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Full Length Article

Dietary Selenium Yeast Supplementation Reduced High Concentrate Diet-Induced Epithelial Injury and Improved Antioxidative Status by Mitigating Apoptotic Genes Expression in Rumen of Goats

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Abstract

Selenium (Se) as a micronutrient in ruminant diet plays a vital role in antioxidant systems and preventing cellular damage. High concentrate diets cause abnormal fermentation in rumen that leads to increased oxidative stress (OS), leading to rumen epithelial tissue damage and apoptosis in goats. This study evaluated the influence of dietary Se on OS, rumen epithelium and apoptosis in goats. Thirty cross-bred goats (12-16 weeks, 10-13 kg) were allocated to group LC (low concentrate diet), HC (high concentrate diet) and HC-SY (HC + selenium yeast at 0.5 mg/kg diet) for 75 days. Rumen epithelial tissue samples were analyzed for the biomarkers of OS and mRNA expression of apoptotic pathway genes. Histomorphology of rumen epithelium showed reduced OS and improved epithelial morphology in group HC-SY than in HC. Malondialdehyde (MDA) raised ($P < 0.0001$) in HC than LC but reduced ($P < 0.001$) in HC-SY than HC. Glutathione peroxidase (GSH-Px) and superoxide dismutase (SOD) activity escalated ($P < 0.05$) in HC-SY compared to HC. mRNA gene expression of apoptotic pathway genes *Bax, Caspase-3,* and *-9* were upregulated ($P < 0.05$) and the *Bcl2* and *Bcl2/Bax* ratio downregulated ($P < 0.05$) in group HC than in LC, however mRNA expression level of *Bax, Caspase-3* and *-9* were downregulated ($P < 0.0001$) while *Bcl2,* and *Bcl2/Bax* ratio were upregulated ($P < 0.01$) in HC-SY. In conclusion, it is suggested that Se addition to high concentrate diet improves ruminal epithelium *via* alleviating diet-induced OS and apoptosis in rumen of goats.

Keywords: Antioxidant; Apoptosis; Goats; Oxidative stress; Rumen epithelium; Selenium

Introduction

Selenium (Se) is an essential micronutrient required by the ruminants in their diet for various metabolic and biochemical processes (Ahsan *et al.* 2014). It plays a vital role in modulating the antioxidative status *via* seleno enzymes such as glutathione peroxidases (GSH-Px) which play vital role to ensure the cellular protection with a defensive role in antioxidation by detoxifying potentially hazardous free radicals like superoxides, peroxides and hydroxyl ions produced during oxidative metabolism (Shah *et al.* 2022; Bano *et al.* 2024). Bioavailability and production GSH-Px is directly dependent on the concentration and form of dietary Se that becomes widely distributed in various parts of animal body such as spleen, pancreas, hepatic and renal organs, musculoskeletal, respiratory, reproductive system and gastrointestinal tract (Samo *et al.* 2018). Supplementing ruminant diet with supranutritional Se levels greater than standard dosage up to 20-30 folds enhances the Se retention in tissues and improves the antioxidative status without causing toxic effects (Samo *et al.* 2020). Feeding caprine with concentrate diets have shown improved Se retention at tissue level as Se travels through the gut, as compared with the animals kept on feeding roughage diet plan (Samo *et al.* 2018). Supplementing the Se content at supranutritional levels exhibited enhanced impact in sheep with better efficiency in growth as well as muscular health in conditions of heat stress, whereas it reduced the degree of oxidative stress (OS) which promoted the GIT

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performance in swine experiencing heat stress in swine (Chauhan *et al.* 2016; Liu *et al.* 2016). In addition, Se has been demonstrated to mitigate OS in a number of toxicity models and illness states of an animal (Zakeri *et al.* 2021).

Fattening ruminants receive high concentrate diets containing starch which is readily fermented by ruminal microbes to produce volatile fatty acids (VFAs) which help to fulfill the energy requirement of the animal in order to maximize its growth and productivity (Boerman *et al.* 2015; Chen *et al.* 2022). Consistently feeding the ruminants with grain-rich diet could result in complex systemic problems and digestive disorders because of the immense quantities of non-structural sugars found in that feed. (Tao *et al.* 2015; Chen *et al.* 2023). Consequently, rumen is predisposed to abnormal changes as well as systemic diseases induced by high concentrate diets due to an unusual rise in fermentation with in the rumen that decreases the pH attributable to the accumulation of immensely build-up short-chain fatty acids (SCFAs) beyond absorption capacity of rumen tissue (Li *et al.* 2019; Shahid *et al.* 2020; Zhang *et al.* 2020). During ordinary circumstances, the penetration of endotoxins and antigens through the ruminal epithelium is limited (Balda and Matter 2009) owing to the physical, microbial, and immune barriers that inhibit the transmission of these substances across the rumen (Shen *et al.* 2019). SCFAs have pronounced effect on physical barriers in rumen, compromising the rumen epithelial tissue integrity for the regeneration of rumen epithelial cells and the expression distribution of tight junction proteins (Greco *et al.* 2018; Ma *et al.* 2021). Decreased pH due to excessive SCFA production against high concentrate diet induces a significant release of lipopolysaccharides (LPS) that alter the gut permeability (Gozho *et al.* 2005; Emmanuel *et al.* 2007) followed by epithelial damage concomitant with escalated concentration of reactive oxygen species (ROS) (Tao *et al.* 2014). Feeding high concentrate diet for an extended period further induces the proinflammatory response resulting in severe epithelial insults in rumen which trigger up the apoptotic cell death (Dai *et al.* 2022). According to an investigation, Se may ameliorate the process of apoptosis and the cell damage caused by cyclophosphamide by altering the apoptotic signaling mechanisms (İpek *et al.* 2023). Considering the potential advantages of dietary Se supplementation, especially in challenging circumstances, we speculated that a grain-rich diet as a high concentrate supplemented with organic Se in goat feeding could mitigate epithelial injuries by improving antioxidant mechanism and preventing the apoptotic condition within the goat rumen. To our knowledge, no studies have assessed the influence of supplemental organic Se-yeast on OS markers, histopathology of rumen epithelium, and apoptosis induced by high concentrate diet in rumen of goats. The present study was conducted to investigate the influence of dietary Se supplementation against the changes in biomarker of OS, histopathological alterations in rumen epithelium, and pro and anti-apoptotic gene expression in rumen of goats induced by the high concentrate diet.

Materials and Methods

Experimental design, animal feeding and management

Present research was carried out at Ruminant Research Unit situated in Livestock Experimental Station, Sindh Agriculture University, Tandojam, Pakistan; prior to approval by the Institutional Ethical Committee under 151st resolution of BASAR as on 13-07-2023 (Approval No.: DAS/1869 of 2023, Dated 08-08-2023). Thirty cross-bred goats aging between 12-16 weeks, having bodyweight of 10-13 kg were kept in individual room spacing of 3 × 3 sq. ft. Animals were divided in three experimental groups as LC: low concentrate (fed as roughage : concentrate 65:35), HC: high concentrate diet (fed as roughage : concentrate 35:65) and HC-SY: Grain rich feed plus selenium yeast supplementation (fed as roughage : concentrate 35:65 + Se at 0.5 mg/kg diet). With an adaptation period of 30 days, the trial lasted for the next 75 days. Routinely goat feeding was practiced on daily basis at 08:00 am and 05:00 pm with open access to fresh drinking water during the whole trial period. Se yeast was added to the LC diet at a rate of 0.115 mg/kg to equalize the content of Se to that in high concentrate diet. However, as treatment in high concentrate diet the Se yeast at 0.5 mg/kg of diet added in HC-SY. In order to estimate the Se content in all diets, the wet microwave digestion was carried out to determine the digestion level against the standards through inductively coupled plasma optical emission spectroscopy (ICP-OES Optima 2100-DV, Perkin Elmer, USA) as per the procedures by Taylor (2005). The composition of diets and level of Se in each diet is presented in Table 1.

Slaughtering

At the end of experimental trial, each goat was slain in isolated slaughtering chamber within the Ruminant Research Unit. Goats were appropriately restrained in lateral recumbency and slaughtered by decapitation. Immediately after the exsanguination, complex stomach was exteriorized through abdominal incision, isolated from other visceral organs, and taken into a clean tub.

Measurement of oxidative stress and antioxidative status

A segment of ruminal wall measuring 5 cm^2 was excised from the atrium ruminis and the rumen epithelium was subsequently separated manually from the underlying muscle layer from the free side of ruminal wall. Following that, the sample was frozen in an Eppendorf tube at –80°C to measure the OS biomarkers, and gene expression of pathways linked to apoptosis. The lysates of rumen epithelial tissues were prepared by homogenizing the samples in a standardized PBS solution (0.1 *M*, 7 pH) and centrifuged at 3000 × g for 15 min and supernatants were collected. Tissue malondialdehyde (MDA), glutathione

Table 1: Composition of different diets for animal feeding

Contents	LC	HC	HC-SY
Ingredients (% of Dry Matter)			
Corn	25.6	25	25
Wheat bran	-	30.7	30.7
Soybean meal	7.4	2.2	2.2
Rapeseed meal	-	4	4
Dicalcium phosphate	0.8	0.7	0.7
Calcium carbonate	0.5	1.5	1.5
Sodium chloride	0.4	0.4	0.4
Premix	0.4	0.4	0.4
Se (mg/kg diet)			
Se content in basal diet	0.035	0.15	0.15
Supplemented Se	0.115	-	0.50
Final content of Se	0.15	0.15	0.65

Dietary groups for animal feeding were LC: low concentrate, HC: high concentrate, HC-SY: HC diet supplemented with Se-yeast

Table 2: Primer sequences and GenBank accession number for real time PCR

Gene	Primer Orientation	Primer sequence (5' – 3')	GenBank accession No.	Amplicon size (bp)
GAPDH	Forward	GGGTCATCATCTCTGCACCT	HM043737.1	180
	Reverse	GGTCATAAGTCCCTCCACGA		
Bcl2	Forward	TCGCCCAAGTCAAACATTA	AY423861.1	208
	Reverse	CACAGGTGAAACTGCCAAGAT		
Bax	Forward	TGCTCACTGCCTCACTCAC	AF163774.1	178
	Reverse	CCAAGACCACTCCTCCCTA		
Caspase-3	Forward	GGTTCATCCAGGCTCTTT	AF068837.1	98
	Reverse	TTCTGTCGCTACCTTTCG		
Caspase-9	Forward	TCCTTTGTTCATCTCCTGCTTG	XM-004013798.1	115
	Reverse	TTTTCCTTGGCTTGGCTTTG		

peroxidase (GSH-Px), superoxide dismutase (SOD), catalase (CAT), and total antioxidant capacity (TAC) were determined with a kit method through ELISA kits (Nanjing Jincheng Bioengineering Institute, China), following the manufacturer's instructions and testing protocol.

Rumen epithelial histomorphology

A 1 cm^2 segment of rumen tissue was excised from the atrium ruminis of each animal, washed with physiological saline solution, and fixed in 4% paraformaldehyde solution for 24 to 48 h to carry out histopathological examination. The samples were suspended in running tap water overnight to remove excessive fixation followed by dehydration in ascending concentrations of ethanol, and clearance in pure xylene. Tissue blocks were prepared by embedding in paraffin wax, making serial sections each having 4 μm thickness, were cut from the blocks using a microtome, and stained with hematoxylin and eosin. Rumen epithelial morphology was observed under the microscope in 4 sections from each goat using the method described by Tao *et al.* (2014).

Measurement of expression of apoptosis-related genes using PCR

Tissue samples of rumen epithelium were processed for total RNA extraction using acid guanidinium thiocyanate-phenol-chloroform method, according to Chomczynski and Sacchi (2006). Total RNA concentration was measured at 260 and 280 nm with a nanodrop spectrophotometer. The absorbance ratio of each sample ranging between 1.91 and 2.29 showed maximum RNA purity. Relative mRNA expression evaluation was done by performing real-time PCR by utilizing the MyiQ2 2-color real-time PCR detection system. Real-time PCR was executed in a final volume of 20 µL containing 1x iQ SYBR Green Supermix (Bio-Rad Laboratories, Inc., Hercules, CA, USA), cDNA template, using a combination of forward and reverse primers for the target genes (Table 2), with sterile water for volume adjustment. To denature the cDNA, a first cycle of 30 sec at 95°C was applied. This procedure followed further with 40 PCR cycles to denature cDNA for 10 s at 95°C and the annealing and extension of primers for 30 sec at 55°C. Prior to execution of the PCR on ready samples, the amplification efficiencies of each primer was precisely calculated using a standard dilution series. At the end of each PCR, a melt curve analysis was performed. Gene expression was normalized to Glyceraldehyde 3-phosphate dehydrogenase, GAPDH ($\Delta Ct = Ct_{target} - Ct_{GAPDH}$). After normalization with GAPDH, the expressions values of relative mRNA for the targeted genes were determined by applying the formula $2^{-\Delta\Delta Ct}$ as illustrated by Livak and Schmittgen (2001).

Data analysis

The data collected was analyzed using statistical software GraphPad Prism, GraphPad Software Inc., version 8.0.1 (for Windows). Results were expressed as mean values ± standard error and differences were considered statistically significant at $P < 0.05$.

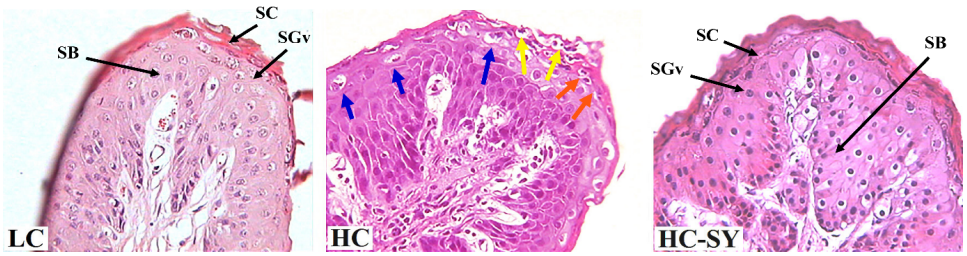

Fig. 1: Representative photographs histological sections of rumen tissue. Comparison of histological alterations in rumen epithelium in groups LC, HC and HC-SY at 10X magnification. Stratum corneum (SC), Stratum germinativum (SGv), and Stratum basale (SB). Rumen tissue damage is represented by yellow, blue and orange arrows depicting injury in rumen epithelium, inflammatory cellular infiltrate and the depletion of SC, respectively

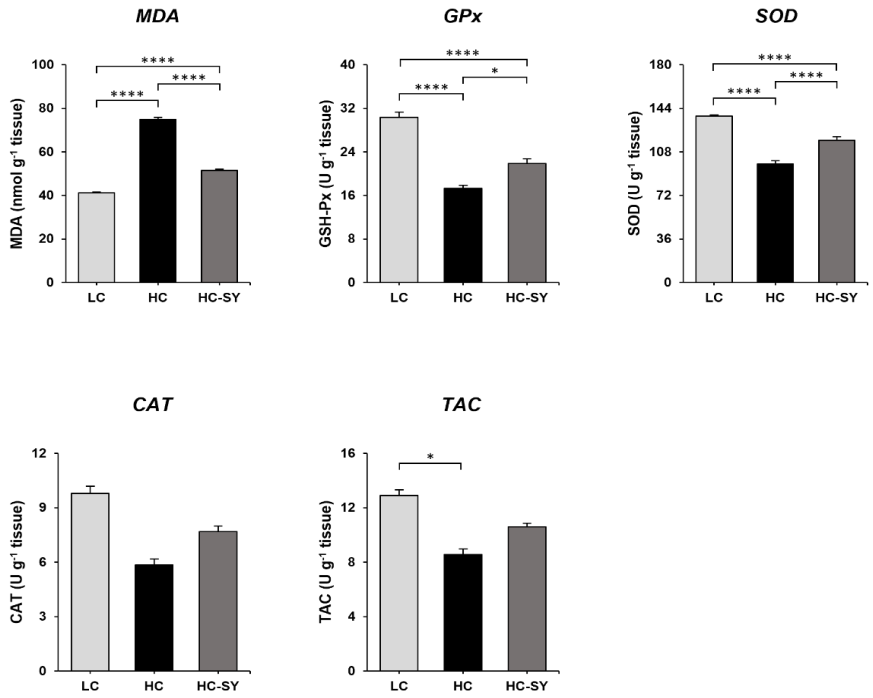

Fig. 2: Representing the antioxidant activity in rumen epithelial tissue in group LC, HC and HC-SY. MDA: Malondialdehyde; GSH-Px: Glutathione peroxidase; SOD: Superoxide dismutase; CAT: Catalase and TAC: Total antioxidant capacity. Values are means ± S.E. Statistical difference among groups: ****$P < 0.0001$; ***$P < 0.001$; **$P < 0.01$; *$P < 0.05$

Results

Histomorphology of rumen epithelial tissue

Goats fed high concentrate diet showed pronounced damage to rumen epithelial tissue characterized by injuries in rumen epithelium, stratum corneum (SC) depletion and inflammatory cellular infiltrate compared to goats fed LC diet. Whereas, HC-SY lowered the severity of these lesions in comparison to group HC (Fig. 1).

Oxidative stress and antioxidative status

Fig. 2 shows the OS and antioxidative status in each group. Group fed HC diet had greater ($P < 0.0001$) MDA level than LC group whereas, in HC-SY it was decreased ($P < 0.0001$) Compared to HC. GSH-Px and SOD activity escalated ($P < 0.05$) in HC-SY than grain-rich HC group. CAT activity was non-significant ($P > 0.05$) among all groups. Compared with LC, TAC activity reduced ($P < 0.05$) in HC group, however group HC-SY showed slight increase compared to HC however showed no significant difference ($P > 0.05$) compared to other groups.

mRNA expression of apoptosis regulator genes in rumen epithelial tissue

The mRNA expression of apoptosis regulator genes *Bcl2* and *Bax* of *Bcl* family and *Caspase-3* and *-9* in rumen epithelial tissue of goats are shown in Fig. 3. Compared

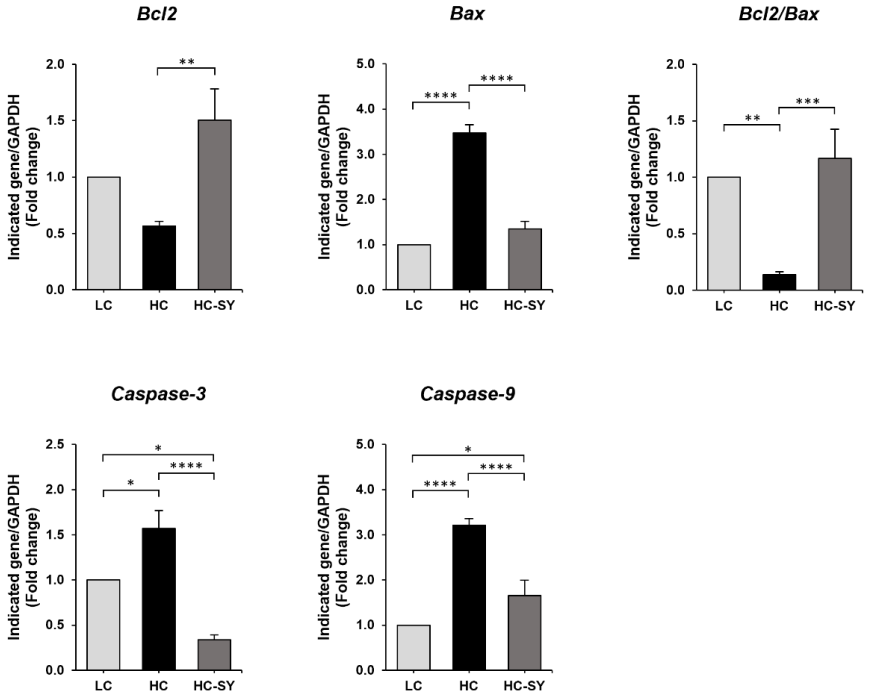

Fig. 3: Representing the mRNA expression of apoptotic genes *i.e*., *Bcl2, Bax* genes in rumen epithelium of goats. *Bcl2*, B-cell lymphoma 2; *Bax*, *Bcl2*-associated X protein; *Caspase-3,* cysteine-aspartic protease-3; *Caspase-9,* cysteine-aspartic protease-9; GAPDH, glyceraldehyde 3-phosphate dehydrogenase. Values are means ± S.E. Statistical difference among groups: ****$P < 0.0001$; ***$P < 0.001$; **$P < 0.01$; *$P < 0.05$

with HC, goats fed HC-SY diet upregulated ($P < 0.01$) the mRNA expression level of *Bcl2*. mRNA expression level of *Bax* was downregulated ($P < 0.0001$) in HC-SY compared to other groups. *Bcl2/Bax* ratio was downregulated in group HC than LC ($P < 0.01$) and HC-SY ($P < 0.001$) however it was non-significant ($P > 0.05$) between LC diet and grain-rich diet group. Compared with LC, goats fed high concentrate diet upregulated the mRNA expression level of *Caspase-3* ($P < 0.05$) and *Caspase-9* ($P < 0.0001$). Whereas group HC-SY downregulated ($P < 0.0001$) the high concentrate diet-induced mRNA expression level of *Caspase-3* and *-9*.

Discussion

Damage to the epithelial barrier is associated with inflammation and OS followed by excessive consumption of high concentrate diet that results to the initiation of NF-κB mechanism of LPS and TLR4 (Toll-like receptor 4) signaling pathway (Zhang *et al.* 2020; Ma *et al.* 2021). Apoptotic changes and the cellular infiltrate contribute to epithelial cell damage in colon and cecum of goats fed on grain-rich diet and hence induced mucosal injuries (Tao *et al.* 2014). This study showed pronounced damage to rumen epithelium in the group fed with high concentrate diet. However, no indicative alterations were observed in the rumen of goats on feeding HC-SY diet, which suggests a mitigating effect of Se on rumen epithelial injuries induced against high concentrate diet feeding plans in goats. Organic Se-yeast has demonstrated to alleviate the mucosal injuries in colon on grain-rich feeding (Samo *et al.* 2020), enhanced GSH-Px level in goats colonic tissue (Abbasi *et al.* 2020), alleviation of cardiac damage in rabbits driven on by a diet enriched with excessive lipids (Mehta *et al.* 2002), eliminated the immuno-toxic response in 7 to 21 days old chickens by diminishing the oxidative impairments (Hu *et al.* 2018).

MDA is a result of ROS-induced lipid peroxidation which when escalated tends to correlate with impaired antioxidative state, hence employed as a biological indicator for OS. (Teama 2018; Elvira *et al.* 2023). Current investigation revealed that the rumen epithelium of goats in group HC had higher levels of MDA and lower levels of GSH-Px and SOD. Our results are supported by previous research showing that a grain-rich diet lowered the ruminal pH and boosted LPS in goat rumen, thereby escalated the MDA content and inhibited the antioxidative action in the gut epithelium, hence resulting in OS and oxidative damage at tissue level (Guo *et al.* 2013; Abaker *et al.* 2017), and the goats given an high concentrate diet had higher MDA levels and lower OS biomarker levels (Ma *et al.* 2021). In present study, Se supplementation augmented the activities of antioxidant enzymes and attenuated lipid peroxidation as measured by MDA levels, hence mitigating the high concentrate diet-induced OS. GSH-Px and SOD have the defensive role against the reactive oxygen species (ROS)

where SOD acts to dismutate the toxic molecules however, GSH-Px has an antioxidative plus preventive action against the lipid peroxidation process in tissue (Ighodaro and Akinloye 2018). Various studies report reduces the OS through antioxidant mechanisms, protecting against various toxicities (Ahsan *et al.* 2014; Zakeri *et al.* 2021). Shi *et al.* (2011, 2018) reported that supplementation of Se at supranutritional level improves the antioxidative status in caprine. In present study, the reduced GSH-Px activity in HC diet group compared to LC exhibit to the variable Se content among both groups, whereas the augment activity GSH-Px in Se treated high concentrate diet was noticed to that of HC group, which suggests the superior availability of Se to suppress the OS and tissue damage by the grain-rich diet. Panev *et al.* (2013) and Čobanová *et al.* (2017) and also illustrated that the increment activity of GSH-Px and lessen content of MDA exhibited due to enhanced Se level in gut microflorae content. Shahid *et al.* (2020) also reported that the increment in activity of GSH-Px up to 25% when treated with Se-yeast supplementation in young goats.

OS induced by the grain-rich diet initiates cell death in the gut epithelial tissue through apoptosis, hence jeopardizing the mucosa's integrity, which ultimately compromises the epithelial lining and the tissue (Tao *et al.* 2015). Apoptosis is a tightly regulated process primarily governed by the *Bcl-2* family proteins and caspases (Elmore 2007). *Bcl-2* family includes two types of proteins, the anti-apoptotic (*Bcl2*) and pro-apoptotic (*Bax*) proteins, which exist in stable condition, but any notable change in the *Bcl2/Bax* ratio might influence the physiological state and shift the apoptotic frequency at the cell level (Droin and Green 2004; Tian *et al.* 2016). A study by Gui and Shen (2016) reported that the grain rich diet having 35% concentrate versus 10% concentrate lowered the mRNA expression of *Bcl2/Bax* ratio by upregulating mRNA gene expression of *Caspase-3, -8, -9, p53* and *Bax*. The current investigation demonstrated that the grain-rich diet led to OS-induced rumen epithelial cell death by apoptosis through upregulation of mRNA gene expression level of *Bax* and declined the *Bcl2/Bax* ratio comparing to LC. However, diet supplemented with Se raised the *Bcl2* and *Bcl2/Bax* ratio, while declined the expression level of *Bax, Caspase-3,* and *-9*. An earlier study by Dieho *et al.* (2017) and Xu *et al.* (2018) illustrates that grain-rich diet feeding in bovine and caprine exhibited rumen and hindgut epithelial tissue deterioration, along with a substantial rise in TUNEL-positive apoptotic cells analogous with regulating *Bcl2* and *Bax* gene expression.

The intrinsic pathway, which is the mitochondrial route and the extrinsic pathway, which is the death receptor route are the two main pathways that trigger the initiation of apoptosis (Khosravi-Far 2004). *Bax* mediates activation of the intrinsic apoptotic pathway in response to cellular stress by promoting mitochondrial outer membrane permeabilization (MOMP). This event facilitates the release of intermembrane space proteins, including cytochrome c (Cyt c) and inhibitor of apoptosis protein (IAP)-binding proteins (Tait and Green 2012). In the cytosol, Cyt c associates with Apaf-1 and ATP to form the apoptosome, which in turn activates *Caspase-9*. Activated *Caspase-9* subsequently initiates a caspase cascade through *Caspase-3*, culminating in the execution of programmed cell death, whereas, in the extrinsic pathway, a ligand connects itself to death receptors, triggering *Caspase-8* to initiate the programmed cell death (Li *et al.* 1997; Taylor *et al.* 2008). In the current research, the intrinsic apoptotic pathway induced against feeding of grain rich diet resulted in apoptotic condition in ruminal epithelial tissue along with the amplification of *Caspase-3* and *-9* concurrently with *Bax*. In contrast, a prior study by Gui and Shen (2016) showed that feeding a grain-rich diet results in an overexpression of *Caspase-3, -9* and *Bax,* as well as a lowered *Bcl2/Bax* expression ratio. These results provide credence to the idea that ruminal epithelial cells related inherent apoptotic mechanism is activated by high-grain diets. Similarly, apoptotic pathways were also activated in the colonic epithelium of goats fed a grain-rich diet, as evidenced by enhanced mRNA expression of *Bax* and *Caspase-3* (Tao *et al.* 2015; Samo *et al.* 2020).

Conclusion

High concentrate diet reduced the antioxidative status which led to elevated OS and mRNA gene expression level of *Bax, Caspase-3* and *-9* concomitantly by decreasing the *Bcl2* and *Bcl2/Bax* ratio inducing rumen epithelial injuries in goats. However, the high concentrate diet containing dietary Se mitigated the OS by escalating the antioxidant activity of GSH-Px, SOD and TAC by reducing the MDA level, and mRNA gene expression level of *Bax, Caspase-3,* and *-9* concomitantly by increasing the *Bcl2* and *Bcl2/Bax* ratio. Hence in conclusion, the supplementation of Se alleviated high concentrate diet-induced epithelial injury thereby reducing the OS and apoptotic genes expression in rumen of goats.

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Author Contributions

ABS: Conducted all the experimental procedures prepared the overall manuscript. MM: Conceived the concept and supported for the experimental plan, data analysis. ABK: Supported for the experimental plan. BB: Aided with technical support in lab work. UA and JKZS: Assisted in manuscript drafting and reviewing. All authors reviewed and approved the final manuscript for publication.

Conflict of Interest

All authors declare no conflict of interest.

Data Availability

Data will be made available on reasonable request.

Ethics Approval

Not applicable to this paper.

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