects of sucrose and saccharin on sperm count, viability and motility in male Wistar rats.
(a, b) ($p < 0.05$) significantly different from the control group and sucrose group, respectively.

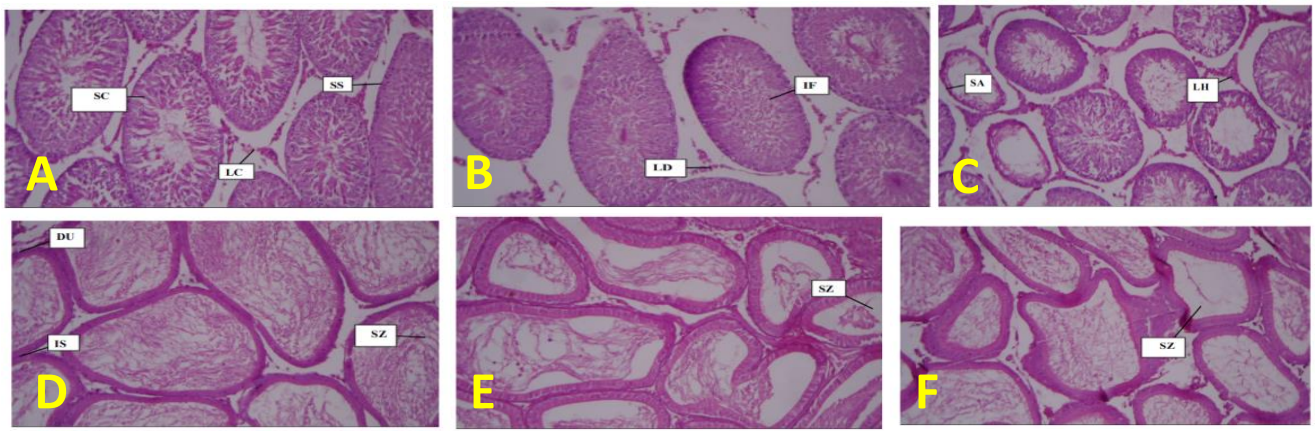


Plate 1

A. Rat testes, group A (control), showing tubules lined with a normal spermatogenic series (SS), Sertoli cells (SCs) and interstitial Leydig (LC) cells: H&E ×100×

B. Rat testes, group B (sucrose), showing Leydig cell degeneration (LD) and a tubular lumen packed with immature forms (IF): H&E 100X.

C. Rat testes, group C (saccharine), showing patchy spermatogenic arrest (SA) and Leydig cell hyperplasia (LH): H&E 100X.

D. Rat epididymis, group A (control), showing the following: normal ducts (DUs), dilated with spermatozoa (SZs), and interstitial space (IS): H&E × 100×.

E. Rat epididymis, group B (sucrose), showing remarkable ductal depletion of spermatozoa (SZ): H&E 100X.

F. Rat epididymis, group C (saccharine), showing patchy ductal depletion of spermatozoa (SZ): H&E 100X.

Testicular StAR gene expression: A significant reduction in testicular StAR gene expression was seen in the rats treated with sucrose and saccharine compared with the control group ($P<0.05$), and a significant increase was observed in the saccharine group compared with the sucrose group ($p<0.05$).

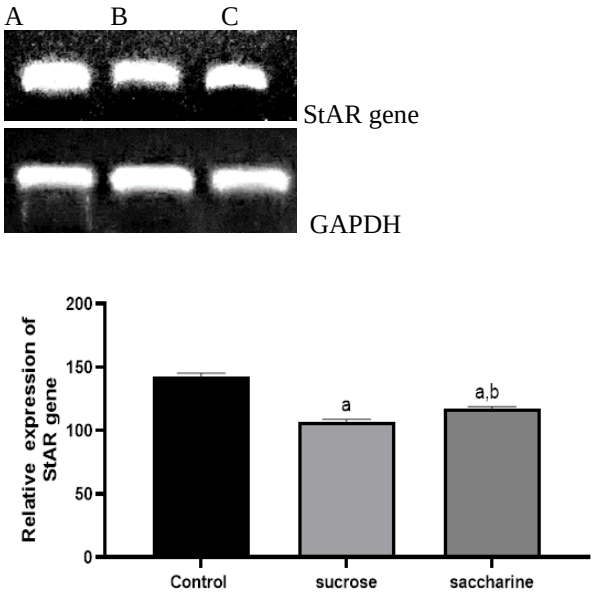


Figure 7:
Effects of sucrose and saccharin on the StAR gene in male Wistar rats.
(a, b) ($p < 0.05$) significantly different from the control group and sucrose group, respectively.

DISCUSSION

Recently, there have been increasing concerns about the effects of sweeteners on different physiological systems of the body (Ghusn *et al*., 2023; Prada *et al*., 2022; Singh *et al*., 2023). The tendency to consume non-nutritive sweeteners for the sweet sensation they add to the diet, without adding extra calories has increased (Faruque *et al*., 2019). This study examines and compares the effects of consuming nutritive (sucrose) and nonnutritive (saccharin) sweeteners on male reproductive functions.

Sucrose, a nutritive sweetener, is derived primarily from the juice of Saccharum officinarum (sugarcane) and the roots of Beta vulgaris (sugar beet) (Gruska *et al*., 2022), whereas saccharin, a nonnutritive sweetener, is manufactured from toluene or anthranilic acid (Azeez *et al*., 2019).

Testosterone plays a crucial role in primary sexual development, which encompasses testicular descent, spermatogenesis, enlargement of the penis and testes, and increased libido (Kalfa *et al*., 2019). In this study, in comparison with the control group, the sucrose group exhibited a significant reduction in testosterone levels, which is consistent with findings from Ashby *et al*. (2003) and Gong *et al*. (2016). Excessive sucrose consumption has been linked to apoptosis through overactivation of the testicular apoptotic pathway, leading to decreased testosterone levels (Glendinning *et al*., 2015).

Luteinizing hormone (LH) is a glycoprotein hormone that is co-secreted with follicle-stimulating hormone (FSH) by gonadotrophin cells in the anterior pituitary gland (Kazmi and Can 2020). The results indicated that both the FSH and LH levels were lower in the sucrose group than in the control group. The cause of this decrease is unclear, as this finding opposes the basic physiological knowledge that expects a negative feedback-induced increase in gonadotropins stimulated by a decrease in testosterone (Marques *et al*., 2000). Additionally, when the saccharin group was compared with the control group, the levels of luteinizing hormone, follicle-stimulating hormone, and testosterone also decreased. This finding suggests that there could be an alteration in the normal hypothalamo-pituitary–gonadal pathway resulting from excessive consumption of both natural and artificial sweeteners. This is in line with the reports of Puica and colleagues in 2009, that aspartame, an artificial sweetener, has a harmful effect on the hypothalamic–pituitary–gonadal axis (Puica *et al*., 2009).

In the study of male infertility, malondialdehyde (MDA) serves as a stable byproduct of polyunsaturated fatty acid

peroxidation and is often associated with protein crosslinking (Aitken *et al*., 1987). Superoxide dismutase (SOD) is the primary detoxifying enzyme and a potent antioxidant within cells, playing a vital role as part of the first line of defense against reactive oxygen species (ROS) (Ighodaro and Akinloye, 2017). Catalase is another key antioxidant enzyme found in nearly all oxygen-utilizing tissues (Ighodaro and Akinloye, 2017). The results showed an increase in the MDA levels in both the sucrose and saccharin groups compared with those in the control group, with the MDA levels being significantly greater in the sucrose group (Adekunbi *et al*., 2016). Elevated lipid peroxidation in the testes due to high sucrose levels may suggest free radical damage, further confirming its detrimental effects on reproductive function (Adekunbi *et al*., 2016). Additionally, noncaloric sweeteners, such as artificial sweeteners, have been reported to increase MDA levels in the testes (Adekunbi *et al*., 2016).

When the sucrose and saccharin groups were compared with the control group, a noticeable decrease in SOD activity was detected. Antioxidant enzymes are typically mobilized in response to rising free radical levels; however, when overwhelmed by excessive free radicals, oxidative stress can occur (Adekunbi *et al*., 2016). Consequently, excessive sucrose consumption may induce oxidative stress in the testes, potentially impairing reproductive performance (Adekunbi *et al*., 2016).

Compared with those in the control group, both the sucrose and saccharin groups exhibited lower catalase activity. Report has shown that administration of sucrose and saccharin led to a reduction in catalase activity (Husni *et al*., 2023). There was a positive relationship between oxidative stress and saccharin consumption; saccharin could release ROS that might be counteracted by the body's ability to produce antioxidants such as catalase, which protects against ROS. This homeostatic mechanism involves a complex antioxidant defense system comprising several enzymes. However, if saccharin consumption continues at elevated doses, leading to increased free radical production, saccharin consumption could overwhelm antioxidant defenses and result in decreased catalase activity (Lobo *et al*., 2010).

Sperm function—reflected by sperm count, motility, viability, and morphology—is a crucial indicator of male fertility potential (Moretti *et al*., 2022). Compared with the control group, the sucrose group showed reductions in sperm count, motility, and viability. High sucrose levels have been shown to significantly disrupt sperm motility (Chiu *et al*., 2014), with lower sperm motility and counts observed in young healthy men who consume high amounts of sugar-sweetened beverages (Chiu *et al*., 2014). Furthermore, Gong *et al*. (2016) reported decreased sperm viability in the sucrose group alongside reductions in motility, characterized by fewer rapid sperm and a greater proportion of nonprogressive sperm. Compared with the control group, the saccharin group also presented decreased sperm count, motility, and viability. These findings align with those of Burke and Small (2015), who reported that high saccharin intake is associated with reduced sperm count and motility. Another study revealed that the total number of sperm significantly decreased in the high-dose saccharin group compared with the control group, while a reduction in rapid sperm and an increase in immotile sperm were noted (Gong *et al*., 2016).

Steroidogenic Acute Regulatory Protein (StAR) is essential for transporting cholesterol into mitochondria for steroid hormone synthesis, including testosterone (Stocco, 2001). The results indicated that StAR gene expression was lower in the sucrose group than in the control group; this finding is consistent with Gong *et al*. (2016), who reported a significant reduction in StAR expression in this group. A similar decrease was observed in the saccharin group relative to the control group. According to Gong *et al*. (2016), the saccharin group also presented lower StAR expression. This decline may correlate with increased cleaved caspase-3 levels—enzymes involved in apoptosis—suggesting potential damage to testicular function (Gong *et al*., 2016).

In the histological examination of the testes, the control group (Figure 8) displayed tubules lined with a normal spermatogenic series (SS), Sertoli cells (SCs), and interstitial Leydig cells (LCs), which are essential for testosterone production in males. In contrast, the sucrose group exhibited degeneration of Leydig cells (LD) and tubular lumens filled with immature forms (IF), as depicted on figure 9. This degeneration may be attributed to sucrose exposure, which can induce oxidative stress. Oxidative stress damages Leydig cells, resulting in their degeneration and a subsequent decline in testosterone production (Aitken and Roman, 2008). Diets high in sugar have been linked to oxidative stress and impaired testicular function, with histological evidence showing disrupted spermatogenesis and the presence of immature germ cells within the tubular lumen (Lei *et al*., 2001).

The saccharin group demonstrated patchy spermatogenic arrest (SA) and Leydig cell hyperplasia (LH), as illustrated in figure 10. This condition may also stem from oxidative stress, resulting in damage to sperm-producing cells (spermatocytes and spermatids), disrupting their development and leading to areas of arrested spermatogenesis (Monageng *et al*., 2023). Elevated reactive oxygen species (ROS) levels can promote Leydig cell hyperplasia by altering the hormonal balance within the testes (Monageng *et al*., 2023).

In the histology of the epididymis, the control group exhibited normal ducts (DU) filled with spermatozoa (SZ) and interstitial space (IS). The sucrose group showed significant depletion of spermatozoa in the ducts (SZ), as shown on figure 12. This depletion may be due to oxidative stress, as sucrose metabolism can generate reactive oxygen species (ROS), leading to oxidative damage. The epididymis is particularly susceptible to oxidative injury due to its high content of polyunsaturated fatty acids and low antioxidant capacity (Kumair and Sait, 2011). Additionally, sucrose intake has been associated with structural changes in the epithelial cells lining the epididymal ducts, including loss of cilia and degenerative alterations (Marcondes, 2018). The saccharin group also exhibited patchy ductal depletion of spermatozoa (SZ), as illustrated on Figure 13, likely due to oxidative stress. Saccharin may compromise antioxidant defense mechanisms in the epididymis, exacerbating oxidative stress and its detrimental effects on epididymal function (Vingren and Kraemer, 2010). Studies have shown that saccharin exposure in animal models leads to elevated levels of ROS and markers of oxidative damage in the

epididymis, which is correlated with the observed histological changes (Lei *et al*., 2001).

In conclusion, both sucrose and saccharin adversely affect reproductive function primarily through mechanisms involving oxidative stress and the inhibition of steroidogenic acute regulatory protein (StAR), resulting in testicular damage, although sucrose had more negative effect when compared to saccharin.

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