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Sodium selenite addition promotes the proliferation of bovine mammary epithelial cells through the Akt-mTOR signalling pathway

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ABSTRACT. The experiment was designed to evaluate the effect of sodium selenite (Na_2SeO_3) on the proliferation of bovine mammary gland epithelial cells (BMECs), and to assess the underlying mechanisms. The addition of Na_2SeO_3 at doses ranging from 18 to 140 nM stimulated BMEC proliferation. Nevertheless, high doses of Na_2SeO_3 (> 70 nM) did not further stimulate BMEC proliferation compared to 35 nM Na_2SeO_3. Therefore, the addition of 35 nM Na_2SeO_3 increased the proportion of epithelial cells undergoing DNA replication, mRNA expression of proliferating cell nuclear antigen (PCNA), Cyclin A2 (CCNA2) and Cyclin D1 (CCND1), as well as protein expression of PCNA and Cyclin A1. Transcription and translation of the B-cell lymphoma 2 (*BCL2*) gene and the ratio of *BCL2* to BCL2-associated X 4 (*BAX4*) genes were significantly elevated, while mRNA and protein expressions of *BAX*, caspase 3 (*CASP3*) and caspase 9 (*CASP9*) genes were significantly reduced as a result of 35 nM Na_2SeO_3 supplementation. Additionally, both the Akt and mTOR signalling pathways were activated by 35 nM Na_2SeO_3. On the other hand, the stimulation of BMEC proliferation, altered expression of proliferative and apoptotic genes and proteins, as well as mTOR signalling pathway activation caused by 35 nM Na_2SeO_3 addition was suppressed by the Akt inhibitor (AKT-IN-1). Likewise, rapamycin-mediated suppression of mTOR completely reversed the 35 nM Na_2SeO_3-stimulated BMEC proliferation and alteration of proliferous gene and protein expressions, without affecting mRNA or protein expression of genes related to apoptosis and the Na_2SeO_3-activated Akt signalling pathway. In conclusion, the results implicated that the proliferation of BMECs was stimulated by 35 nM Na_2SeO_3 through the Akt-mTOR signalling pathway.

Introduction

The main goal of dairy industry development is the production of more high-quality milk by healthy heifers with well-functioning mammary glands with the capacity to synthesise and secrete large milk quantities (Akers, 2017). Proper development and functional differentiation of the bovine mammary glands is the key to reaching this goal. The mammary glands of dairy cows consist of teats, ducts, alveoli containing secretory cells, and sustentacular tissue (Akers, 2002). The mammary gland alveoli are lined with two layers of epithelial secretory cells that synthesize and secrete milk and are considered the functional unit of the mammary gland (Akers, 2002). Milk production is commonly attributed to

the number of mammary epithelial cells and their secretory function (Kim and Wu, 2009). Therefore, the correct development of the bovine mammary gland during pregnancy is crucial for improving milk performance of dairy cows. Many factors have been shown in the literature to influence mammary epithelial cell proliferation, including nutrition (Molenaar et al., 2020), hormones (Macrina et al., 2011) and environment (McCabe et al., 2021).

Selenium (Se), an essential trace element for normal growth, development and health of dairy cows, plays an essential role in numerous metabolic processes of tissues and organs due to its antioxidant function (Xiao et al., 2021; Zhong et al., 2022). Se is involved in the formation of many Se-dependent enzymes, including glutathione peroxidase (GSH-Px), thioredoxin reductase, selenoprotein-P, and thioredoxin. These oxidoreductases regulate the intracellular inflammatory and apoptotic signalling cascades to maintain the internal environment (Huang et al., 2012; Li et al., 2014). Numerous studies have highlighted the relationship between Se and production performance of dairy cows. Se supplementation was shown to increase the production of milk (Wang et al., 2009; Ullah et al., 2019), milk fat and protein (Najafnejad et al., 2013), nutrient digestibility (Najafnejad et al., 2013), improve neutrophil function and adaptive immunity (Souza et al., 2012), as well as reduce the incidence of mastitis and embryonic mortality (Xiao et al., 2021; Zhang et al., 2021). Moreover, previous works indicated the role of Se in the regulation of several protein kinases and intracellular signalling pathways involved in cell proliferation and apoptosis (He et al., 2016). Recent studies have demonstrated that Se addition promoted the proliferation of lens epithelial cells (HLECs), after damage induced by ultraviolet radiation B (Zhong et al., 2022), as well as proliferation of sheep spermatogonial stem cells (Shi et al., 2020). Furthermore, Zhang et al. (2020) reported that the addition of 50 nmol/l Se significantly increased cell viability and relative growth rate of bovine mammary epithelial cells (BMECs). Therefore, it can be concluded that Se is involved in the stimulation of BMEC proliferation.

Both the Akt and mTOR pathways were shown to be involved in cell proliferation (Zhang et al., 2018). Other studies reported that the PI3K/Akt (De Cicco et al., 2016; Meng et al., 2017) and mTOR signalling pathways (Jiang et al., 2017; Li et al., 2017; Pan et al., 2017) were involved in cell proliferation. To the best of our knowledge, the effect of Na_2SeO_3 on the Akt and mTOR signalling pathways with respect to BMEC proliferation remains unclear. Therefore, the current study aimed to evaluate the impact of Na_2SeO_3 on BMEC proliferation and to explore the role of related signalling pathway in this process, as well as provide a new method of Na_2SeO_3 supplementation as a nutritional agent to promote mammary gland development in dairy cows.

Material and methods

The experimental protocol was approved by the Animal Welfare Committee of Shanxi Agriculture University (Taiyuan, Shanxi Province, China, IACUC Issue No. SXAU-EAW-2021C.FU.00301401).

Reagents

Na_2SeO_3 used in the study was of cell grade and was purchased from Sigma-Aldrich (Cat. no. S5261; St. Louis, MI, USA). Foetal bovine serum (FBS) and Dulbecco's modified Eagle's medium F12 (DMEM-F12) were purchased from Gibco BRL (Gaithersburg, MA, USA). Akt inhibitor-1 (AKT-IN-1) and mTOR inhibitor, rapamycin (Rap), were purchased from MedChemExpress LLC (Monmouth Junction, NJ, USA). The Cell Counting Kit 8 (CCK-8) was purchased from Dojindo Laboratories (Kyushu, Japan). The Cell-Light™ EdU *in vitro* kit was purchased from Ribobio Biological Technology Co., Ltd (Guangzhou, GD, China). Cyclin A1 was targeted using a mouse anti-cyclin A1 monoclonal antibody (Cat. no. NB100-2660; Novus Biologicals LLC, Centennial, CO, USA), proliferating cell nuclear antigen (PCNA) was targeted using a mouse anti-PCNA monoclonal antibody (Cat. no. 2586; Cell Signaling Technology, Inc., Danvers, MA, USA), Akt and β-actin were targeted with rabbit monoclonal antibodies against Akt and β-actin (Cat. no. 9272 and 4970; Cell Signaling Technology, Inc., Danvers, MA, USA), phosphorylated p-Akt_{Ser473}, p-$mTOR_{Ser2448}$ and mTOR were targeted with rabbit polyclonal antibodies against p-Akt_{Ser473}, p-$mTOR_{Ser2448}$ and mTOR (Cat. no. bs-0876R, 3495R and 1992R; BIOSS, Beijing, China), B-cell lymphoma 2 (BCL2), Caspase-3 and Caspase-9 were targeted with rabbit polyclonal antibodies against BCL2, Caspase-3 and Caspase-9 (Cat. no. bs-20351R, 0081R and 0049R; BIOSS, Beijing, China), BCL2-associated X (BAX) was targeted with a mouse anti-BAX polyclonal antibody (Cat. no. bs-0127M; BIOSS, Beijing, China); goat anti-rabbit (Cat. no. bs-0295G) and goat anti-mouse (Cat. no. bs-0296G) secondary antibodies were purchased from BIOSS (Beijing, China). Plastic culture plates were manufactured by Corning Inc. (New York, USA).

BMEC isolation and culture

BMECs were isolated based on the method described by Zhao et al. (2010). Briefly, three multiparous Holstein cows (45 ± 4.1 months, 56 ± 2.4 days in milk, 638 ± 14.3 kg body weight; mean ± SD) were used to provide mammary gland tissue. The samples were cut into 1 mm^3 pieces using sterile surgical scissors and then washed several times in PBS buffer, and incubated in DMEM-F12 (10% foetal bovine serum, 1% glutamine, 1% penicillin-streptomycin, 5 µg/ml insulin, 5 µg/ml transferrin, 5 µg/ml prolactin, 1 µg/ml hydrocortisone and 10 ng/ml epidermal growth factor) (Gibco BRL, Gaithersburg, MA, USA) at 37 °C in a humidified 5% CO_2 atmosphere. Tissues were removed when cells had migrated from them and covered up to 80% of the flask bottom surface. Approximately 1 × 10^6 cells were cultured in 10 cm^2 plastic dishes in complete DMEM-F12.

Cell Counting Kit 8 assay and EdU incorporation assay

The effect of Na_2SeO_3 on BMEC proliferation was assessed using CCK-8 and Cell-Light™ EdU according to the manufacturer's protocol. Briefly, for the CCK8 assay, exactly 1 × 10^3 BMECs were seeded in 96-well plates and exposed to different supplementary Na_2SeO_3 doses (0, 9, 18, 35, 70, 140, 280 and 560 nM) for 3 days, with eight replicate wells per dose, then 10 µl of CCK8 solution and 100 µl of FBS-free DMEM-F12 were added to each well. Subsequently, the plates were cultured for 1 h at 37 °C in a humidified atmosphere of 5% CO_2. The number of viable cells was evaluated by determining the absorbance at 450 nm using a microplate reader (Infinite 200 PRO, Tecan Austria GmbH, Grödig, Austria). BMEC proliferation was performed with Cell-Light™ EdU based on the procedure of Zhang et al. (2018). Briefly, exactly 1 × 10^3 BMECs were seeded in 96-well plates and exposed to 0 and 35 nM Na_2SeO_3 for 3 days, with six replicate wells per treatment. BMECs were cultured with 50 µM EdU for 3 h, washed 3 times with PBS, fixed in 4% paraformaldehyde for 30 min at 25 °C, and then stained with Apollo staining reaction solution and Hoechst 33342 (Ribobio Biological Technology Co., Ltd, Guangzhou, GD, China) reaction solution in the dark at 25 °C for 30 min. Subsequently, cell images were acquired using an inverted microscope (100 × magnification, kFluor488: excitation 495 nm, emission 520 nm; Leica Microsystem CMS GmbH, Wetzlar, Germany). The rate of BMEC proliferation was estimated as the proportion of EdU-positive nuclei to total nuclei in five high-power fields for each well.

Real-time quantitative PCR

Exactly 4 × 10^4 BMECs were seeded in 12-well plates and exposed to 0 and 35 nM Na_2SeO_3 for 3 days, 6 replicate wells per dose. The transcripts of cyclin A2 (*CCNA2*), and cyclin D1 (*CCND1*), *PCNA*, *BCL2*, BCL2-associated X 4 (*BAX4*), caspase 3 (*CASP3*) and caspase 9 (*CASP9*) genes were analysed by real-time quantitative PCR. Briefly, total RNA was extracted using the RNA extraction kit (Magen Biotechnology Co., Ltd, Guangzhou, GD, China) based on the manufacturer's specifications. Complementary DNA (cDNA) was synthesized using 2 µg of total RNA, reverse transcriptase (M-MLV, RR047A; TaKaRa, Tokyo, Japan) and random primers based on the manufacturer's specifications. *GAPDH* was used as a housekeeping gene. Real-time quantitative PCR was performed in an ABI QuantStudio5 instrument (Thermo Fisher Scientific, Fremont, NY, USA). The 20 µl reaction mixture contained 10 µl SYBR Green Real-time PCR Master Mix reagents (RR820A; TaKaRa, Tokyo, Japan), 2 µl DNA template, 0.8 µl forward primer (10 µM), 0.8 µl reverse primer (10 µM), 0.4 µl of ROX Reference Dye II (TaKaRa, Tokyo, Japan), and 6.0 µl dH_2O. The PCR assay conditions were as follows: 1 cycle at 95 °C for 20 s, 40 cycles at 95 °C for 15 s, annealing at 55 °C for 20 s, followed by melting curve analysis. The primer sequences used for real-time quantitative PCR are listed in Table 1.

Western blot analysis

Exactly 8 × 10^4 BMECs were seeded in 6-well plates and exposed to 0 and 35 nM Na_2SeO_3 for 3 days, 3 replicate wells per dose. The protein content was analysed using the Thermo Scientific Pierce™ BCA Protein Assay Kit (23225, Thermo Fisher Scientific, Waltham, MA, USA) based on the manufacturer's specifications. β-actin was used as a loading control, equal quantities of protein (20 µg) were loaded onto 12% SDS/PAGE and the separated proteins were transferred onto nitrocellulose membranes (1704271; BioRad, Hercules, CA, USA). Membranes were blocked with 6% (w/v) bovine serum albumin (BSA) in Tris-HCl buffer solution + Tween (TBST) for 2 h at room temperature. Membranes after transfer were incubated with primary antibodies for: Cyclin A1 (1:2000), PCNA (1:2000), Akt (1:2000), p-Akt_{Ser473} (1:2000), mTOR (1:2000), p-$mTOR_{Ser2448}$ (1:2000), BCL2 (1:2000), BAX (1:2000), Caspase-3 (1:2000), Caspase-9 (1:2000) and β-actin (1:10000) diluted with TBST, and incubated overnight at 4 °C. Membranes were washed five times in 1 × TBST for 5 min to remove excess

Table 1. Primer sequences used in real-time quantitative PCR

Target species	(**F**orward/**R**everse) Sequence 5'→3'	GeneBank accession no.	Amplification length, bp
CCNA2	F: ACCACAGCACGCACAACAGTC R: AGTGTCTCTGGTGGGTTGAGGAG	NC_037333.1	87
CCND1	F: GCCGAGGAGAACAAGCAGATCATC R: CATGGAGGGCGGGTTGGAAATG	NC_037356.1	96
PCNA	F: ACATCAGCTCAAGTGGCGTGAAC R: GCAGCGGTAAGTGTCGAAGCC	NC_037340.1	101
BCL2	F: TGTGGATGACCGAGTACCTGAA R: AGAGACAGCCAGGAGAAATCAAAC	NC_037351.1	221
BAX4	F: TTTTGCTTCAGGGTTTCATCCAGGA R: CAGCTGCGATCATCCTCTGCAG	NC_037345.1	231
CASP3	F: AGAACTGGACTGTGGCATTGAG R: GCACAAAGCGACTGGATGAAC	NC_037354.1	163
CASP9	F: CCAGGACACTCTGGCTTCAT R: CGGCTTTGATGGGTCATCCT	NC_037343.1	70
GAPDH	F: CCTGGAGAAACCTGCCAAGT R: AGCCGTATTCATTGTCATACCA	NC_037332.1	120

CCNA2 – cyclin A2, *CCND1* – cyclin D1, *PCNA* – proliferating cell nuclear antigen, *BCL2* – B cell lymphoma 2, *BAX4* – BCL2-associated X 4, *CASP3* – caspase 3, *CASP9* – caspase 9, *GAPDH* – glyceraldehyde-3-phosphate dehydrogenase

antibodies, and incubated with secondary antibodies at 25 °C for 2 h.

Statistical analysis

CCK8 data were analysed using the mixed procedure of SAS (Proc Mixed; SAS Institute, 2002), and linear and quadratic orthogonal contrasts were analysed using the CONTRAST statement of SAS with coefficients estimated based on the Na_2SeO_3 dose. Analysis of other experimental data was performed using the SigmaPlot statistical analysis package version 12.5 (Systat Software, Inc., San Jose, CA, USA). Statistical differences in means between treatments were analysed using Student's *t*-test (*t* test), or analysis of variance (ANOVA) with Dunnett's post-hoc test; the level of statistical significance was set at $P < 0.05$. Data in graphs are presented as means ± standard error of the mean.

Results

Effect of Na_2SeO_3 on BMEC proliferation

To determine the effect of supplemental Na_2SeO_3 on BMEC proliferation, the cells were cultured in DMEM-F12 and different supplementary Na_2SeO_3 doses (0, 9, 18, 35, 70, 140, 280 and 560 nM) for 3 days. CCK8 assay results confirmed that the proliferation of BMECs increased quadratically ($P < 0.05$) due to Na_2SeO_3 addition. Although Na_2SeO_3 doses ranging from 18 to 140 nM significantly promoted ($P < 0.05$) the proliferation of BMECs compared to control, higher doses of Na_2SeO_3 (> 70 nM) did not further stimulate BMEC proliferation compared to 35 nM Na_2SeO_3 (Figure 1A). Therefore, a Na_2SeO_3 concentration of 35 nM was selected and used in the following experiments. At the same time, the results of EdU incorporation indicated that the proportion of EdU-positive cell increased ($P < 0.05$) after 35 nM Na_2SeO_3 supplementation (Figure 1B, C).

The *CCNA2* and *CCND1* genes encode Cyclin A2 and D1, respectively. Supplementary Na_2SeO_3 at 35 nM significantly enhanced mRNA expressions of genes involved in cell proliferation, such as *PCNA* ($P < 0.01$), *CCNA2* ($P < 0.01$) and *CCND1* ($P < 0.01$) (Figure 1D). PCNA ($P < 0.01$) and Cyclin A1 ($P < 0.05$) protein expression were also enhanced as a result of 35 nM Na_2SeO_3 supplementation (Figure 1E, F). The level of transcripts of genes associated with apoptosis inhibition, such as *BCL2* ($P < 0.01$), as well as the *BCL2* to *BAX4* ratio ($P < 0.01$) were significantly elevated by 35 nM Na_2SeO_3 supplementation, while mRNA expression of apoptosis-related genes, such as *BAX4*, *CASP3* and *CASP9* were reduced ($P < 0.05$) (Figure 2A). BCL2 protein expression and the BCL2 to BAX ratio were significantly elevated ($P < 0.01$) by the 35 nM Na_2SeO_3 dose, whereas BAX ($P < 0.01$), Caspase-3 ($P < 0.05$) and Caspase-9 ($P < 0.05$) protein expressions were reduced (Figure 2B, C).

Effect of Na_2SeO_3 on Akt and mTOR signalling pathways in BMECs

The ratios of the p-Akt/Akt and p-mTOR/mTOR were highly increased ($P < 0.01$) when BMECs were exposed to 35 nM Na_2SeO_3, suggesting that the activation of Akt/mTOR signalling pathways was stimulated by 35 nM Na_2SeO_3 (Figure 3A, B).

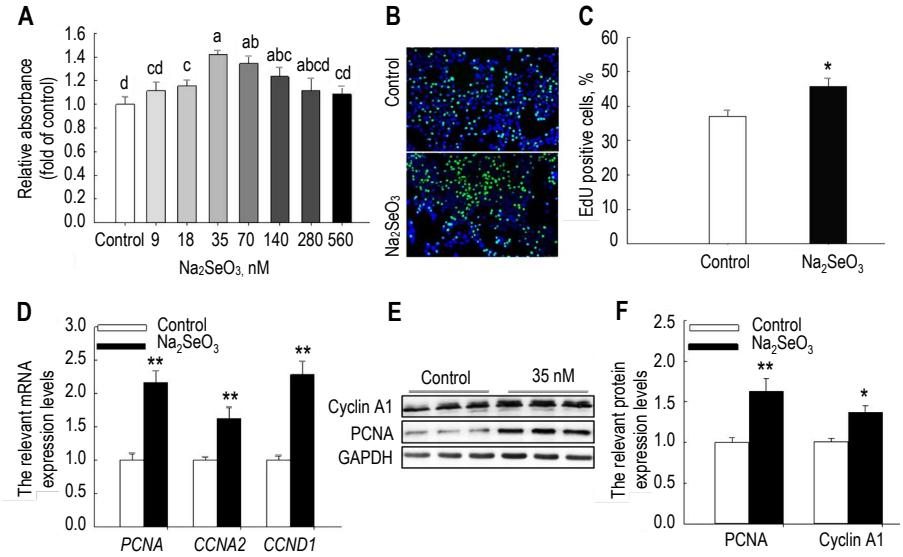

Figure 1. Effect of sodium selenite (Na_2SeO_3) on the proliferation of bovine mammary gland epithelial cells (BMECs). (A) Effect of different doses of Na_2SeO_3 (0, 9, 18, 35, 70, 140, 280 and 560 nM) on the proliferation of BMECs after 3-day culture as determined by the Cell Counting Kit 8 (CCK-8) assay. (B) Effect of Na_2SeO_3 (0 and 35 nM) on BMEC proliferation as determined by the EdU incorporation assay. (C) Analysis of EdU-positive cell percentage in panel B. (D) mRNA expression levels of proliferating cell nuclear antigen (*PCNA*), cyclin A2 (*CCNA2*) and cyclin D1 (*CCND1*) in response to 35 nM Na_2SeO_3. (E) Western blot analysis of PCNA and Cyclin A1 proliferation markers in BMECs after 3-day culture; β-actin was used as a loading control. (F) Mean ± SEM (standard error of the mean) of immunoblotting bands of PCNA and Cyclin A1, and band intensities are expressed in arbitrary units. Bars that do not share the same capital letter are significantly different ($P < 0.01$). * $P < 0.05$ and ** $P < 0.01$ compared to the control group.

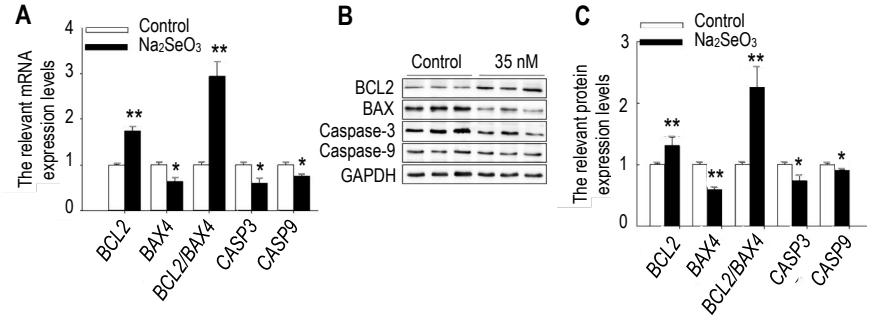

Figure 2. Effect of sodium selenite (Na_2SeO_3) on the apoptosis of bovine mammary gland epithelial cells (BMECs). (A) mRNA expression levels of B cell lymphoma 2 (*BCL2*), BCL2-associated X 4 (*BAX4*), *BCL2/BAX4*, caspase 3 (*CASP3*) and caspase 9 (*CASP9*) in response to 35 nM Na_2SeO_3. (B) Western blot analysis of BCL2, BAX, BCL2/BAX, caspase-3 and caspase-9 apoptotic markers in BMECs after 3-day culture; β-actin was used as a loading control. (C) Mean ± SEM (standard error of the mean) of immunoblotting bands of BCL2, BAX, BCL2/BAX, Caspase-3 and Caspase-9, and band intensities of the bands are expressed in arbitrary units. * $P < 0.05$ and ** $P < 0.01$ compared to the control group.

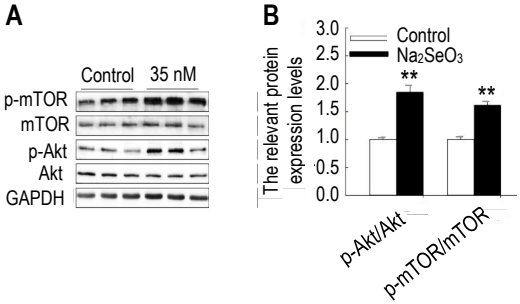

Figure 3. Effect of sodium selenite (Na_2SeO_3) on the Akt-mTOR signalling pathway in bovine mammary gland epithelial cells (BMECs). (A) Western blot analysis of p-Akt, Akt, p-mTOR and mTOR in BMECs treated with Na_2SeO_3 (0 and 35 nM) after 3-day culture; β-actin was used as a loading control. (B) Mean ± SEM (standard error of the mean) of immunoblotting bands of p-Akt/Akt and p-mTOR/mTOR, and band intensities are expressed in arbitrary units. ** $P < 0.01$ compared to the control group.

These findings indicated that Akt/mTOR signalling pathways could be involved in Na_2SeO_3-induced BMEC proliferation.

Akt suppression reversed the stimulation of BMEC proliferation and the activation of mTOR signalling pathways modulated by Na_2SeO_3

The results of the CCK-8 assay suggested that AKT-IN-1 had no effect on BMEC proliferation. AKT-IN-1 could completely block ($P < 0.01$) BMEC proliferation stimulated by 35 nM Na_2SeO_3 (Figure 4A). In turn, significant increases in mRNA levels of *PCNA* ($P < 0.01$), *CCND1* ($P < 0.01$), *CCNA2* ($P < 0.01$), *BCL2* ($P < 0.01$) and

BCL2/BAX-4 ($P < 0.05$) genes, and significant decreases ($P < 0.05$) in *BAX4*, *CASP3* and *CASP9* mRNA levels promoted by 35 nM Na_2SeO_3 were reversed ($P < 0.05$) by 30 nM AKT-IN-1 (Figure 4B); the expression of PCNA, Cyclin A1, BCL2, BAX, BCL2/BAX, Caspase-3 and Caspase-9 proteins was also induced ($P < 0.05$) after 30 nM AKT-IN-1 addition (Figure 4C, D). In addition, the significantly elevated ratios of p-Akt/Akt ($P < 0.01$) and p-mTOR/mTOR ($P < 0.01$), as a response to 35 nM Na_2SeO_3 stimulation also decreased ($P < 0.01$) after 30 nM AKT-IN-1 addition (Figure 4C, D). It should be noted that Na_2SeO_3-modulated activation of Akt, a downstream target of mTOR, was reversed by AKT-IN-1, indicating that mTOR was likely involved in the Na_2SeO_3-stimulated AKT signalling cascade. For this reason, subsequent experiments focused on mTOR.

Suppression of the mTOR signalling pathway eliminated Na_2SeO_3- stimulated BMEC proliferation

CCK-8 assay results showed that there was no effect of Rap on the proliferation of BMECs. The stimulation of BMEC proliferation induced by 35 nM Na_2SeO_3 was abolished ($P < 0.05$) by 50 pM Rap (Figure 5A). Moreover, changes in *PCNA* ($P < 0.01$), *CCNA2* ($P < 0.05$) and *CCND1* ($P < 0.05$) mRNA transcript levels (Figure 5B), as well as the expression of Cyclin A1 ($P < 0.05$) and PCNA ($P < 0.05$) (Figure 5C, D) proteins were also inhibited ($P < 0.05$) by 50 pM Rap. Moreover, the significantly elevated ratios of p-mTOR/mTOR ($P < 0.05$) by 35 nM Na_2SeO_3 decreased ($P < 0.05$) after 50 pM Rap addition (Figure 5C, D). Notably, mTOR suppression had no influence on the expression of mRNA or proteins associated with cell apoptosis and the Na_2SeO_3-activated Akt signalling pathway (Figure 5B, C, D).

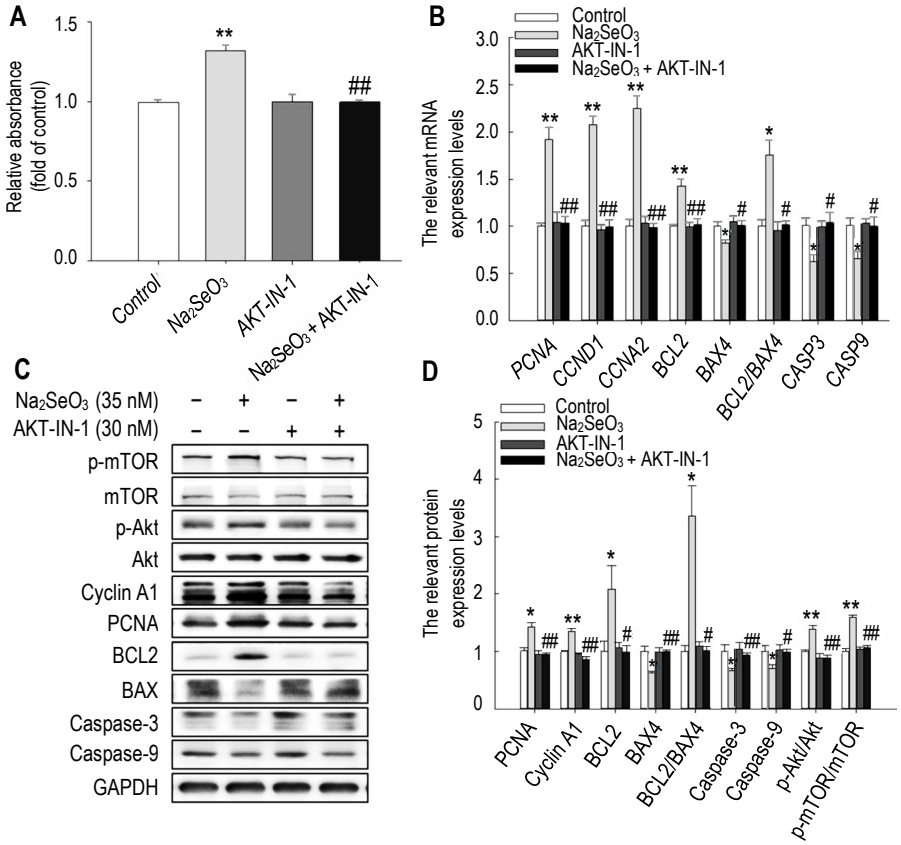

Figure 4. Suppression of Akt reversed the stimulation of bovine mammary gland epithelial cell (BMEC) proliferation and activation of the mTOR signalling pathway modulated by 35 nM sodium selenite (Na_2SeO_3). (A) Effect of 30 nM Akt inhibitor-1 (AKT-IN-1) on the proliferation of BMECs after 3-day incubation was determined using the Cell Counting Kit 8 (CCK-8) assay. (B) mRNA expression levels of proliferating cell nuclear antigen (*PCNA*), cyclin A2 (*CCNA2*) and cyclin D1 (*CCND1*), B cell lymphoma 2 (*BCL2*), BCL2-associated X 4 (*BAX4*),*BCL2*/*BAX4*, caspase 3 (*CASP3*) and caspase 9 (*CASP9*) in response to 35 nM Na_2SeO_3 and/or 30 nM AKT-IN-1. (C) Western blot analysis of PCNA, Cyclin A1, BCL2, BAX, BCL2/BAX, Caspase-3, Caspase-9, p-Akt, Akt, p-mTOR and mTOR in BMECs after 3-day culture in the presence of 35 nM Na_2SeO_3 and/or 30 nM AKT-IN-1; β-actin was used as a loading control. (D) Mean ± SEM (standard error of the mean) of immunoblotting bands of PCNA, Cyclin A1, BCL2, BAX, BCL2/BAX, Caspase-3, Caspase-9, p-Akt/Akt and p-mTOR/mTOR, and band intensities are expressed in arbitrary units. * $P < 0.05$ and ** $P < 0.01$ compared to the control group, # $P < 0.05$ and ## $P < 0.01$ compared to the Na_2SeO_3 group.

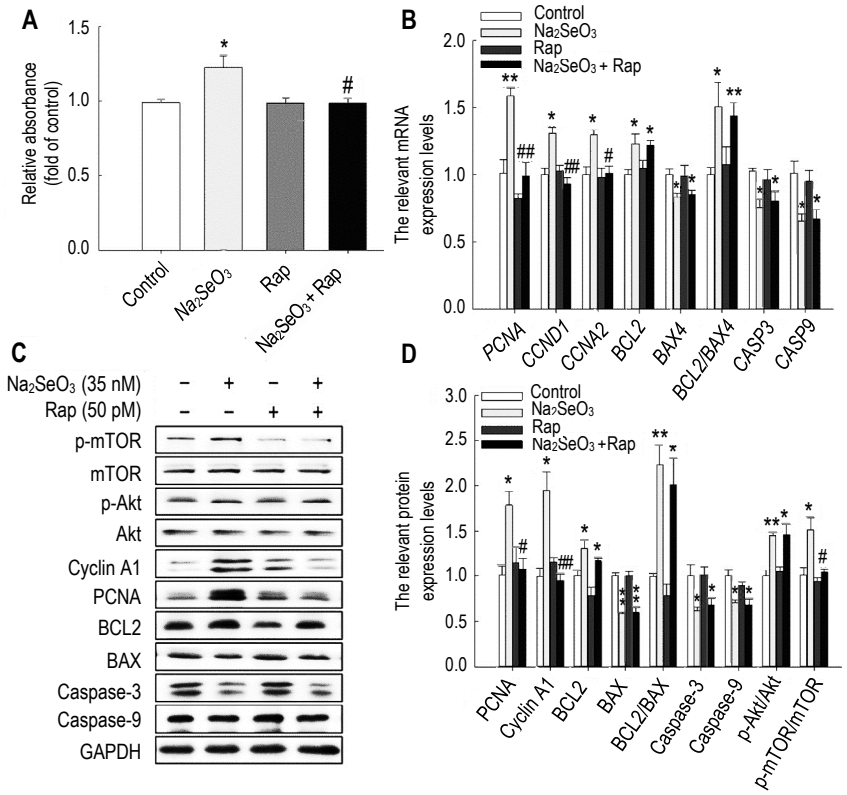

Figure 5. Suppression of the mTOR signalling pathway eliminated the promotion of bovine mammary gland epithelial cell (BMEC) proliferation modulated by 35 nM sodium selenite (Na_2SeO_3). (A) Effect of 50 pM rapamycin (Rap), an inhibitor of mTOR, on the proliferation of BMECs after 3-day incubation was determined using the Cell Counting Kit 8 (CCK-8) assay. (B) mRNA expression levels of proliferating cell nuclear antigen (*PCNA*), cyclin A2 (*CCNA2*) and cyclin D1 (*CCND1*), B cell lymphoma 2 (*BCL2*), BCL2-associated X 4 (*BAX4*), *BCL2*/*BAX4*, caspase 3 (*CASP3*) and caspase 9 (*CASP9*) in response to 35 nM Na2SeO3 and/or 50 pM Rap. (C) Western blot analysis of PCNA, Cyclin A1, BCL2, BAX, BCL2/BAX, caspase-3, caspase-9, p-Akt, Akt, p-mTOR and mTOR in BMECs after 3-day culture in the presence of 35 nM Na_2SeO_3 and/or 50 pM Rap; β-actin was used as a loading control. (D) Mean ± SEM (standard error of the mean) of immunoblotting bands of PCNA, Cyclin A1, BCL2, BAX, BCL2/BAX, Caspase-3, Caspase-9, p-Akt/Akt and p-mTOR/mTOR, and band intensities are expressed in arbitrary units; * $P < 0.05$ and ** $P < 0.01$ compared to the control group, # $P < 0.05$ and ## $P < 0.01$ compared to the Na_2SeO_3 group.

Discussion

In the current study, Na_2SeO_3 supplementation promoted the proliferation of BMECs and suppressed the expression of mRNA or proteins involved in BMEC apoptosis, supporting previous findings regarding increased milk production (Wang et al., 2009; Ullah et al., 2019), and milk fat and protein yields (Najafnejad et al., 2013) after Se supplementation. The present study determined the effect of different concentrations of supplemental Na_2SeO_3 on BMEC proliferation. Although Na_2SeO_3 supplementation at doses ranging from 4 to 160 nM stimulated the proliferation of BMECs, high doses of Na_2SeO_3 (> 70 nM) did not further stimulate BMEC proliferation compared to 35 nM Na_2SeO_3. These results indicated that the appropriate dose of Na_2SeO_3 for BMEC proliferation was 35 nM, and the proliferation of BMECs increased quadratically due to Se supplementation. Similarly, Zhang et al. (2020) reported that the addition of 50 to 200 nM Se significantly increased cell viability and relative growth rate of BMECs, with 50 nM Se being the optimum dose. Compared to the present study, the dose of supplementary Se (0, 10, 20, 50, 100, 150 and 200 nM, an equivalent to 0, 22, 44, 110, 220, 330 and 440 nM Na_2SeO_3) in the study of Zhang et al. (2020) was higher. Based on all the above results, it was speculated that Na_2SeO_3 may had a dual effect on BMECs. Zhang et al. (2020) also found that the positive effect of high Se concentrations on antioxidant function was gradually reduced in healthy BMECs.

Maximising the number of secretory cells in the mammary gland by stimulating proliferation and suppressing apoptosis should be a critical check point for extending lactation in dairy cows (Bae et al., 2020). The expression levels of proliferation marker genes, such as *PCNA* and cyclins (*CCNA2* and *CCND1*) were determined to illustrate the

regulatory effect of Na_2SeO_3 on the proliferation of BMECs. Cyclin D modulates the transition from the G1 phase of the cell-cycle to the S phase, and Cyclin A is considered essential for the initiation and termination of DNA synthesis during the S phase (Bertoli et al., 2013). Cyclin A2 is ubiquitously expressed in all proliferating cells and has functions in both S-phase and mitosis (Bertoli et al., 2013). Additionally, *PCNA* has its main function in DNA replication and repair mechanism (Park et al., 2016). Meanwhile, Cyclin D3 and PCNA have been implicated in previous studies in the regulation of mammary epithelial cell proliferation (Meng et al., 2017; Zhang et al., 2018). In the current study, supplementary 35 nM Na_2SeO_3 elevated mRNA expression levels of the *PCNA*, *CCNA2* and *CCND1* genes, as well as PCNA and Cyclin A1 protein levels, indicating that supplementary 35 nM Na_2SeO_3 stimulated the expression of proliferation markers in BMECs. Apoptosis is executed in cells via a highly complex signalling cascade involving two pathways, i.e. mitochondrial and death receptor pathways, and is induced by BCL2 family proteins. Furthermore, the latter proteins take part in the modulation of apoptotic cell death, including anti-apoptotic (BCL2, etc.) and pro-apoptotic (BAX, etc.) members of this family (Shamas-Din et al., 2013). Since BCL2 prevents apoptosis by inhibiting the activity of BAX(-4), a high BCL2/BAX(-4) ratio is an indicator of suppressed apoptosis. Therefore, the ratio of BCL2/BAX protein expression levels may indicate a state of cellular apoptosis. Both mitochondrial and death receptor pathways converge in the common pathway of executioner caspases, namely CASP3 and CASP9 (Pisani et al., 2020); these two caspases play a crucial role in the execution phase of cell apoptosis. Thus, reducing its expression inhibits the initiation of apoptosis. In the present study, increased mRNA and protein levels of the *BCL2* gene, and the *BCL2* to *BAX(-4)* ratio, reduced mRNA levels of *BAX4*, *CASP3* and *CASP9* genes, as well as lower expression of BAX, CASP3 and CASP9 proteins after 35 nM Na_2SeO_3 supplementation suggested that its addition promoted the expression of apoptotic markers in BMECs. Therefore, the results illustrated that Na_2SeO_3 modulated proliferation by stimulating the expression of proliferative markers and inhibiting the expression of apoptotic markers.

Recently, there have been many implications that the Akt signalling pathway is involved in modulating the proliferation and apoptosis of different cell types, e.g. breast cancer cells (Huang et al., 2021) or HC11 cells (Meng et al., 2017). Consistent with these results, in the current study, the Akt signalling pathway was activated by 35 nM Na_2SeO_3 during BMEC proliferation. This was further supported by the findings that suppression of the Akt signalling pathway with AKT-IN-1 reversed Na_2SeO_3-promoted cell viability and the expression of proliferation and apoptotic markers in this study. The protein expression of the p-Akt/Akt and p-mTOR/mTOR ratios were decreased by 36.1% and 33.7% respectively. The findings provided evidence that activation of the Akt-mTOR signalling pathway could be involved in pro-proliferative effects of Na_2SeO_3 on BMECs.

The mTOR signalling pathway was also shown to participate in regulating the proliferation of various cells (Li et al., 2017). Moreover, the mTOR signalling pathway was activated when BMECs were exposed to 35 nM Na_2SeO_3 in the current study. Moreover, suppression of the mTOR signalling pathway by Rap reversed the Na_2SeO_3-modulated stimulation of BMEC proliferation and altered the expression of proliferation markers. The protein expression of p-mTOR/mTOR ratio was decreased by 31.2%. These observations demonstrated that the mTOR signalling pathway could be involved in the stimulatory effect of Na_2SeO_3 on BMEC proliferation.

Based on the above results, the upstream and downstream relationships between Akt and mTOR should be determined to further elucidate the signalling pathway involved in BMEC proliferation affected by supplementary Na_2SeO_3. In the current study, the protein expression of p-Akt/Akt and p-mTOR/mTOR ratio were decreased by AKT-IN-1. Meanwhile, the protein expressions of p-mTOR/mTOR ratio were decreased by Rap. Nevertheless, there was no impact of Rap on the ratio of p-Akt/Akt. Therefore, Na_2SeO_3 supplementation stimulated BMEC proliferation by modulating the Akt-mTOR signalling pathway. Taken together, the results provided new information that Na_2SeO_3 was able to stimulate BMEC proliferation by regulating the Akt-mTOR signalling pathway.

Conclusions

Se supplementation stimulated BMEC proliferation by promoting mRNA and protein expression of proliferative and inhibitory apoptotic markers by regulating the Akt-mTOR signalling pathway. The findings may provide new information on the

potential applications of Se as a nutritional regulator in the stimulation of mammary gland development.

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Conflict of interest

The Authors declare that there is no conflict of interest.

References

Akers R.M. (Editor), 2002. Lactation and the mammary gland. 1st Edition. Wiley-Blackwell. Hoboken, NJ (USA), https://doi.org/10.1002/9781119264880

Akers R.M., 2017. A 100-Year Review: mammary development and lactation. J. Dairy Sci. 100, 10332–10352, https://doi.org/10.3168/jds.2017-12983

Bae D., Chon J.W., Kim D.H., Kim H., Seo K.H., 2020. Effect of folic acid supplementation on proliferation and apoptosis in bovine mammary epithelial (MAC-T) cells. Anim. Biotechnol. 33, 13–21, https://doi.org/10.1080/10495398.2020.1758123

Bertoli C., Skotheim J.M., de Bruin R.A.M., 2013. Control of cell cycle transcription during G1 and S phases. Nat. Rev. Mol. Cell Biol. 14, 518–528, https://doi.org/10.1038/nrm3629

De Cicco P., Panza E., Ercolano G., Armogida C., Sessa G., Pirozzi G., Cirino G., Wallace J.L., Ianaro A., 2016. ATB-346, a novel hydrogen sulfide-releasing anti-inflammatory drug, induces apoptosis of human melanoma cells and inhibits melanoma development in vivo. Pharmacol. Res. 114, 67–73, https://doi.org/10.1016/j.phrs.2016.10.019

He S.L., Guo X., Tan W.H., Su X.H., Li J.P., Pan W., Qiu H.Y., 2016. Effect of selenium deficiency on phosphorylation of the AMPK pathway in rats. Biol. Trace Elem. Res. 169, 254–260, https://doi.org/10.1007/s12011-015-0427-z

Huang J., Qin Y.Y., Lin C.F., Huang X.G., Zhang F.R., 2021. MTHFD2 facilitates breast cancer cell proliferation via the AKT signaling pathway. Exp. Ther. Med. 22, 703, https://doi.org/10.3892/etm.2021.10135

Huang Z., Rose A.H., Hofmann P.R., 2012. The role of selenium in infammation and immunity: from molecular mechanisms to therapeutic opportunities. Antioxid. Redox. Signal. 16, 705–743, https://doi.org/10.1089/ars.2011.4145

Jiang D., Cho W.C., Li Z., Xu X., Qu Y., Jiang Z., Guo L., Xu, G., 2017. MiR-758-3p suppresses proliferation, migration and invasion of hepatocellular carcinoma cells via targeting MDM2 and mTOR. Biomed. Pharmacother. 96, 535–544, https://doi.org/10.1016/j.biopha.2017.10.004

Kim S.W., Wu G., 2009. Regulatory role for amino acids in mammary gland growth and milk synthesis. Amino Acids 37, 89–95, https://doi.org/10.1007/s00726-008-0151-5

Li F., Lutz P.B., Pepelyayeva Y., Arnér E.S.J., Bayse C.A., Rozovsky S., 2014. Redox active motifs in selenoproteins. Proc. Natl. Acad. Sci. U. S. A. 111, 6976–6981, https://doi.org/10.1073/pnas.1319022111

Li L., Liu L., Qu B., Li X., Gao X., Zhang M., 2017. Twinfilin 1 enhances milk bio-synthesis and proliferation of bovine mammary epithelial cells via the mTOR signaling pathway. Biochem. Biophys. Res. Commun. 492, 289–294, https://doi.org/10.1016/j.bbrc.2017.08.130

Macrina A.L., Kauf A.C.W., Kensinger R.S., 2011. Effect of bovine somatotropin administration during induction of lactation in 15-month-old heifers on production and health. J. Dairy Sci. 94, 4566–4573, https://doi.org/10.3168/jds.2011-4394

McCabe C.J., Suarez-Trujillo A., Teeple K.A., Casey T.M., Boerman J.P., 2021. Chronic prepartum light-dark phase shifts in cattle disrupt circadian clocks, decrease insulin sensitivity and mammary development, and are associated with lower milk yield through 60 days postpartum. J. Dairy Sci. 104, 2422–2437, http://dx.doi.org/10.3168/jds.2020-19250

Meng Y., Zhang J., Zhang F. et al., 2017. Lauric acid stimulates mammary gland development of pubertal mice through activation of GPR84 and PI3K/Akt signaling pathway. J. Agric. Food Chem. 65, 95–103, https://doi.org/10.1021/acs.jafc.6b04878

Molenaar A.J., Maclean P.H., Gilmour M.L., Draganova I.G., Symes C.W., Margerison J.K., Mcmahon C.D., 2020. Effect of whole-milk allowance on liveweight gain and growth of parenchyma and fat pads in the mammary glands of dairy heifers at weaning. J. Dairy Sci. 103, 5061–5069, https://doi.org/10.3168/jds.2019-17126

Najafnejad B., Aliarabi H., Tabatabaei M.M., Alimohamady R., 2013. Effects of different forms of selenium supplementation on production performance and nutrient digestibility in lactating dairy cattle. In: Proceedings of the Second International Conference on Agriculture and Natural Resources. 25-26 December 2013. Kermanshah (Iran)

Pan J., Li K., Huang W., Zhang X., 2017. MiR-137 inhibited cell proliferation and migration of vascular smooth muscle cells via targeting IGFBP-5 and modulating the mTOR/STAT3 signaling. PLoS One 12, e0186245, https://doi.org/10.1371/journal.pone.0186245

Park S.Y., Mi S.J., Chang W.H., Yu H.S., Jang S.B., 2016. Structural and functional insight into proliferating cell nuclear antigen. J. Microbiol. Biotechnol. 26, 637–647, https://doi.org/10.4014/jmb.1509.09051

Pisani C., Ramella M., Boldorini R., Loi G., Billia M., Boccafoschi F., Volpe A., Krengli M., 2020. Apoptotic and predictive factors by Bax, caspases 3/9, Bcl-2, p53 and Ki-67 in prostate cancer after 12 Gy single-dose. Sci. Rep. 10, 7050, https://doi.org/10.1038/s41598-020-64062-9

SAS Institute, 2002. SAS/STAT®9.0 User's Guide. SAS Institute Inc. Cary, NC (USA)

Shamas-Din A., Kale J., Leber B., Andrews D.W., 2013. Mechanisms of action of Bcl-2 family proteins. Cold Spring Harbor Perspect. Biol. 5, a008714, https://doi.org/10.1101/cshperspect.a008714

Shi L., Duan Y., Yao X., Song R., Ren Y., 2020. Effects of selenium on the proliferation and apoptosis of sheep spermatogonial stem cells *in vitro*. Anim. Reprod. Sci. 215, 106330, https://doi.org/10.1016/j.anireprosci.2020.106330

Souza F.N., Blagitz M.G., Latorre A.O., Mori C.S., Sucupira M.C.A., Della Libera A.M.M.P., 2012. Effects of *in vitro* selenium supplementation on blood and milk neutrophils from dairy cows. Pesq. Vet. Bras. 32, 174–178, https://doi.org/10.1590/S0100-736X2012000200014

Ullah H., Khan R.U., Mobashar M., Ahmad S., Sajid A., Khan N.U., Usman T., Khattak I., Khan H., 2019. Effect of yeast-based selenium on blood progesterone, metabolites and milk yield in Achai dairy cows. Ital. J. Anim. Sci. 18, 1445–1450, https://doi.org/10.1080/1828051X.2019.1683475

Wang C., Liu Q., Yang W.Z., Dong Q., Yang X.M., He D.C., Zhang P., Dong K.H., Huang Y.X., 2009. Effects of selenium yeast on rumen fermentation, lactation performance and feed digestibilities in lactating dairy cows. Livest. Sci. 126, 239–244, https://doi.org/10.1016/j.livsci.2009.07.005

Xiao J., Khan M.Z., Ma Y., Alugongo G.M., Ma J., Chen T., Khan A., Cao Z., 2021. The antioxidant properties of selenium and vitamin E; their role in periparturient dairy cattle health regulation. Antioxidants 10, 1555, https://doi.org/10.3390/antiox10101555

Zhang B.Q., Guo Y.M., Yan S.M., Guo X.Y., Zhao Y.L., Shi B.L., 2020. The protective effect of selenium on the lipopolysaccharide-induced oxidative stress and depressed gene expression related to milk protein synthesis in bovine mammary epithelial cells. Biol. Trace Elem. Res. 197, 141–148, https://doi.org/10.1007/s12011-019-01961-7

Zhang J., Ye J., Yuan C., Fu Q., Zhang F., Zhu X., Wang L., Gao P., Shu G., Jiang Q., Wang S., 2018. Exogenous H_2S exerts biphasic effects on porcine mammary epithelial cells proliferation through PI3K/Akt-mTOR signaling pathway. J. Cell. Physiol. 233, 7071–7081, https://doi.org/10.1002/jcp.26630

Zhang Y., Xu Y., Chen B., Zhao B., Gao X.J., 2021. Selenium deficiency promotes oxidative stress-induced mastitis via activating the NF-κB and MAPK pathways in dairy cow. Biol. Trace Elem. Res. 200, 2716–2726, https://doi.org/10.1007/s12011-021-02882-0

Zhao K., Liu H.Y., Zhou M.M., Liu J.X., 2010. Establishment and characterization of a lactating bovine mammary epithelial cell model for the study of milk synthesis. Cell Biol. Int. 34, 717–721, https://doi.org/10.1042/CBI20100023

Zhong J.X., Jin S.S., Wu K.S., Yu G.C., Tu L.L., Liu L., 2022. Effect of nano-selenium loaded with lycium barbarum polysaccharide on the proliferation of lens epithelial cells after UVB damage in vitro. Int. J. Ophthalmol. 15, 9–14, https://doi.org/10.18240/ijo.2022.01.02