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The role of quercetin in primary culture of ovine spermatogonial stem cells

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Abstract: The aim of the present study was to examine the effect of quercetin on the survival and primary culture of ovine spermatogonial stem cells (SSCs). The two-time enzymatic digestion process was employed to obtain SSCs from lamb testes. In the next step, the use of filtration and differential plating methods caused an increase in the number of SSCs in the cell suspension resulting from enzymatic and mechanical digestions. Mitomycin-C-treated Sertoli cells were used to prepare the feeder layer. The stem cells were then cultured on the Sertoli cell feeder layer. The identification of the colonies was done through alkaline phosphatase staining methods and specific gene expression of ram's SSCs (*nanog* and *Plzf*). The results of methylthiazolyldiphenyl-tetrazolium bromide assay on SSCs 72 h after culture with different treatments of quercetin demonstrated that the highest percentage of survival was for 5 μM and 10 μM concentrations, respectively; however, compared to the control, no significant difference was observed. In comparison with the control, the concentration equal to and greater than 20 μM quercetin caused a significant decrease in the survival of SSCs ($P < 0.05$). Seven days after culture, 40 μM quercetin caused a substantial reduction in the mean number of colonies, compared to the control ($P < 0.05$). The results demonstrated that, compared to the control, 5 μM to 40 μM of quercetin significantly reduced *Plzf* gene expression. Furthermore, the concentration equal to and higher than 10 μM quercetin significantly decreased *bcl-2* gene expression in the cells under study ($P < 0.05$). Based on the findings of the present study, the use of quercetin for the primary culture of ovine SSCs is not recommended. It is suggested that the function of this antioxidant should be investigated on the differentiation of SSCs.

Keywords: spermatogonial stem cell; sheep; stem cell culture

In the germline niche, spermatogonial stem cells (SSCs) are distinguished by two definite and noticeable functions: (1) production of differentiating spermatogonia towards spermatogenesis (differentiation); (2) production of SSCs themselves (self-renewal) (Takashima and Shinohara 2018). SSCs have already obtained a considerable attention, as they have a special ability for self-renewal and also differentiation which is able to transmit paternal genetic information to offspring. SSCs are capable of being genetically modified and further differentiate into spermatozoa *in vitro*. Afterwards, SSCs can lead to the development of genetically modified offspring by means of intracytoplasmic sperm injection or SSC transplantation into a recipient testis (Kanatsu-Shinohara et al. 2011; Sehgal et al. 2014; Feng et al. 2016). The basement membrane and Sertoli cells as well as hormonal and paracrine

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factors are the main components of specialized microenvironment called a niche that promotes the self-renewal of germ cells (Majidi Gharenaz et al. 2020). Considering that SSCs constitute 0.02–0.03% of the total germ cells in testes, the enrichment and culture of SSCs would be the primary prerequisite for any use of these cells (Goharbakhsh et al. 2013).

Quercetin is a compound belonging to flavonoids. In recent years, many studies have verified that a fair dose of quercetin has a number of biological functions, including anti-inflammatory, anti-oxidant and anti-cancer effects (Shen et al. 2016; Lan et al. 2019; Ma et al. 2020; Tang et al. 2020). Furthermore, many recent researches have demonstrated that the differentiation of osteoblasts is facilitated by quercetin (Pang et al. 2018). Bone marrow-derived mesenchymal stem cells can be protected from erastin-induced ferroptosis by quercetin, possibly via the antioxidant pathway (Li et al. 2020). In addition, the findings of Chang et al. (2013) indicated that self-renewal, stemness signature expression, and the migratory ability of head and neck cancer-derived sphere cells were meaningfully decreased by quercetin (Chang et al. 2013). Mechanisms of quercetin triggered apoptosis including activation of caspases, upregulation of Bax and downregulation of Bcl-2 and inhibition of telomerase enzyme activity (Gokbulut et al. 2013). Quercetin has been demonstrated to improve the proliferation of neural stem cells; but the mechanism of action remains elusive (Sarkoohi et al. 2019).

Considering the properties of quercetin, here, we assessed the effects of quercetin on survival, maintenance and apoptotic gene expression of ovine SSCs during the primary culture.

MATERIAL AND METHODS

Chemicals

If not otherwise specified, all the chemicals employed in the present study were bought from Sigma (St. Louis, MO, USA). Additionally, the plastics were purchased from Sorfa life science research Co., Ltd. (Zhejiang, China).

Isolation and culture of SSCs

The protocol for animal use in the present research was approved by the Iranian Research Organization for Science and Technology (IROST), Agricultural Institute of Animal Ethics, Care and Use (approval number: 2019-3). The testes of three rams of Shal breed, aged 3–5 months and weighing about 27.1 ± 4.1 kg on average, were collected from the local slaughterhouse and delivered to the laboratory within 2 h after slaughter. In order to isolate SSCs, the researchers employed a modified form of the two-time enzymatic digestion process which was illustrated by Izadyar et al. (2002). Dulbecco's Modified Eagle Medium (DMEM) (Inoclon, Karaj, Iran) was used as a medium to suspend the minced seminiferous tissue for the first enzymatic digestion. The medium contained 1 mg/ml trypsin (Inoclon, Karaj, Iran), 1 mg/ml hyaluronidase type II, 1 mg/ml collagenase, and 5 µg/ml DNase. A shaker incubator (200 cycles/min) was used to incubate the minced seminiferous tissue at 37 °C for 45 minutes. The dispersed tissue was collected and centrifuged at 30 *g* for 2 minutes. Afterwards, the analysts collected the supernatant and used DMEM to wash the pellet. DMEM was also used as a medium to suspend the pellet for the second enzymatic digestion. This time, the medium contained 1 mg/ml hyaluronidase type II, 1 mg/ml collagenase, and 5 µg/ml DNase without trypsin. A shaker incubator (200 cycles/min) was employed to incubate the pellet for 30 minutes. Subsequently, the researchers centrifuged the suspension at 30 *g* for 2 minutes.

Enrichment of SSCs

For enrichment of SSCs, nylon net filtration and differential plating were used as previously described by Jasour et al. (2017). To enrich SSCs, an 80-µm and a 60-µm nylon net filter were consecutively used to filter the supernatant. The filtered cells were subsequently transferred to 60-mm petri dishes coated with lectin-bovine serum albumin (BSA), which were developed by dissolving the lectin (5 µg/ml) from *Datura stramonium* agglutinin in Dulbecco's phosphate-buffered saline (DPBS). The lectin-BSA coated dishes were held at room temperature for 2 h and then washed with BSA (0.6% BSA in DPBS). Afterwards, for the coating with BSA, the analysts kept the dishes at room temperature for another 2 hours. To enable Sertoli cells to get attached to the lectin-BSA, the suspension, containing the Sertoli and germ cells, was incubated between 5 h and 6 h at 37 °C in a CO_2 incuba-

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tor with 5% CO_2 in air. Thereafter, the researchers collected and transferred the remaining medium to a 15-ml tube. It was anticipated that the medium, which was subsequently centrifuged at 30 *g* for 5 min, would contain SSCs. After this process, the supernatant was thrown away and the pellet was suspended in DMEM once again.

Preparation of feeder layers

Preparation of feeder layers was performed as described previously by Jafarnejad et al. (2018) with minor modifications. Fresh DMEM supplemented with 10% foetal bovine serum (FBS) revitalized the remaining cells in the lectin-BSA coated dishes (Gibco, Life Technologies, Rockville, MD, USA). In order to allow the residual cells, which were anticipated to be Sertoli cells for the most part, to grow until a confluent monolayer was developed, a CO_2 incubator with 5% CO_2 in air was employed for incubating the cells at 37 °C for 2–3 days. Having been disaggregated with 0.25% trypsin-EDTA, the cells were subcultured in a 50-ml cell culture flask for propagation. To prepare a feeder layer, the researchers inactivated the Sertoli cells by applying a 10 µg/ml mitomycin-C treatment for 3 hours. After that, DPBS and DMEM supplemented with 10% FBS were used to wash the cells five times.

MTT reduction assay

A kit (Thermo Fisher Scientific, Rockford, IL, USA), as per manufacturer's protocol, was used for determining cell viability after a seventy-two-hour cell culture with 0.6 µM, 1.25 µM, 2.5 µM, 5 µM, 10 µM, 20 µM and 40 µM quercetin in 96-well dishes (5 000 cells per well). At first, 1 ml of sterile PBS was added to 5 mg of methylthiazolyldiphenyl-tetrazolium bromide (MTT), leading to the preparation of a 12 mM MTT stock solution. 10 µl of the 12 mM MTT stock solution was then added to each well. It has to be mentioned that each well contained a negative control of 10 µl of the MTT stock solution added to 100 µl of the medium alone. The stock solution in question subsequently underwent the four-hour incubation with 5% CO_2 at 37 °C. In the next step, the researchers added 100 µl of sodium dodecyl sulfate-HCl solution (10 ml of 0.01 M HCl + 1 g of sodium dodecyl sulfate) to each well. A humidified chamber was used for overnight incubation of the microplate at 37 °C. The formazan concentration was defined at 570 nm by optical density. To remove the effect of Sertoli cells, a coated 96-well dish without SSCs was finally employed for each treatment.

Culture of SSCs

The isolated SSCs were cultured on the Sertoli cell feeder layer in gelatin-based 50-ml cell culture flasks which contained DMEM supplemented with 10% FBS, 10 µg/ml glial cell line-derived neurotrophic factor (GDNF), 100 IU/ml penicillin, and 50 mg/ml streptomycin. This procedure was performed with 1.25 µM, 2.5 µM, 5 µM, 10 µM, 20 µM and 40 µM quercetin based on the results of MTT assay. Subsequently, a CO_2 incubator with 5% CO_2 in air was used to incubate the SSCs at 37 °C. After a period of seven days of culture, the SSC colonies were counted after alkaline phosphatase (AP) staining under a stereomicroscope (Nikon SMZ 745, Tokyo, Japan).

To characterize the SSCs, the analysts employed AP staining and the expression of *nanog* and *Plzf* genes. For AP staining, DPBS was used to wash SSC colonies twice. After that, the SSC colonies were stained by means of an AP kit (Catalogue No. 86C; Sigma Chemicals, St. Louis, MO, USA) as per manufacturer's protocol.

RNA isolation, reverse transcription and real-time PCR

Based on the results of SSC colony formation, the expression of SSC specific markers and apoptotic gene expression were analysed with more effective concentration of quercetin (5 µM, 10 µM, 20 µM and 40 µM). Trizol reagent was used to isolate total RNA (Invitrogen Corp., Carlsbad, CA, USA). To prevent any DNA contamination, total RNA was then treated with DNase (Ambion Inc., Houston, TX, USA). Moloney murine leukemia virus enzyme and oligo dT primers were used to perform reverse transcription (Takara, Maebashi, Japan). Real-time PCR was employed to examine the variations in the expression of specific markers and apoptosis-related genes. The re-

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searchers established the PCR at a final volume of 10 µl with 5 µl Syber Green (Genaxxon bioscience GmbH, Ulm, Germany), 1.4 µl nuclease-free water, 0.8 µl of each primer (forward and reverse), and 2 µl template. With the purpose of activating the polymerase, the real-time PCR process was initiated with a preliminary melting cycle at 94 °C for 15 minutes. Forty amplification cycles of denaturation at 95 °C for 10 s, and the annealing of specific primers at 60 °C for 15 s and at 72 °C for a twenty-second extension were the subsequent steps to this procedure. A final extension at 72 °C for 5 min brought the reactions to an end.

In order to analyse real-time PCR gene expression, the present researchers used the custom primer sequences mentioned below:

β actin [5'-ACCCAGCACGATGAAGATCA-3' (forward) and 5'-GTAACGCAGCTAACAGTCCG-3' (reverse)]; *nanog* [5'-CCGAAGCATCCAACTCT-3' (forward) and 5'-GAGACAGTGTCCGTGTCGAG-3' (reverse)]; *Plzf* [5'-CCTCAGATGACAATGACACG-3' (forward) and 5'-CGCCTTGGTGGGACTCA-3' (reverse)]; *bcl-2* [5'-GATGACTTCTCTCGGCGCTA-3' (forward) and 5'-GACCCCTCCGAACTCAAAGA-3' (reverse)]; *bax* [5'-GTGAGACCTCTAACCCCACC-3' (forward) and 5'-GGTCAGAGGTCATGAGGAGG-3' (reverse)].

The comparative threshold cycle (CT) method was employed to analyse the data. In this analysis, β actin was used as an endogenous control, *nanog* and *Plzf* genes acted as SSC markers and *bcl-2* and *bax* genes were used as apoptotic genes.

Statistical analysis

Each experiment was replicated at least three times and a statistical software program IBM SPSS Statistics v16 (IBM® SPSS®, Chicago, IL, USA) was used to analyse the data. With the purpose of comparing multiple numeric datasets, the present researchers employed one-way ANOVA followed by Duncan's multiple-range test. The results were expressed as mean ± SEM. In addition, statistical significance was accepted at $P < 0.05$.

RESULTS

The results of MTT assay on SSCs 72 h after culture with different treatments of quercetin demonstrated that the highest percentage of survival was related to the concentrations of 5 µM and 10 µM, respectively; however, compared to the control, no significant difference was observed. In comparison with the control, 40 µM quercetin caused a significant decrease in the survival of SSCs ($P < 0.05$) (Figure 1).

Figure 2 displays the effect of different concentrations of quercetin on SSC colony formation seven days after the feeder layer-based culture. The results showed that, compared to other treatments, 40 µM quercetin significantly reduced the mean number of colonies. The images of the colonies formed after undergoing treatments with the control and with 10 µM, 20 µM and 40 µM quercetin, and after alkaline phosphatase staining as a specific marker of the stem cells, are presented in Figure 3.

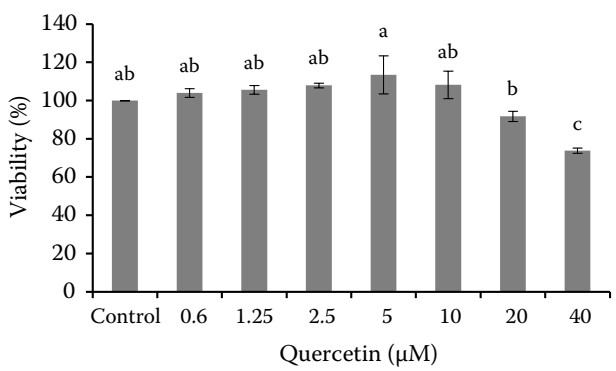

Figure 1. The effect of different concentrations of quercetin (0.6 µM to 40 µM) on the survival of spermatogonial stem cells 72 h after the primary culture

[a–c]Indicate statistical difference ($P < 0.05$) by the Duncan test

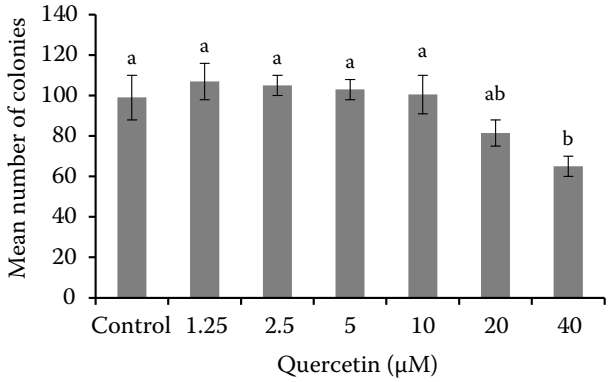

Figure 2. The effect of different concentrations of quercetin (1.25 µM to 40 µM) on spermatogonial stem cell colony formation seven days after the primary synchronous culture

[a,b]Indicate statistical difference ($P < 0.05$) by the Duncan test

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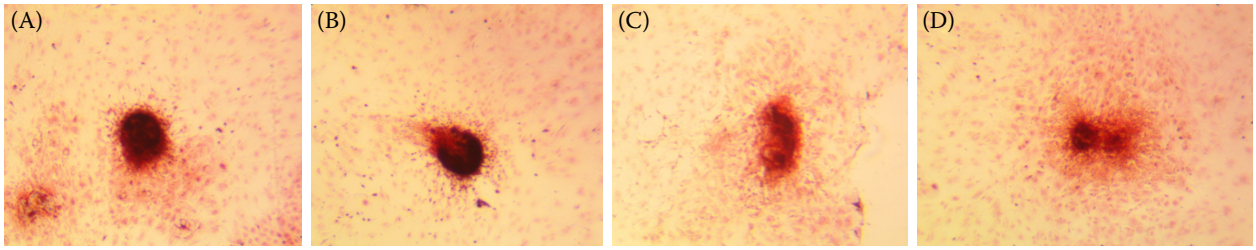

Figure 3. Alkaline phosphatase staining in colonies formed after undergoing treatments with (A) control, (B) 10 µM quercetin, (C) 20 µM quercetin and (D) 40 µM quercetin

The analysis conducted on the expression of *Plzf* and *nanog* genes, which are considered as ram's SSC specific genes, is shown in Figure 4. The results demonstrated that, compared to the control, 5–40 µM quercetin did not have a significant effect on *nanog* gene expression. On the other hand, *Plzf* gene expression was reported to decrease significantly in comparison with the control.

Having analysed the gene expression of the pro-apoptotic gene, *bax*, and the anti-apoptotic gene, *bcl-2*, in SSCs under 5–40 µM quercetin treatments, it was realized that *bax* gene expression increased at 20 µM and 40 µM treatments; however, no significant difference was observed. In the cells under study, a concentration equal to and greater than 10 µM quercetin caused a substantial decrease in *bcl-2* gene expression ($P < 0.05$) (Figure 5).

DISCUSSION

In the primary culture of SSCs, 40 µM quercetin for 72 h caused a significant decrease in the cell survival. One week after culture with 40 µM quercetin, the mean number of colonies was meaningfully reduced in comparison with the control ($P < 0.05$). Quercetin (20–60 µM) decreased human embryonic neural stem cell viability in a concentration-dependent manner

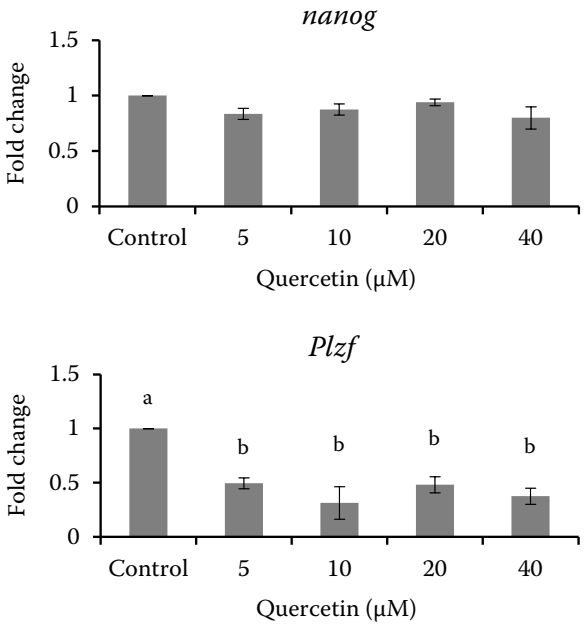

Figure 4. The effect of 5 µM, 10 µM, 20 µM, and 40 µM quercetin on specific gene expression of ram's spermatogonial stem cells seven days after culture on the feeder layer

[a,b]Indicate statistical difference ($P < 0.05$) by the Duncan test

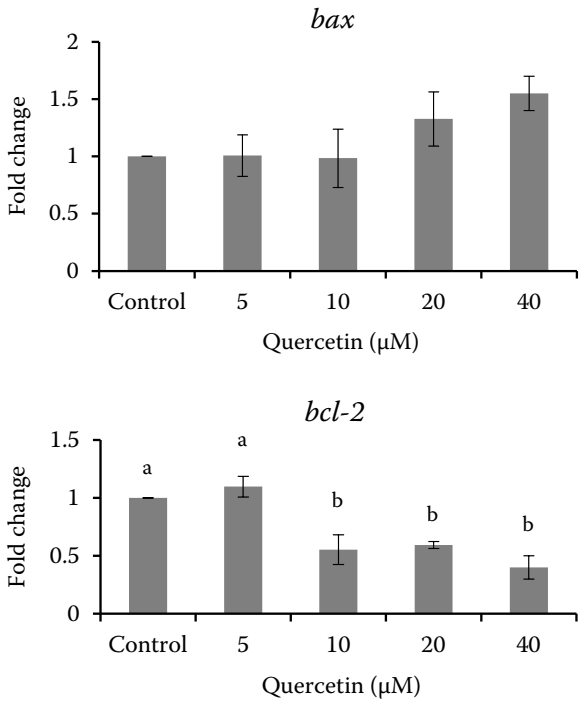

Figure 5. The effect of 5 µM, 10 µM, 20 µM, and 40 µM quercetin on *bax* and *bcl-2* gene expression seven days after culture on the feeder layer

[a,b]Indicate statistical difference ($P < 0.05$) by the Duncan test

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(Sarkoohi et al. 2019). In Gokbulut et al. (2013) study, it was shown that cell proliferation and cell cycle progression were inhibited by quercetin. It was also demonstrated that apoptosis in chronic lymphocytic leukemia cells is induced by quercetin. The half maximal inhibitory concentration value of quercetin was 24 μM. Quercetin was reported to exert an anti-proliferative effect on U937 cells at a concentration of 20 μM (Lee et al. 2006). Cell proliferation was inhibited by quercetin in a dose-dependent way. There was no influence on cell viability in the process. Several scholars have referred to the quercetin-induced inhibition of cell proliferation for various cancer cells (Kim et al. 2006; Mojsin et al. 2013; Lee et al. 2015). Moreover, several studies have demonstrated the low toxicity of quercetin (Caltagirone et al. 2000; Alia et al. 2006). According to some studies, the deleterious effects of quercetin could be ascribed to its pro-oxidant activities through mutagenic and DNA-damaging activities (Johnson and Loo 2000; van Duursen et al. 2004). On the contrary, according to the findings of Sarkoohi et al. (2019) the proliferation capacity of mice embryonic neural stem/progenitor cells of ganglionic eminence was improved by quercetin in a concentration-dependent way. In addition, the findings demonstrated that the proliferation of mice hippocampal neural progenitor cells and human embryonic neural stem cells were enhanced by CTN986 (a quercetin derivative) and Q3GA (a quercetin metabolite), respectively (Zhang et al. 2009; Baral et al. 2017). As reported by Sarkoohi et al. (2019) and Abarikwu et al. (2012), the decreased cell viability and germ cell loss were prevented by quercetin. The redox state of the cell and the dose used might be responsible for quercetin inconsistent effects (Mi and Zhang 2005). The hormetic role of quercetin in the viability and proliferation of different cell types may account for the contradiction observed in the result. It would indicate that quercetin stimulates cell proliferation at a low concentration. On the other hand, cell proliferation is inhibited by quercetin at a high concentration (Sarkoohi et al. 2019). Nonetheless, our results did not find any significant positive effect of quercetin on SSC viability and survival.

The results demonstrated that, in comparison with the control, *Plzf* gene expression was substantially reduced by 5 μM to 40 μM quercetin ($P < 0.05$) and we did not find any significant effect of quercetin on *nanog* expression. *Plzf* is specific to cultured spermatogonia characterization and its expression is higher in SSCs than in somatic feeder cells (Wang et al. 2014; Azizi et al. 2019). The role of *Plzf* in spermatogonia is the maintenance of an undifferentiated state (Qasemi-Panahi et al. 2018). Borjigin et al. (2010) stated that *Plzf* plays the role of a molecular marker of sheep SSCs. *Nanog* is a transcriptional factor for regulating self-renewal and pluripotency (Han et al. 2009). According to the findings of Tsai et al. (2016), the expression of cancer stem cell markers CD44, ABCG2, Sox2 and Nanog was decreased by quercetin. It was also stated that quercetin significantly reduced the cancer stem cell associated spheroid formation. The JNK signalling pathway was inhibited by quercetin. This fact would explain quercetin effects on stemness, vasculogenic mimicry, and metastatic potential. Earlier studies demonstrated that JNK kinase activity was instrumental in preserving the stemness of glioblastoma and other cells (Matsuda et al. 2012; Yoon et al. 2012).

The results of the present study demonstrated that the expression of *bcl-2* gene was considerably attenuated by the concentrations equal to and greater than 10 μM quercetin ($P < 0.05$) and did not find any significant effect on *bax* expression. Quercetin is a flavonoid with remarkable anti-cancer potentials and a variety of action mechanisms such as cell cycle arrest and apoptosis induction, variations in oncogenic gene expression, tumour suppressor genes, and apoptosis-related genes like *bcl-2* and caspases (Atashpour et al. 2015; Lan et al. 2019). Shen et al. (2016) have confirmed that the apoptosis of gastric cancer stem cells can be induced by quercetin. Quercetin triggered caspase-3, -8, -9, inspired the expression of Bax Bad, and downregulated the anti-apoptotic proteins like Bcl-XL, Bcl-2 and Mcl-1 during the intrinsic apoptotic pathway (Primikyri et al. 2018). Simultaneously, the release of cytochrome-c from the mitochondria to the cytoplasm was enhanced by quercetin (Primikyri et al. 2014). Additionally, such findings elucidate the point that quercetin-stimulated apoptosis might be directly linked with Bcl-2 (Tang et al. 2020). According to the studies conducted earlier, tumour cell necrosis was induced by quercetin after the improvement of ROS through activating caspase-3 (Chan et al. 2018). Members of the Bcl-2 family of proteins regulate the mitochondrial apoptotic pathway in mam-

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mals. Likewise, in Shen et al. (2016), the involvement of the mitochondrial apoptotic pathway was indicated by the collapse of the membrane potential, caspase-3 and caspase-9 activation, and cytochrome-c upregulation after treatment with quercetin. Furthermore, the hypothesis that quercetin-induced growth inhibition occurs through mitochondrial-dependent signalling in gastric cancer stem cells is further highlighted by the downregulation of the anti-apoptotic protein, Bcl-2, and the upregulation of the pro-apoptotic protein, Bax (Shen et al. 2016).

To overcome the limitation of the present study, it is suggested to analyse ROS levels and/or oxidative stress markers to have better understanding of the mechanism of quercetin action on maintenance of SSCs. Based on the results of quercetin on the expression of *nanog* and *Plzf* markers, it is also suggested that the role of this antioxidant in differentiation of SSCs should be studied. The small sample size (n = 3) is another limitation of the study.

CONCLUSION

Based on the results of this study, the action of quercetin on ovine SSCs is more similar to its inhibitory function of cancer cells and does not work in maintenance of SSCs. It is suggested that its function should be investigated in relation to the differentiation of SSCs.

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Conflict of interest

The authors declare no conflict of interest.

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