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Dietary Selenium Supplementation Reduces Oxidative Stress and Attenuates Apoptotic Gene Expression in Testes of Pre-Pubertal Male Goat

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Selenium, Seleno-proteins, Oxidative Stress, Apoptosis, Goat, Testis

ABSTRACT

Selenium (Se) nutrition improves reproductive health in ruminants. We hypothesized that pre-pubertal Se supplementation in feed would improve testicular growth by modulating oxidative stress (OS) and apoptosis. To test this hypothesis, twenty young cross-bred male goats (3-3.5 months old, 11-13 kg) were divided into control (C, n=10) and Se-Yeast (SY, n=10) groups, receiving the same diet without (C) or with (SY) Se at 0.3 mg/kg diet for 10 weeks. OS markers and apoptotic genes expressions were measured through kits, and RT-qPCR, respectively. Results showed significant increase ($P < 0.05$) in testicular weight, width, thickness, and circumference in SY compared to C. Testicular volume tended to increase ($P = 0.08$) in SY compared with C, however, length showed no significant difference between the groups. Analysis of anti-oxidative markers revealed significant increase ($P < 0.05$) in activities of glutathione peroxidase (GSH-Px), superoxide dismutase (SOD) and catalase (CAT) and simultaneous decrease ($P < 0.05$) in malondialdehyde (MDA) content in both serum and testicular tissues in SY compared to C. Concurrent with reduction in OS, Se supplementation significantly reduced ($P < 0.05$) expression of pro-apoptotic genes including *caspase 3*, *caspase 9* and *Bax* by 0.47- fold, 0.44-fold, 0.52-fold, respectively, while increased ($P < 0.05$) anti-apoptotic gene (*Bcl-2)* expression and *Bcl-2/Bax* ratio by 0.48-fold and 2.3-fold, respectively in SY compared to C. This study demonstrates that dietary Se supplementation enhanced testicular growth as evidenced by increase in its dimensions by attenuating OS and apoptosis in testicular tissue of pre- pubertal male goat.

INTRODUCTION

Selenium (Se) is a vital trace mineral essential for various biological functions, including antioxidant defence, immune response modulation, and reproductive health (Schomburg, 2019).

It is an essential component of enzymes like glutathione peroxidase (GSH-Px), plays a pivotal role in protecting cells from oxidative stress (OS)-induced damage and maintaining cellular redox balance (Bano *et al*., 2023). In livestock nutrition, adequate Se intake is pivotal for optimal health and productivity, particularly in enhancing reproductive performance and mitigating the detrimental effects of OS on fertility (Liao *et al*., 2020).

In the process of testicular growth, mature cells maintain equilibrium in their number through growth and differentiation, maturation and death (Ashraf *et al*., 2022). Regarding testicular growth, various gross morphological dimensions are important for young bucks to gauge their testicular development and functioning. These attributes are directly proportional to spermatogenesis along with

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general reproductive fitness (Bano *et al*., 2019). Proper selenium (Se) requirement permits full growth and establishment of the testicles because Se is involved in antioxidant defence mechanisms as well as cellular redox state that maintain normality of the testicles (Zoidis *et al*., 2018). Previous studies have shown that supplementation of Se affects the structure of the testicle in several species. Liao *et al*. (2020) showed that supplementing breeders roosters with Se increases not just its size but also weight indicating it might have similar effect in other species including ruminants.

Neutralizing reactive oxygen species (ROS) and safeguarding the testicular cells from oxidation is a primary function of antioxidants such as GSH-Px, superoxide dismutase (SOD) and catalase (CAT) (Zoidis *et al*., 2018). Selenium in seleno-proteins, such as GSH-Px, strengthens antioxidant defence mechanisms that alleviate oxidative stress within the testes thereby maintaining cellular structure and function (Shahid *et al*., 2020). Some investigations have found that Se supplementation promotes antioxidant enzyme activities in testicular tissues. Zhang *et al*. (2019) discovered that organic Se sources could increase poultry's GSH-Px activity, which suggests that similar results might be obtainable in goats. According to Hawkes *et al*. (2019), Se supplementation alters porcine embryo's anti-oxidant status and gene expression indicating a wider effect on oxidative stress management across species.

The somatic and germ cell homeostasis is maintained through proliferation and apoptosis (Ashraf *et al*., 2022). Se-induced testicular improvement consequently increase anti-oxidative status by modulating apoptotic gene expression which may be attributed to anti-apoptotic effect of Se (Xu *et al*., 2023). Apoptosis occurs through both either mitochondrial (intrinsic) or death receptor (extrinsic) pathways through activation of caspases family has been associated with modulation in pro-apoptotic and anti-apoptotic gene expression. The fate of the germinal cells within testis depends on the equilibrium between pro-apoptotic factors such as Caspase 3, Caspase 9 and Bax, and anti-apoptotic factors like Bcl-2 and Bcl-2/Bax ratio. Apoptosis involves activation of caspase enzymes that cause cellular component atrophy leading to organ death (Soomro *et al*., 2018). In testicular tissue under various conditions including oxidative stress and hormonal regulation, decreased expression of apoptotic processes involving caspase 3, caspase 9 and Bax have been documented (Liao *et al*., 2020). In contrast, anti-apoptotic proteins such as Bcl-2 work to inhibit caspases activation hence promoting cell viability (Samo *et al*., 2020). There exists a delicate balance between these entities for sustainable seminiferous epithelium integrity and ordinary spermatogenesis.

A deficiency of selenium in livestock diets is a significant issue that can reduce fertility and productivity. Testicular growth is a sophisticated biochemical process in which cellular homeostasis is maintained by proliferation, differentiation, maturation, and apoptosis of the cells (Ashraf *et al*., 2022). Se-induced histological improvement consequently increases parenchymal tissue mass in testes, which may be attributed to anti-apoptotic effects of Se in pre-pubertal goats (Xu *et al*., 2023). Cellular apoptosis consists of pro-apoptotic genes such as caspases, bax, and anti-apoptotic genes such as bcl-2 (Soomro *et al*., 2018). We speculated that Se-induced alteration in parenchymal tissue might have been associated with decreased OS and modulation in apoptotic genes expression. The present study was, therefore, designed to investigate the effects of dietary Se supplementation on testicular gross morphology, anti-oxidative markers and apoptotic gene expression in testes of pre-pubertal goat.

MATERIALS AND METHODS

Animal assortment and feeding management

Twenty young cross-bred male goats, aged 3-3.5 months and weighing 11-13 kg body weight (BW), were purchased and sent to the livestock experimental station at SAU Tandojam. After two weeks of adaption, the animals were randomly separated into two groups: control (C, n=10) and Se-Yeast (SY, n=10), and they were fed the same diet without (C) or with Se supplementation. Se was added from an organic source, namely Se-yeast, commercially available as Selemax (Biorigin®, São-Paulo, Brazil), at a dosage of 0.3 mg/kg diet. The diet consisted of a 35:65 concentrate to roughage ratio (Table I) and was served twice a day, with water always provided before the animals. Se concentration in diets was analysed by using inductively coupled plasma mass-spectrometry (Perkin Elmer-Optima, 2100-DV) as illustrated by Samo *et al*. (2020). The trial lasted for 10 weeks.

Table I. Ingredients and nutrient level in diet fed to experimental animals.

Ingredients (% of DM)		Nutrient level	
Berseem	65	DM (%)	85.41
Corn	25.6	Crude protein (% of DM)	16.74
Soybean meal	7.4	Crude fat (% of DM)	3.81
Lime stone	0.5	Crude fiber (% of DM)	6.85
Calcium phosphate dibasic	0.8	Crude ash (% of DM)	8.11
Salt	0.4	ME (MJ/kg of DM)	10.88
Mineral Premix[1]	0.4		

DM, Dry matter; ME, Metabolizable energy. [1]Per kg of premix= Vitamin A 6 000U; Vitamin D2 500U; Vitamin E 80 mg; Cu 6.25 mg; Fe 62.5 mg; Zn 62.5 mg; Mn 50 mg; I 0.125 mg; Co 0.125 mg; Mo 0.125 mg.

Blood sample collection and enzymatic analysis activity

On the day of the trial's completion, a 5 mL blood sample was obtained from each animal's jugular vein and placed in a sterile vacutainer. Blood was centrifuged at 3000 g for 10 min and kept at -20°C to measure oxidative stress markers. Enzymatic activity was evaluated using an ELISA test kit (Blue Gene, Shanghai, China) according to the manufacturer's instructions.

Slaughter and tissue sample collection

Following live BW record, the goats were slaughtered via Halal Method. The testicles along with epididymis were hygienically detached. Immediately, the epididymis was gently separated from each testis and weighed, and then two testicular tissues sampling was performed. First, a few tissue pieces from left testis were taken and fixed in standardized phosphate buffer solution (PBS) buffer solution for oxidative stress markers evaluation. Another tissue sample from right testis was taken in Eppendorf tube and stored frozen for RNA extraction and PCR analysis.

Testicular gross biometry

The physical characteristics of the fresh testis were observed and documented. A technique known as water displacement was used to quantify testicular volume (Bano *et al*., 2019). In summary, this was performed by filling a measuring cylinder with normal saline to a pre-set starting point, and then measuring the level of normal saline at the topmost area of the meniscus. The final volume was determined by subtracting the last range from the initial range of normal saline. The images of the testes were taken using a digital camera (Samsung ES95, 16.2 megapixel). The testicle weight was measured at triple beam balance machine. According to Bano *et al*. (2019) measurement of testicular length was determined through placing the fixed arm of vernier calliper at on proximal ending point, while other arm was moved to distal end of testes. Thickness of depth was measured by placing the fixed arm of calliper anteriorly (dosrsal aspect) and sliding arm at the posteriorly (ventral aspect) at its maximum level. Although the measurement of width in each testis was determined by keeping the caliper arm at medial aspect, while the sliding arm at the lateral point hence measured (medio-laterally) at the level of maximum width. However, the circumference was determined by wrapping a thread around each testis at the midpoint, which was then levelled and measured on a meter rule, as well as by enclosing calculating tape directly on the surface of the testicles.

Malondialdehyde (MDA) level and anti-oxidant activity assay in serum and tissue

The testicular tissue lysates were prepared by homogenizing in standardized PBS. The pro-oxidant MDA concentration and anti-oxidant activity of GSH-Px, SOD and CAT were measured using enzyme-linked immuno-sorbent assay kits (Blue Gene, Shanghai, China) (Shah *et al*., 2022). Standard processes and procedures as indicated by the manufacturer were followed.

Isolation of whole RNA and real-time qRT-PCR

The acid guanidinium thiocyanate-phenol-chloroform (GTC) method was used to extract total RNA from testicular tissue (Soomro *et al*., 2018; Malhi *et al*., 2013). Using the nanodrop spectrometer (Thermo Scientific™), the RNA concentration at 260 and 280 nm was determined. All of the samples had absorbance ratios between 1.72 and 1.84, which is a sign of very pure RNA. Using a combination of forward and reverse primers (Table II), 1xiQ SYBR Green supermix (Bio-Rad Laboratories, Inc., Hercules, CA), a particular volume of sterile water, and one cDNA template, real-time PCR (Thermo Scientific™) was performed in a 20 µl total volume. To denaturize the cDNA,

Table II. Primers used in quantitative real-time PCR analysis.

Gene	Primer sequence 5′ to 3′	Accession number	Size (bp)
Caspase 3	AGCCATGGTGAAGAAGGAATCA ACCACAGTCCAGTTCTGTGCCT	NM-001077840.1	156
Caspase 9	TCCTTTGTTCATCTCCTGCTTG TTTTCCTTGGCTTGGCTTTG	XM-004013798.1	115
Bcl-2	GATGACCGAGTATCTGAACCG GACAGCCAGGAGAAATCAAACA	NM-001166486.1	120
Bax	TCTGACGGCAACTTCAACTG TGGGTGTCCCAAAGTAGGAG	NM-173894.1	205
GAPDH	TTGTCTCCTGCGACTTCA CCACCACCCTGTTACTGTT	HM043737.1	135

Caspase 3 and 9; Bax, Bcl-2-associated X protein; *Bcl-2*, B-cell lymphoma 2; *GAPDH mRNA*, Glyceraldehyde 3-phosphate dehydrogenase; bp, base pairs. The first primer listed for each gene is the forward primer and the sec primer is reverse primer.

a 30-sec cycle at 95 °C was employed. Following that, 40 PCR cycles were run, with primer annealing and extension at 55 °C for 30 sec and denaturation at 95 °C for 10 sec. Prior to performing PCRs on experimental materials, the amplification efficiencies of all primers were evaluated using standard dilution series. Every sample was examined three times, and a melt analysis was done following the PCR analysis. GAPDH was used to normalize gene expression (ΔCt = Ct target–Ct GAPDH). The method $2^{-\Delta\Delta Ct}$ was utilized to calculate the relative expression values, following the instructions provided by (Soomro *et al*., 2018; Malhi *et al*., 2013).

Statistical analysis

The data were analysed using the Student's t test (unpaired) in the statistical program SPSS25.0 (Stat Soft, Tulsa, OK, USA). The results were shown as Means ± SEM, with significant differences at $P < 0.05$.

RESULTS

Testicular gross morphology

The effect of diet without and with selenium (Se) supplementation on gross morphology of testes, and testicular weight, length, width, thickness, circumference and volume of goat is presented in Table III. The testicular weight (9.01 ± 0.34 g in SY vs 7.78 ± 0.26 g in C), width (1.78 ± 0.05 cm in SY vs 1.54 ± 0.08 cm in C), thickness (2.36 ± 0.16 cm in SY vs 1.97 ± 0.06 cm in C) and circumference (3.06± 0.9 cm in SY vs 2.78 ± 0.09 cm in C) significantly greater ($P < 0.05$) in SY related with C. However, length (5.54 ± 0.29 cm in SY vs 5.27 ± 0.33 cm in C) in SY compared to C showed no any significant difference ($P < 0.05$), while volume of testis (3.81± 0.71 cm^3 in SY vs 3.39± 0.13 cm^3 in C) showed tendency to increase ($P = 0.08$) in SY compared with C.

Table III. Effect of dietary organic selenium supplementation on gross morphology of testis in male goat.

Items	Groups		P value
	C	SY	
Weight (g)	7.78 ± 0.26	9.01 ± 0.34*	0.0138
Length (cm)	5.27 ± 0.33	5.54 ± 0.29	0.5402
Width (cm)	1.54 ± 0.08	1.78 ± 0.05*	0.0305
Thickness (cm)	1.97 ± 0.06	2.36 ± 0.16*	0.0386
Circumference (cm)	2.78 ± 0.09	3.06 ± 0.9*	0.0518
Volume	3.39 ± 0.13	3.81 ± 0.17	0.0796

* values (mean ± SE) differ at $P < 0.05$ between two groups.

Pro-oxidant and Anti-oxidant markers

Oxidative stress markers in serum of male goat

The effect of diet without and with Se addition in blood serum anti-oxidant glutathione peroxidase (GSH-Px), super-oxide dismutase (SOD) and catalase (CAT) activity as depicted in Figure 1. The GSH-Px (27.20 ± 0.85 U/ml in SY vs 23.09 ± 0.67 U/ml in C), SOD (33.13 ± 0.45 U/ml in SY vs 28.50 ± 0.63 U/ml in C) and CAT (30.06 ± 0.42 U/ml in SY vs 23.13± 0.64 U/ml in C) activities were significantly greater ($P < 0.05$) in SY related with C. However, pro-oxidant marker malondialdehyde (MDA) level in serum (9.75 ± 0.18 nmol/ml in SY vs 14.33 ± 0.81 nmol/ml in C) was declined significantly ($P < 0.05$) in SY related with C.

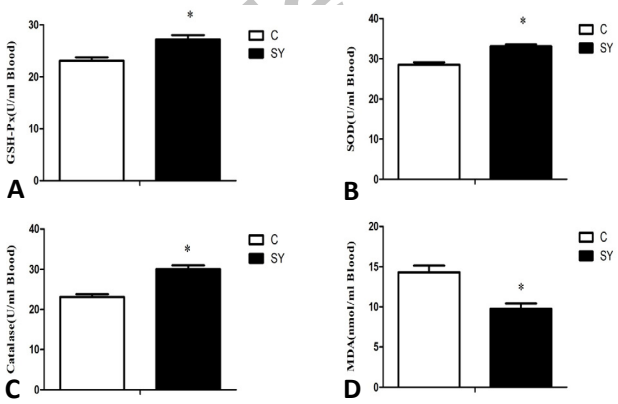

Fig. 1. The effect of dietary SY supplementation on glutathione peroxidase (GSH-Px), super oxide dismutase (SOD) Catalase (CAT) activity (U/ml) and Melondialdehyde (MDA) activity (nmol/ml) in serum of goat. Goats were fed diet without (C) or with (SY) selenium at the rate of 0.3 mg kg^{-1} diet for 10 weeks. *Values are mean ± S.E on the bars exhibit the differences between groups with $P < 0.05$.

Oxidative stress markers in testis of male goat

The evaluation of the effects of diet without and with Se addition in testis on OS markers is shown in Figure 2. The GSH-Px (32.76 ± 0.43 U/g in SY vs 29.26 ± 0.72 U/g in C), SOD (226.44 ± 0.21 U/g in SY vs 215.06 ± 0.37 U/g in C) and CAT (20.92 ± 0.36 U/g in SY vs 16.51 ± 0.76 U/g in C) was significantly higher ($P < 0.05$) in SY related with C. However, the MDA content in testis (12.33 ± 0.16 nmol/g in SY vs 19.49 ± 0.13 in C) was declined significantly ($P < 0.05$) in SY related with C group.

Apoptotic gene expressions in testicular tissue

Concurrent with changes in gross morphology and anti-oxidative stability, dietary organic Se produced significant effect on the expression of pro-apoptotic

Caspase 3, Caspase 9, Bax and anti-apoptotic *Bcl2 and Bcl2/Bax* genes in the testicular tissue as depicted in Figure 3. The pro-apoptotic genes including *caspase 3*, *caspase 9* and *bax* downregulated ($P < 0.05$) by 0.47- fold, 0.44-fold, 0.52-fold, respectively, while, anti-apoptotic genes including *bcl-2* and the *bcl-2/bax* ratio upregulated ($P < 0.05$) by 0.48-fold and 2.3-fold, respectively in SY group compared with C group.

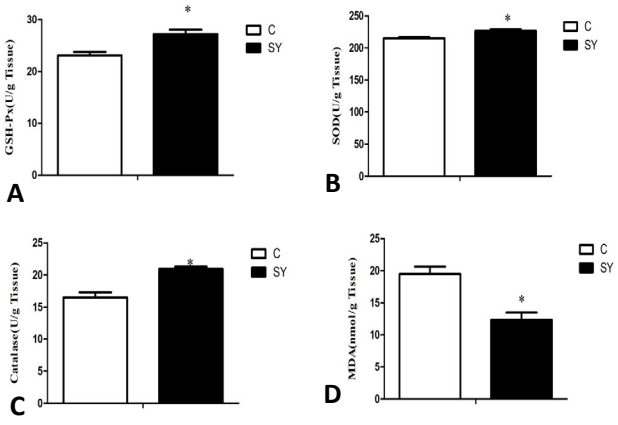

Fig. 2. The effect of dietary SY supplementation on glutathione peroxidase (GSH-Px), super oxide dismutase (SOD) Catalase (CAT) activity (U/g) and Melondialdehyde (MDA) activity (nmol/g) in testis of goat. Goats were fed diet without (C) or with (SY) selenium at the rate of 0.3 mg kg^{-1} diet for 10 weeks. *Values are mean ± S.E on the bars exhibit the differences between groups with $P < 0.05$.

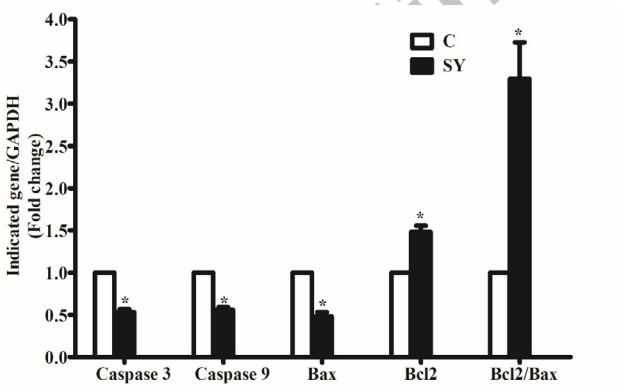

Fig. 3. Effect of dietary organic selenium supplementation on apoptosis related genes *Caspase 3 and 9; Bax, Bcl-2*-associatedX protein; *Bcl-2*, B-cell lymphoma 2; *mRNA* expression in testicular tissue of goat. Goats were fed diet without (C) or with (SY) selenium at the rate of 0.3 mg kg^{-1} diet for 10 weeks. Gene expression level was calculated with real-time PCR in comparison with *GAPDH RNA*. *Values are mean ± S.E on the bars exhibit the differences between groups with $P < 0.05$.

DISCUSSION

Se addition in the pre-pubertal diet increases reproductive life of ruminants by lowering the time to attain puberty. The progressive effects of Se on male puberty involves the stimulation of enhanced body growth with simultaneous testicular development and growth. The present study was intended to understand the underlying anti-oxidative and anti-apoptotic effects of Se on pre-pubertal testicular growth.

Our results demonstrate that dietary Se supplementation improved testis weight, width, thickness and circumference. Consistent with our results, previous investigations reported that feeding Se-supplemented diet in young kids increased testis weight, width and circumference in goat (Bano *et al*., 2019). Improved testis weight and other dimensions are important reproductive indicators determine the antioxidant capability in animals. (Mojapelo and Lehloenya, 2019). The increased testicular volume in the treated animals also support the idea that Se helps promote testicular health, possibly by encouraging cell growth and reducing cell death. Qazi *et al*. (2020) proposed that adding of Se might improve testicular development by controlling pathways related to oxidative stress (OS) and cell death.

The ROS are highly active oxidizing factors that cause OS that decrease anti-oxidant defense system (Jamali *et al*., 2019). Due to the lipid-peroxidation (LPO), the malondialdehyde (MDA) is three carbon compound that is considered as OS marker produce cell deterioration and cell death (Ma *et al*., 2018). The MDA concentration in tissue reflects the pro-oxidant level hence used as OS marker (Samo *et al*., 2020). Earlier study have demonstrated that feeding of high concentrate (HC) diet enhance MDA content in blood and body tissue (Bano *et al*., 2023). Although the effect of Se supplementation on testicular tissue MDA content has not been much studied before however, in existing study the organic Se supplementation at the dose rate of 0.3 mg/kg diet declined MDA content in serum and tissue.

All living organisms endogenously produced essential component of anti-oxidant defence system are GSH-Px, SOD and CAT enzymes that provide shielding effect against the ROS (Ahmed *et al*., 2016). In addition, GSH-Px plays pivotal role in prevention of oxidative damage via inhibiting LPO through converting in their corresponding alcohols (Čobanová *et al*., 2017; Samo *et al*., 2020). Our findings demonstrated that adding Se to the feed decreased the amount of MDA in the serum testes of the treated goats, while also increasing the activities of GSH-Px, SOD, and CAT. Consistent with our findings previous studies also show increased serum and tissue GSH-Px, SOD and CAT

content in SY supplemented diet, similarly MDA content showed decline in a group who is supplemented with organic selenium (Bano *et al*., 2019, 2023). Moreover, from current findings it is demonstrated that improved anti-oxidant stability might be due to improved tissue Se concentration eventually resulted in attenuation of OS and epithelial damage (Samo *et al*., 2018). Our findings suggest that Se supplementation increases antioxidant enzyme activity and decreases OS markers are consistent with these findings. Furthermore, a study by Shah *et al*. (2022) suggested that the role of Se in protection against oxidative damage in hepatic cells.

Oxidative metabolism results in lipid peroxides production, which leads to OS and may ultimately cause cell death in the testes epithelium by engaging an apoptotic pathway that compromises epithelial damages (Samo *et al*., 2020). Apoptosis, is an extremely specialized process regulated by the *Bcl-2* family of regulatory proteins and caspases. These proteins are normally present in a stable form; however, slight modification in the rate of their expression may cause change in the *Bcl-2/Bax* ratio, which would modify the apoptotic rate, and eventually influence the cell phenotype (Soomro *et al*., 2018). A balance between apoptosis and cell proliferation is essential for maintaining tissue homeostasis and normal growth. These two opposing processes are connected by shared molecular machinery depending on the cellular environment (Soomro *et al*., 2018). In current study, the testes epithelium of a pre-pubertal male goat supplemented with SY caused down-regulation of pro-apoptotic viz., *caspase 3 caspase 9* and *Bax* genes and upregulation of anti-apoptotic viz., *Bcl-2* gene and the *Bcl-2/Bax* ratio. Previous studies have shown that adequate amount of Se in diet is required for normal reproductive health and testicular development in animal, whereas the diet either deficient or excess in Se induced testicular damage through oxidative stress mediated modulation in proliferative and apoptotic genes in rats and roosters (Xu *et al*., 2023; Yan *et al*., 2024).

The modulation of apoptotic genes (*Caspase 3, Caspase 9, Bax*) and the anti-apoptotic gene *Bcl-2* within the testicular tissues of the SY indicates that Se supplementation impacts apoptosis pathways. Similarly, *Bax* promotes apoptosis by means of antagonizing *Bcl-2*, which has anti-apoptotic effects (Samo *et al*., 2020). When the pro-apoptotic *Bax* gene is activated in a stress-mediated state, it attaches to the mitochondrial outer membrane permiabilization (MOMP) and releases cytochrome C (cyt, C). This process starts the mitochondrial or intrinsic route (Kumar *et al*., 2018). The apoptotic pathway forms a complex called cyt C, in which *caspase 9* activates executioner *caspase 3*, which in turn causes cell death (Shi *et al*., 2018). Moreover, Se affects the metabolic capacity of epithelial cells in testes in concentration dependent manner and modulates the expression of genes linked to cell apoptosis through the regulation of histone deacetylase (Adimulam *et al*., 2021; Gui and Shen, 2016).

CONCLUSION

This study demonstrates that supplementation of Se in diet enhanced testicular growth as evidenced by increase in its dimensions such as weight, width, thickness and circumference by attenuating OS and apoptosis in testicular tissue of pre- pubertal male goat.

DECLARATIONS

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IRB approval

The research design was approved by the Board of Advanced Studies and Research (BASR); Directorate of Advance Research (DAS) under reference number (DAS/2464) in the year 2023.

Ethical approval

The procedures performed in this research such as handling of animals, sampling, and analytical methods, received prior approval in 152nd meeting of board of advanced studies and research (No. DAS/2464/of 2023), Sindh Agriculture University (SAU), Tandojam.

Statement of conflict of interest

The authors have declared no conflict of interest.

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