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The Effects of Saccharin and Hydroalcoholic Extract of *Stevia Rebaudiana* on the Reproductive System in Adult Male Rats

Mohammad Babaei[a,*], **Ghazal Samavat**[b], **Ali Karimi-Goudarzi**[b], **Hossein Erik-Aghaji**[c]

[a] Department of Basic Sciences, Faculty of Veterinary Medicine, Bu-Ali Sina University, Hamedan, Iran.
[b] Department of Basic Sciences, SR.C., Islamic Azad University, Karaj, Iran.
[c] Department of Basic Sciences, SR.C., Islamic Azad University, Tehran, Iran.

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Abstract

The increasing consumption of artificial sweeteners such as saccharin has raised concerns regarding their potential adverse effects on male reproductive health. Natural alternatives like Stevia rebaudiana may offer safer options. This study aimed to investigate the effects of saccharin and hydroalcoholic extract of Stevia rebaudiana, both individually and in combination, on reproductive parameters in adult male rats. Twenty-four adult male Wistar rats (200-250 g) were randomly divided into four groups (n=6): control (distilled water), saccharin (5 mg/kg body weight), stevia (400 mg/kg body weight), and saccharin+ stevia (5 mg/kg + 400 mg/kg body weight) administered via gavage for 48 days. Following treatment, testicular biometric parameters, sperm parameters (count, viability, motility, DNA damage, morphology), histomorphometry (tubular differentiation index, repopulation index, spermiogenesis index, seminiferous tubule diameter, germinal epithelium height, testicular capsule diameter), and malondialdehyde (MDA) levels were evaluated. Saccharin treatment significantly decreased sperm count, viability, and motility ($p<0.05$), increased DNA damage and abnormal sperm percentage ($p<0.05$), reduced tubular differentiation index, repopulation index, and spermiogenesis index ($p<0.05$), decreased seminiferous tubule diameter and germinal epithelium height ($p<0.05$), and elevated MDA levels ($p<0.05$) compared to the control group. Co-administration of stevia with saccharin ameliorated these adverse effects, with parameters approaching control group levels. Saccharin induced oxidative stress and detrimental effects on male reproductive parameters in rats. Stevia rebaudiana extract demonstrated protective properties, potentially through antioxidant mechanisms and improved testicular blood flow, suggesting its suitability as a safer alternative sweetener with reproductive health benefits.

1. Introduction

In the last decade, there has been a substantial increase in the consumption of food products containing calorie-free sweeteners to address health problems associated with obesity and diabetes. Numerous research studies have focused on sweetener consumption in obese and diabetic patients, primarily aiming to reduce calorie

*Corresponding author: M. Babaei (Associate Professor)
E-mail address: mohammad.babaei@basu.ac.ir
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intake while maintaining dietary satisfaction (Gardner *et al.*, 2012).

The growing prevalence of chronic diseases such as diabetes and obesity has amplified the importance of sugar substitutes as alternatives to sucrose. Sugar substitutes are food additives that replicate or exceed the sweetness of sucrose with minimal or no caloric content. They are broadly classified into artificial sweeteners—including sucralose, saccharin, aspartame, and cyclamate—and natural sweeteners such as stevia (Tandel, 2011). The Food and Drug Administration (FDA) has approved five artificial sweeteners: saccharin, acesulfame, aspartame, neotame, and sucralose. Additionally, stevia has been recognized as a low-calorie natural sweetener (Mats and Popkin, 2009).

The human body responds to artificial sweeteners in complex ways. Non-nutritive sweeteners possess sweetness intensities significantly greater than sucrose or high-fructose corn syrup. Frequent consumption of these artificial sweeteners can overstimulate sugar receptors, potentially reducing tolerance for naturally sweet foods such as fruits and non-sweet foods like vegetables (Farid *et al.*, 2020).

Animal studies have demonstrated addictive properties associated with artificial sweeteners (Yang, 2010). Lenoir *et al.* (2007) reported that the intense sweetness of saccharin could exert more powerful rewarding effects than cocaine, even in drug-sensitized and addicted rats (Lenoir *et al.*, 2007). Mathor *et al.* (2020) found that individuals with type 2 diabetes who consumed artificial sweeteners exhibited greater insulin resistance compared to non-consumers (Mathor *et al.*, 2020). Furthermore, research has indicated that artificial sweeteners, depending on dosage and duration of consumption, can induce pro-inflammatory effects alongside digestive disorders, correlating with alterations in digestive hormones that regulate bowel motility (Bueno-Hernández *et al.*, 2019). Emamat *et al.* (2020) further emphasized that artificial sweeteners play a significant role in altering gut microbiota and inducing dysbiosis (Emamat *et al.*, 2020).

Male reproductive health has experienced significant deterioration in recent years, with the quality and function of male reproductive cells declining substantially. Male infertility rates have increased by approximately 50% (Esmaelzadeh *et al.*, 2007). Male reproductive health is influenced by numerous environmental and genetic factors, with nearly every aspect of modern lifestyle potentially affecting male fertility. Among the most critical lifestyle factors is nutrition, which serves as a determining factor in normal reproductive function. Substantial evidence suggests a direct relationship between poor nutrition and reproductive problems.

Over the past decade, the escalating use of food additives has become a growing concern. Although many food additives are generally recognized as safe (GRAS) for human consumption, considerable concerns remain regarding their potential health effects. Multiple studies have documented adverse effects of food additive consumption on the reproductive system, making it essential to investigate these compounds' effects on male reproductive function for preserving male fertility.

Several hypotheses have been proposed regarding the effects of non-caloric sweeteners. One hypothesis suggests that low-energy sweet foods help prevent weight gain, while alternative theories propose that consuming non-caloric sweetened beverages increases appetite and subsequent food intake. Numerous studies provide conflicting results regarding the effects of artificial sweeteners on food and beverage consumption, body mass increases, and blood glucose levels. However, limited research has examined the effects of artificial sweeteners on the male reproductive system and testicular morphology.

Therefore, our objective is to investigate the effects of saccharin on the male reproductive system and evaluate its potential replacement with a natural sweetener such as stevia, while adhering to ethical principles and animal rights.

2. Materials and Methods

2.1. Animals

In this study, 24 adults male Wistar rats weighing 200-250 g were obtained from the Laboratory Animal Center of the Faculty of Veterinary Medicine, Bu-Ali Sina University, Hamadan, Iran. All rats were transferred to the animal facility one week prior to experimental commencement for environmental adaptation. Animals were housed individually in standard cages with ad libitum access to food and water, maintained at a temperature of 22-26°C, relative humidity of 40-70%, and a 12-hour light/12-hour dark photoperiod. Rats were randomly allocated into four groups, with six animals per group (Zeynab Saberi *et al.*, 2021).

2.2. Experimental Groups

Doses were determined based on acceptable daily intake guidelines for saccharin and the hydroalcoholic extract of Stevia rebaudiana.

Group 1 (Control): Received 3 ml of distilled water via oral gavage daily for 48 days.

Group 2 (Saccharin): Received 5 mg/kg body weight of saccharin via oral gavage daily for 48 days.

Group 3 (Stevia): Received 400 mg/kg body weight of Stevia rebaudiana hydroalcoholic extract via oral gavage daily for 48 days.

Group 4 (Saccharin+ stevia): Received 5 mg/kg body weight of saccharin combined with 400 mg/kg body weight of Stevia rebaudiana hydroalcoholic extract via

oral gavage daily for 48 days (Zeynab Saberi *et al.*, 2021).

2.3. Sampling Procedures

One day after the end of the treatment period, the rats were weighed again. After anesthesia with ketamine (100 mg/kgBW) and xylazine (10 mg/kgBW) and euthanasia by cervical dislocation, the abdominal cavity was opened and blood samples were collected directly from right ventricle. To examine the morphometric indices of the testes, after opening the abdominal cavity with a scalpel, forceps, and scissors, and retracting the intestines to visualize the testes, the left and right testes, along with the epididymis and vas deferens, were removed from the body, washed in physiological saline, and their weights were measured and recorded using a digital balance with 0.01 sensitivity (Babaei *et al.*, 2015).

By determining their weight, the ratio of testicular weight to body weight (GSI x 100) was determined. Also, the thickness, length, and width of the testes were measured, determined, and recorded with a digital caliper.

Investigation of sperm parameters: In order to study the number, viability, motility and morphology of sperm, epididymal tails were cut and transferred to a microtube containing 1 ml of pre-heated HTF medium. Sperm suspension was used to analyze different sperm parameters. The carried-out investigations include counting the mean number of sperms per unit volume and constant dilution using hemocytometer slides, determining the percentage of sperm motility, the percentage of sperm viability (Eosin-Nigrosin staining), the amount of DNA damage (using acridine orange staining method) and nucleus maturation rate (using aniline blue staining method). **Histological evaluations**: Testicular tissue samples fixed in 10% buffered formalin and after tissue passage and preparation of tissue sections (7 μm thickness), hematoxylin-eosin astaining was obtained, and all samples were evaluated in terms of tissue and morphometric changes in multiple magnifications (400x and 1000x) (20). The mean seminiferous tubules diameter (STsD), seminiferous tubules lumen diameter (STsLD), germinal epithelium height (GEH), testicular capsule diameter (TCD) in micrometers were analyzed by Dino-Lite microscope and DinoCapture software (version 2). In order to check the tubular differentiation index (TDI), the percentage of seminiferous tubules with more than three layers of differentiated germ cells from type A spermatogonia were counted and TDI was considered positive. Moreover, to calculate the sperm repopulation index (RI), the ratio of active spermatogonia (type B spermatogonia with dark nuclei) to inactive spermatogonia (type A spermatogonia with light nuclei) was calculated in seminiferous tubules. In order to determine the spermatic index (SI), the percentage of seminiferous tubules with normal spermatogenesis was considered as positive SI. In addition, the tubular degeneration percentage (TDP) was calculated in different experimental groups (Babaei *et al.*, 2018).

Oxidative parameter: Reactive Oxygen Species (ROS) attack polyunsaturated fatty acids present in cell membranes, leading to lipid peroxidation. To determine the extent of lipid peroxidation, MDA production levels were measured in serum samples. Malondialdehyde measurement will be based on reaction with thiobarbituric acid, extraction with normal butanol, spectrophotometric absorption measurement, and comparison of absorption with a standard curve. (Babaei *et al.*, 2018)

Data analysis: The results of this study were statistically evaluated using SPSS version 19 and the results were expressed as mean±standard deviation. To compare between groups, one-way analysis of variance followed by Tukey's supplementary test was used and $p<0.05$ was considered significant.

2.4. Ethical Considerations

The ethical approval for this study was issued from the Committee on Research Ethics of Bu-Ali Sina University, based on the Ethical Guidelines of Research and in accordance with institutional and national guidelines for the care and use of laboratory animals. All animal experiments complied with ARRIVE guidelines (https://arriveguidelines.org) concerning the use of animals in research.

3. Results

3.1. Biometric Parameters

Comparison of body weight, left testicular weight, gonadosomatic index (GSI), and left testicular dimensions (length, width, diameter) among control and experimental groups revealed no significant differences ($p>0.05$) (Table 1).

3.2. Sperm Parameters

Data analysis demonstrated that sperm count in the saccharin-treated group significantly decreased compared to the control and other treatment groups ($p<0.05$). Sperm viability in the saccharin-receiving group significantly decreased compared to the control, stevia, and saccharin+ stevia groups ($p<0.05$). Sperm motility in the saccharin-receiving group exhibited a significant decrease compared to the control and other experimental groups ($p<0.05$). Although sperm motility showed an increasing trend in the stevia group compared to the control and saccharin+ stevia groups, this increase was not statistically significant ($p>0.05$) (Table 2).

Table 1
Comparison of mean testicular biometric parameters in experimental groups.

	BW (gr)	Left Testis W (gr)	GSI (%)	Left testis length (mm)	Left testis wide (mm)	Left testis diameter (mm)
Control	$210/8 \pm 33/43^a$	$1/39 \pm 0/09^a$	$0/006 \pm 0/001^a$	$19/2 \pm 1/82^a$	$11/34 \pm 1/03^a$	$10/51 \pm 0/53^a$
Saccharin	$241 \pm 6/55^a$	$1/39 \pm 0/04^a$	$0/006 \pm 0^a$	$20/66 \pm 0/18^a$	$11/69 \pm 0/34^a$	$10/33 \pm 0/98^a$
Stevia	$201/32 \pm 32/05^a$	$1/2 \pm 0/17^a$	$0/006 \pm 0/0006^a$	$19/08 \pm 1/76^a$	$10/84 \pm 0/97^a$	$10/04 \pm 1/08^a$
Saccharin+ stevia	$222/33 \pm 17/21^a$	$1/34 \pm 0/18^a$	$0/005 \pm 0/0006^a$	$19/6 \pm 1/41^a$	$11/77 \pm 0/35^a$	$10/08 \pm 0/22^a$

Non-similar English letters indicate significant differences in each column ($p<0.05$). **BW**: Body Weight; **W**: Weight; **GSI**: Gonadosomatic Index.

Table 2
Comparison of mean sperm parameters in experimental groups.

	Count (10^6 ml)	Viability (%)	Motility (%)	Maturity (%)	DNA Damage (%)	Abnormality (%)
Control	$62/6 \pm 3/21^a$	$83/2 \pm 3/42^a$	$79/6 \pm 4/83^a$	$99/2 \pm 0/84^a$	$5/6 \pm 2/7^a$	$4/6 \pm 2/7^a$
Saccharin	$54 \pm 4/47^b$	$62 \pm 5/34^b$	$55/8 \pm 2/77^b$	$96/2 \pm 1/78^b$	$12 \pm 2/55^b$	$10/4 \pm 1/14^b$
Stevia	$64/4 \pm 3/19^a$	$83/2 \pm 3/96^a$	$81/6 \pm 6/43^a$	$98/8 \pm 1/3^{ab}$	$6/8 \pm 3/27^a$	$5/4 \pm 2/75^a$
Saccharin+ stevia	$63 \pm 2/91^a$	$84/2 \pm 4/15^a$	$79/6 \pm 5/13^a$	$98/4 \pm 1/82^{ab}$	$8 \pm 3/32^{ab}$	$7/8 \pm 3/11^{ab}$

Non-similar English letters indicate significant differences in each column ($p<0.05$).

Table 3
Comparison of mean histomorphometric parameters in experimental groups.

	TDI (%)	RI (%)	SI (%)	STsD (μm)	GEH (μm)	TCD (μm)	DTP (μm)
Control	$88/8 \pm 1/35^a$	$86/82 \pm 4/78^a$	$92/5 \pm 2/78^a$	$365/83 \pm 8/25^a$	$128/57 \pm 10/65^a$	$19/68 \pm 2/84^a$	$5/2 \pm 2/39^a$
Sakharin	$70 \pm 3/57^b$	$67 \pm 4/59^b$	$70/8 \pm 3/58^b$	$303/75 \pm 11/39^b$	$99/59 \pm 8/99^b$	$28/61 \pm 2/95^b$	$10/4 \pm 1/14^b$
Stevia	$86/2 \pm 6/22^a$	$82/9 \pm 2/88^a$	$85/2 \pm 4/98^a$	$371/25 \pm 11/45^a$	$132/41 \pm 11/86^a$	$20/84 \pm 1/95^a$	$6/8 \pm 1/92^a$
Sakharin+ Stevia	$83/3 \pm 3/95^a$	$83/2 \pm 2/77^a$	$86/5 \pm 4/97^a$	$365/18 \pm 13/63^a$	$137/75 \pm 7/7^a$	$21/11 \pm 0/84^a$	$7/6 \pm 3/64^{ab}$

Non-similar English letters indicate significant differences in each column ($p<0.05$). **TDI**: Tubular Differentiation Index; **RI**: Repopulation Index; **SI**: Spermiogenesis Index; STsD: Seminiferous Tubule Diameter; **GEH**: Germinal Epithelium Height; **TCD**: Testicular Capsule Diameter; **DTP**: Degenerated Tubule Percentage.

Evaluation of sperm nuclear maturity percentage revealed a significant decrease in the saccharin-receiving group compared to the control group, indicating a higher proportion of immature sperm in the saccharin group ($P<0.05$). However, no significant differences were observed with other treatment groups ($P>0.05$). Assessment of sperm DNA damage demonstrated a significant increase in the saccharin-receiving group compared to the control and stevia groups ($p<0.05$). Conversely, the level of sperm with damaged DNA in the saccharin+ stevia group showed a decrease compared to the saccharin group, though this difference was not statistically significant ($p>0.05$) (Table 2).

Measurement of abnormal sperm count revealed a significant increase in the saccharin-receiving group compared to the control and stevia-receiving groups ($p<0.05$). The level of abnormal sperm in the saccharin+ stevia group decreased compared to the saccharin group, but this difference was not statistically significant ($p>0.05$) (Table 2).

3.3. Histomorphometry

Comparison of tubular differentiation index (TDI), repopulation index (RI), and spermiogenesis index (SI) among different experimental groups revealed that saccharin administration caused a significant decrease compared to the control, stevia, and saccharin+ stevia groups ($p<0.05$) (Table 3).

Seminiferous tubule diameter values in the saccharin-treated group exhibited a significant decrease compared to the control, stevia, and saccharin+ stevia groups ($p<0.05$). Germinal epithelium height in the saccharin-treated group demonstrated a significant decrease compared to the control, stevia, and saccharin+ stevia groups ($p<0.05$). Testicular capsule thickness in the saccharin-receiving group significantly increased compared to the control, stevia, and saccharin+ stevia groups ($p<0.05$) (Table 3).

Measurement of degenerated tubule percentage revealed a significant increase in the saccharin-receiving group compared to the control and stevia-receiving groups ($p<0.05$). The percentage of degenerated

tubules in the saccharin+ stevia group was lower than in the saccharin group, though this difference was not statistically significant ($p>0.05$) (Table 3).

3.4. Histological Examination

Histological evaluation of tissue sections from different experimental groups revealed distinct patterns. In the control group, cellular structures appeared normal with spermatids appropriately positioned within seminiferous tubule lumens. Germ cell layers exhibited normal architecture (Fig. 2A). In the saccharin group, germinal epithelium height within seminiferous tubules was markedly reduced, accompanied by an increased number of TDI-negative tubules, indicating impaired spermatogenesis (Fig. 2B). In the stevia group, cellular structures maintained normal morphology with spermatids properly distributed within seminiferous tubule lumens. Germ cell layers displayed normal architecture (Fig. 2C). In the saccharin+ stevia group, cellular structure showed improvement with fewer TDI-negative tubules compared to the saccharin group. Germinal epithelium height was greater than in the saccharin group, approaching control group values (Fig. 2D).

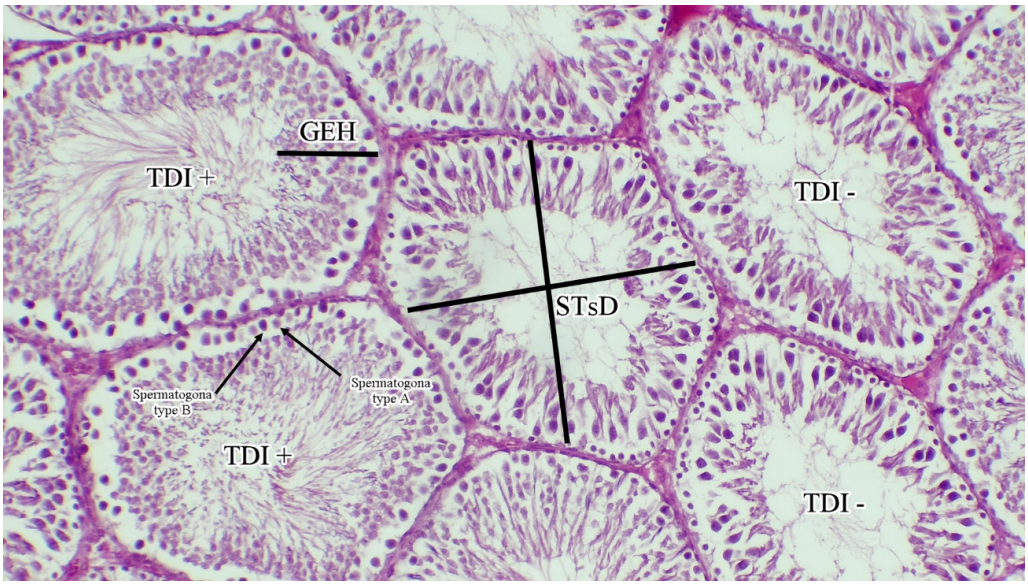

Fig. 1. Histomorphometric parameters in testis. The mean seminiferous tubules diameter (STsD), germinal epithelium height (GEH), tubular differentiation index (TDI), (H&E staining, ×400 magnification).

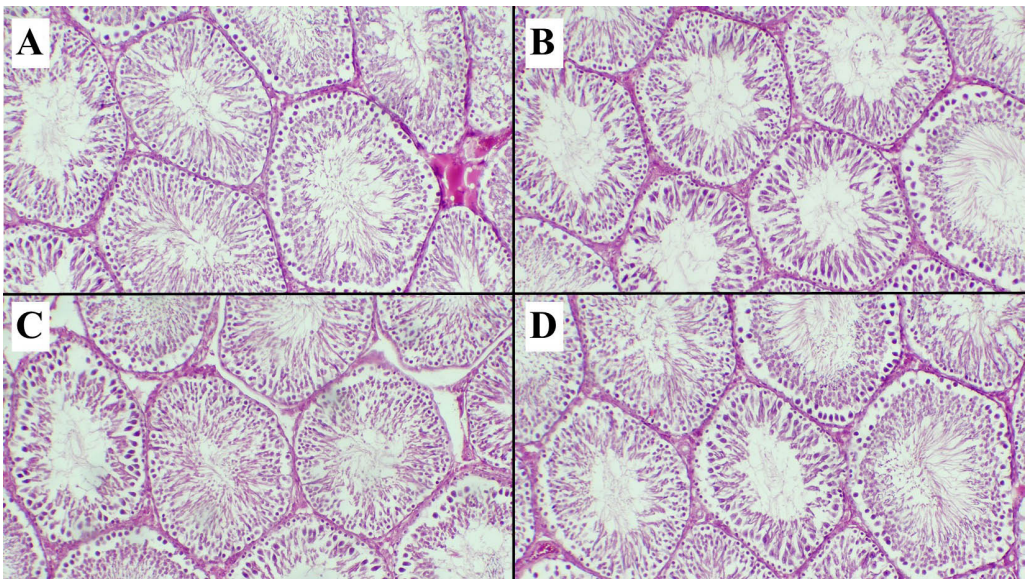

Fig. 2. Histological sections of testis in different experimental groups. A: Control group, B: Saccharin group, C: Stevia group, D: Stevia+Saccharin group. (H&E staining, ×400 magnification).

3.5. Malondialdehyde (MDA) Levels

Investigation of MDA levels in serum across different groups revealed that saccharin administration caused a significant increase in MDA concentration compared to all other experimental groups ($p<0.05$). MDA levels in the stevia+saccharin group were significantly lower than in the saccharin group and significantly higher than in the control and stevia groups ($p<0.05$) (Fig. 3).

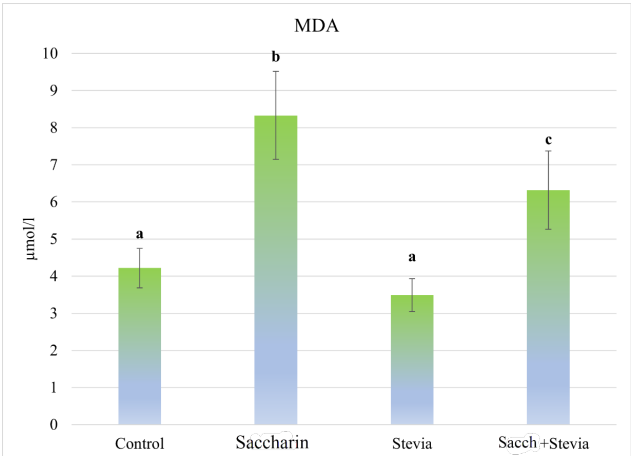

Fig. 3. MDA values in different experimental groups. Non-similar English letters indicate significant differences ($p<0.05$).

3.6. Discussion

Infertility and related reproductive issues are recognized as significant problems affecting quality of life. In recent years, considerable concerns have emerged regarding declining male fertility potential and reproductive problems, particularly in industrialized nations. One of the primary causes of male infertility is compromised sperm quality and men's inability to produce healthy, motile sperm. Multiple factors can adversely affect sperm production and contribute to infertility. The most common cause of male infertility is insufficient production of healthy, active sperm. Sperm fertility depends on various parameters including count, motility, and morphology (Esmaelzadeh *et al.*, 2007).

Stevia rebaudiana, as a natural, carbohydrate-free sweetener, was investigated in this study to compare its effects both individually and in combination with saccharin, an artificial sweetener. Results obtained in this study demonstrated that hydroalcoholic extract of Stevia rebaudiana can ameliorate adverse effects induced by sodium saccharin on multiple parameters related to the male reproductive system.

The sweetening properties of stevia derive from compounds such as Rebaudioside A and steviol (Ashwell, 2015). Numerous therapeutic properties have been documented for stevia, including antioxidant, anti-diabetic, vasodilatory, anti-hypertensive, diuretic, immunomodulatory, antimicrobial, memory-enhancing, cardioprotective, weight-reducing, and antidepressant effects (Momtazi-Borojeni *et al.*, 2017; Ashwell, 2015; Gupta *et al.*, 2014; Shahi and Mohammadyfar, 2017).

Previous studies have shown that stevia increased testosterone and estrogen levels (Juan *et al.*, 2008), while other research demonstrated that stevia extract significantly reduced weights of testes, seminal vesicles, and cauda epididymis (Melis, 1999). Additionally, Rebaudioside A administration in F0 and F1 generations had no adverse effects on reproductive parameters including mating, fertility, pregnancy duration, estrous cycle, sperm motility, and morphology (Curry *et al.*, 2008). In contrast, the present study demonstrated that stevia improved sperm quality in saccharin-receiving rats.

Multiple findings have shown that stevia induces vasodilation and increases blood flow (Melis, 1996), which may enhance blood perfusion to testicular vessels. By increasing blood supply to testicular tissue, stevia may improve reproductive function in male rats.

Examination of sperm abnormalities across control and experimental groups revealed that abnormal sperm count in the stevia group did not differ significantly from the control group. The saccharin+ stevia group also showed no significant difference from the control group. Zhang *et al.* (2017) investigated toxicity of hydroalcoholic extract of Stevia rebaudiana Bertoni leaves on sperm abnormalities in mice, reporting no adverse effects on sperm morphology (Zhang *et al.*, 2017), consistent with the present study's findings. In this study, stevia not only exhibited no adverse effects but also, by potentially increasing testicular tissue blood supply, ameliorated saccharin's adverse effects on sperm parameters to an acceptable degree.

One study demonstrated that ethanolic extract of Stevia rebaudiana exerts superior antioxidant properties and anti-proliferative effects in tumor cells compared to its diterpene glycoside stevioside (Lopez *et al.*, 2016). Interestingly, stevioside and Rebaudioside A, the primary sweetening metabolites in stevia leaves, showed lower oxygen radical absorbance capacity compared to whole plant extracts (Bender *et al.*, 2015). This activity for stevioside likely stems from the absence of phenyl aromatic rings with free radical scavenging capacity in steviol glycosides, indicating that stevia's antioxidant activity depends on other compound types such as polyphenols. Particularly, chlorogenic acids (CGA), well-known for their efficient antioxidant activities, accumulate primarily in ethanolic extract of Stevia rebaudiana Bertoni leaves (Shivanna *et al.*, 2013). These findings support stevia's promising role as a natural antioxidant source with multiple applications in food supplements and pharmaceutical industries (Lopez *et al.*, 2016).

In the present study, no significant difference in

serum antioxidant status was observed between stevia and control groups. However, antioxidant status in the stevia+saccharin group improved compared to the saccharin group, attributable to the antioxidant properties of hydroalcoholic extract of Stevia rebaudiana.

Ghaheri *et al.* (2018) demonstrated in diabetic rats that stevia consumption improved reproductive behaviors, reduced blood glucose, and increased testosterone levels and Leydig cell count (Ghaheri *et al.*, 2018). In the present study, stevia effectively counteracted adverse effects induced by saccharin. Sperm motility, viability, count, DNA integrity, and nuclear maturity were significantly compromised in the saccharin group compared to other groups. Stevia partially mitigated saccharin's adverse effects through its antioxidant properties and enhanced testicular tissue blood supply. Comparison of spermatogenesis indices (TDI, RI, SI) among groups revealed stevia's capacity to improve these parameters.

In the present study, body and testicular weights decreased in the stevia-receiving group, though not significantly, while the gonadosomatic index remained comparable to the control group. Stevia may beneficially contribute to weight management by reducing carbohydrate intake and through its antioxidant properties. Helal *et al.* (2019) found that sodium saccharin adversely affected physiological parameters in rats including body weight and insulin levels (Helal *et al.*, 2019). The present study obtained similar results, with body weight in the saccharin-receiving group showing the highest values, consistent with Helal *et al.*'s findings.

Abdi *et al.* (2023) demonstrated that diabetes caused decreased body and testicular weights and increased fasting blood glucose. Stevia consumption significantly increased body and testicular weights and decreased serum fasting blood glucose compared to the diabetic group. Furthermore, stevia significantly elevated blood testosterone levels compared to the diabetic group. Sperm parameters were significantly improved with stevia treatment compared to the diabetic group, and stevia administration significantly increased in vitro fertilization success rates and in vitro development of fertilized oocytes compared to the diabetic group (Abdi *et al.*, 2023). While body and testicular weights decreased in rats receiving stevia in the present study—inconsistent with Abdi *et al.*'s study—the improvement in sperm parameter quality and spermatogenesis parameters by stevia in the saccharin+ stevia group was consistent with Abdi *et al.*'s findings.

Ganjiani *et al.* (2020) demonstrated that stevia improved adverse effects of testicular torsion including elevated MDA levels and decreased glutathione peroxidase and superoxide dismutase activities, effectively ameliorating adverse effects of ischemic injury in testis (Ganjiani *et al.*, 2020). In the present study, MDA level examination across control and experimental groups showed that MDA levels in the saccharin-receiving group exceeded other groups. Simultaneous stevia consumption with saccharin reduced MDA levels and improved antioxidant status.

Stevia's antioxidant properties have been documented in numerous studies, demonstrating that stevia's antioxidant effects can reduce endothelial damage and protect Leydig cell integrity (Hurtado de Catalfo and De Gómez Dumm, 2017; Wankeu-Nya *et al.*, 2014). Research has shown that stevia prevents Leydig cell degradation in treated diabetic mice compared to untreated diabetic mice. As a calorie-free, anti-diabetic, vasodilatory, antioxidant sweetener, stevia can effectively treat fertility disorders by reducing blood glucose and through its aphrodisiac properties (Ghaheri *et al.*, 2018).

Oxidative stress is a condition caused by increased cellular damage due to oxygen and oxygen-derived free radicals, known as reactive oxygen species (ROS). Reactive oxygen species (ROS), also known as free radicals, have at least one unpaired electron, which makes them highly reactive molecules due to the unpaired electron in their outer shell (Miranda-Vilela *et al.*, 2010; Henkel, 2011). ROS represent a broad collection of free radicals (e.g., hydroxyl ion (OH), superoxide ion (O2), nitric oxide (NO), peroxyl (RO2), lipid peroxyl (LOO), and thiol (RS)) and non-radical molecules (e.g., singlet oxygen (1O2), hydrogen peroxide (H2O2), hypochlorous acid (HOCL), lipid peroxide (LOOH), and ozone (O3)) (Bansal and Bilaspuri, 2011). In a healthy body, pro-oxidants and antioxidants are in balance. Pathological problems occur when ROS overcome antioxidant defense systems and disrupt the complex balance between ROS and the antioxidant system. Depending on the nature, number, and duration of ROS dominance, significant damage occurs in biomolecules such as lipids, proteins, nucleic acids, and carbohydrates (Agarwal and Prabakaran, 2005). Sperm are equipped with antioxidant defense mechanisms and can neutralize ROS. Therefore, germ cells and mature sperm are protected against oxidative stress. However, under disease conditions, ROS production exceeds the capacity of seminal plasma, and oxidative stress occurs (Henkel, 2011; Trussell 2013).

Sperm were the first cell type to show high sensitivity to oxidative stress. Sperm are unable to repair damage caused by oxidative stress because they lack the necessary enzymatic-cytoplasmic repair systems. This is one of the characteristics that leads to increased sensitivity of sperm to oxidative stress (Saleh *et al.*, 2002). On the other hand, the cell membrane of sperm is rich in polyunsaturated fatty acids (PUFAs), making them highly susceptible to oxygen-induced damage and subsequent lipid peroxidation. Subsequently, the rapid loss of intracellular ATP due to lipid peroxidation causes neurological damage, reduced sperm viability, and an increase in morphological defects in the

midpiece of sperm, all of which lead to reduced sperm motility (Bansal and Bilaspuri, 2011; Gharagozloo and Aitken, 2011). With the progression of peroxidation in sperm, approximately 60% of the membrane fatty acids are lost. Hence, sperm function is affected by reduced fluidity, increased non-specific ion permeability, and inactivation of membrane receptors and enzymes (Tremellen, 2008). Oxidative stress is considered a major cause of male infertility. Furthermore, there is ample evidence that increased oxidative stress significantly impairs sperm function (Agarwal *et al.*, 2012).

Semen parameters such as sperm count, motility, and morphology are commonly used to determine the fertilizing potential of sperm. Although these parameters provide an overview of sperm quality, they do not provide information regarding DNA, which is one of the main components of reproduction. Single- or double-strand DNA breaks can be the source of differences in fertility potential between fertile and infertile men. It has been reported that chromatin in the sperm nucleus is vulnerable to oxidative damage, leading to DNA fragmentation (Zribi *et al.*, 2011).

The results of sperm parameter analysis showed a significant decrease in sperm count, motility, and viability in the saccharin group compared to the control group. Also, the number of immature and morphologically abnormal sperm increased in the saccharin group mice compared to the control group. Our data showed that the extent of DNA damage in the saccharin group was significantly higher than in the control group.

Studying spermatogenesis indices is a suitable criterion for evaluating the activity of testicular tissue. Among these, some indices can be used to properly estimate the activity of cells involved in spermatogenesis. Three indices: tubular differentiation index (TDI), spermiogenesis index (SI), and repopulation index (RI) can be useful in understanding the activity of testicular tissue (Shetty *et al.*, 2000). The tubular differentiation index (TDI) indicates the rate of cell division and the ability of cells to increase in number and differentiate. A decrease in this index indicates a decrease in the cell population of the seminiferous tubule wall.As a result of the decrease in the population of germ cell lines in the wall of the seminiferous tubules, structural changes will also be observable, among the most important of which are the reduction in the height of the germinal epithelium and the disruption of the order and arrangement of intercellular junctions. The disruption of intercellular junctions can cause cells to be released into the lumen of the seminiferous tubule, which will lead to a decrease in the tubular differentiation index. Therefore, a decrease in this index can indicate a disorder in intercellular junctions. In the process of normal spermatogenesis, cells are usually drawn in a row from the basal part and seminiferous tubules towards the lumen of the tubules. This indicates the normalcy of the cell division process (Meistrich *et al.*, 2003). Any disruption in the cell division process, such as the elimination and loss of germ cell lines, can reduce the tubular differentiation index. Therefore, a decrease in the mentioned index can be due to a decrease in cell population caused by abnormal elimination in germ cell lines (Shetty *et al.*, 2000; Meistrich *et al.*, 2003). A decrease in the tubular differentiation index is directly related to a decrease in the population of germ cells. Therefore, a decrease in this index can indicate a decrease in the population of germ cell lines, especially spermatocytes. In this regard, it should be noted that a decrease in cell population can be due to a decrease in division power or an increase in cell loss. The spermiogenesis index (SI) indicates the capability and ability of spermatid cells to differentiate into spermatozoa cells. The repopulation index (RI) indicates the number of active spermatogonia cells in the wall of the seminiferous tubules of the testicular tissue. An increase in the number of active spermatogonia cells can lead to an increase in the population of spermatogenesis cell lines (Shetty *et al.*, 2000). A drop in the IR index can be one of the reasons for a decrease in the TDI index. In the present study, saccharin had adverse effects on spermatogenesis parameters, but the consumption of stevia simultaneously with saccharin improved these parameters compared to the control. Stevia, due to its antioxidant effects, can enhance the spermatogenesis process and prevent oxidative damage. Also, given that stevia can act as a vasodilator, by increasing blood supply, it can improve the adverse effects of saccharin.

From the results obtained in this study, it can be concluded that stevia, as an energy-free sweetener that also has antioxidant properties, can be a suitable substitute for the artificial and industrial sweetener sodium saccharin, which, due to its adverse effects on the male reproductive system, is recommended not to be used in industry, and stevia, due to its availability and low cost, can be used as a sweetener and antioxidant.

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Conflict of Interest

The authors declare no conflicts of interest related to this work.

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