doi: 10.17221/76/2017-CJAS

Protection of Bovine Mammary Epithelial Cells from Hydrogen Peroxide-Induced Oxidative Cell Damage by Selenium

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ABSTRACT

Guo Y.M., Gong J., Zheng Y.G., Shi B.L., Guo X.Y., Yan S.M. (2018): **Protection of bovine mammary epithelial cells from hydrogen peroxide-induced oxidative cell damage by selenium**. Czech J. Anim. Sci., 63, 94–102.

The uncontrolled release of arachidonic acid (ARA) and its metabolism by lipoxygenase (LOX) pathway can induce and aggravate cellular oxidative stress. Selenium (Se) is an integral part of some antioxidative selenoproteins and may protect cells from oxidative damage by modulating ARA release and metabolism. The present study aimed to investigate the protective response of Se against hydrogen peroxide (H_2O_2)-induced oxidative damage in bovine mammary epithelial cells (BMECs). The BMECs were incubated for 24 h in serum-free medium and then divided into four groups randomly. The cells in groups 1 and 2 were subsequently incubated for 30 h in serum-free medium containing 0 (control) and 50 nM Se (Se treatment group). The cells in groups 3 and 4 were incubated for 24 h in serum-free medium containing 0 and 50 nM Se, and then treated with 600 μM H_2O_2 for 6 h (H_2O_2 damage group and Se prevention group). The results showed that Se attenuated the H_2O_2-induced production of reactive oxygen species and the decrease of antioxidative enzymes as glutathione peroxidase (GPX), thioredoxin reductase (TrxR), selenoprotein P (SelP), superoxide dismutase, and catalase in BMECs. The preventive effects of Se on the decrease of selenoprotein activity were demonstrated further by the increase of mRNA expression for *GPX1*, *TrxR1,* and *SelP*, and protein expression for GPX1 and TrxR1. Pretreatment of cells with Se inhibited the H_2O_2-induced increase of mRNA expressions and activities for cytosolic phospholipase A2 and 5-lipoxygenase, ARA release, and 15-hydroperoxyeicosatetraenoic acid production. Se also blocked the H_2O_2-induced activation of p38 mitogen-activated protein kinase (MAPK) and c-Jun N-terminal kinase but not that of extracellular signal-regulated kinase. These results suggested that Se may protect BMECs against H_2O_2-induced oxidative damage by increasing selenoproteins synthesis, inhibiting MAPK pathway, and then decreasing ARA release and its metabolism by LOX pathway.

Keywords: selenomethionine; selenoprotein synthesis; arachidonic acid metabolism; mitogen-activated protein kinase

List of abbreviations: ROS = reactive oxygen species, Se = selenium, GPX = glutathione peroxidase, TrxR = thioredoxin reductase, SelP = selenoprotein P, ARA = arachidonic acid, cPLA2 = cytosolic phospholipase A2, LPS = lipopolysaccharides, LOX = lipoxygenase, HPETE = hydroperoxyeicosatetraenoic acid, HETE = hydroxyeicosatetraenoic acid, MAPK = mitogen-activated protein kinase, BMECs = bovine mammary epithelial cells, PBS = phosphate balanced solution, DMEM = Dulbecco's modified Eagle medium, FBS = fetal bovine serum, SeMet = selenomethionine, MTT = 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, DMSO = dimethyl sulfoxide, SOD = superoxide dismutase, CAT = catalase, ERK = extracellular signal-regulated kinase, JNK = c-Jun N-terminal kinase K

Supported by the National Natural Science Foundation of China (Project No. 31560650).

Guo Y.M. and Gong J. contributed equally to this article.

doi: 10.17221/76/2017-CJAS

The widespread incidence of mastitis causes economic and animal welfare problems, such as reduced milk production and quality, high rates of animal culling, and even animal death. Dairy cows are more susceptible to mastitis pathogens during the periparturient period (especially between late pregnancy and early lactation) compared with peak lactation (Sordillo et al. 2007). Mammary metabolic demands associated with parturition and initiation of lactation in the periparturient period would be expected to increase the production of reactive oxygen species (ROS) (Sordillo 2005; Jin et al. 2016). The uncontrolled accumulation of ROS leads to oxidative stress, membrane lipid peroxidation, and cellular damage. Oxidative stress induced by ROS is believed to be a primary contributing factor to the incidence of various cattle diseases including mastitis.

The essential trace element selenium (Se), in the form of selenoproteins, plays a pivotal role in the antioxidant defense system of cell. There are at least 55 selenoproteins that have been identified in mammals. Some antioxidative selenoproteins, such as glutathione peroxidase (GPX), thioredoxin reductase (TrxR), and selenoprotein P (SelP), play a key role in removing potentially damaging lipid hydro-peroxides and hydrogen peroxides and protecting cell from oxidative damage. Some studies indicated that Se supplementation reduced the incidence and severity of mastitis (Yang and Li 2015) and a significant negative correlation existed between whole blood GPX activity and bulk tank milk somatic cell counts (Weiss et al. 1990). Se protected bovine aortic endothelial cells from oxidative damage induced by *t*-butyl hydroperoxide by increasing GPX1, GPX4, and TrxR1 activities (Miller et al. 2001). The mammary tissue of dairy cattle can express GPX1, GPX4, and TrxR1 (Bruzelius et al. 2007) and there exists a significant positive correlation between the gene expression for these antioxidative enzymes and proinflammatory cytokines (Aitken et al. 2009). These studies suggested that Se may attenuate oxidative stress and decrease the incidence of mastitis through manipulating the gene expression and activities of the key antioxidant selenoprotein enzymes in mammary tissue or mammary epithelial cells of dairy cows. However, the exact mechanism is unclear and needs to be further investigated.

Arachidonic acid (ARA) is the common precursor of the eicosanoids, and exists in phospholipids of cell membrane. ARA can be released from phospholipids by the activation of cytosolic phospholipase A2 (cPLA2) and phospholipase C induced by ROS, lipopolysaccharides (LPS), cytokines, and nitric oxide (Piotrowska-Tomala et al. 2012). The study showed that the uncontrolled release of ARA activated the calcium channel on cell membrane and then NADPH oxidase, which results in the excessive production of ROS (Jagnandan et al. 2007). The free ARA also induced ROS production through inhibiting the activities of complexes I and III in mitochondrial respiratory chain and increasing the liquidity of mitochondrial membrane (Schonfeld and Wojtczak 2007). Moreover, ARA can easily be peroxidized, and generate free radicals such as superoxide anion (Seguineau et al. 2011). The metabolism of excessive ARA through lipoxygenase (LOX) pathway caused the accumulation of peroxidized lipids such as 5-, 12-, and 15-hydroperoxyeicosatetraenoic acid (HPETE). These oxidative metabolic products can aggravate cellular oxidative stress and induce cell apoptosis (Maccarrone et al. 2000). Se supplementation in bovine endothelial cells exhibited a significantly higher 15-hydroxyeicosatetraenoic acid (HETE) to 15-HPETE ratio compared with Se-deficient cells (Funk and Cyrus 2001). Some studies showed that Se-dependent GPX is directly involved in the reduction of 5-HPETE to 5-HETE in human peripheral blood monocytes (Straif et al. 2000) and TrxR reduced cPLA2 activity and ARA content in mouse fibrosarcoma cells (Kurosawa et al. 2009). The cPLA2 is a downstream substrate of mitogen-activated protein kinase (MAPK) pathway, which can be activated by ROS (Camps et al. 2000). Se may attenuate oxidative stress through inhibiting the activation of MAPK pathway, and then decreasing the release and oxidative metabolism of ARA. However, little is known about the regulation of Se on MAPK pathway and ARA release and metabolism in bovine mammary epithelial cells (BMECs).

Therefore, the objective of this study was to evaluate how selenium protects BMECs from hydrogen peroxide (H_2O_2)-induced oxidative damage by regulating selenoproteins synthesis, MAPK pathway, and ARA release and metabolism.

MATERIAL AND METHODS

Isolation and culture of BMECs. Healthy mammary tissue samples from 3 multiparous lactating

doi: 10.17221/76/2017-CJAS

Holstein cows were obtained at a local slaughterhouse immediately after slaughter, kept on ice, and transported to the laboratory without delay. The BMECs were isolated from mammary tissue as described previously (Miranda et al. 2009) with slight modifications. Briefly, in a laminar flow hood, the pieces (about 30 g) of the deeper mammary tissue were washed 3 times in the phosphate balanced solution (PBS) and once in 75% ethanol, trimmed of all visible nonepithelial parenchyma, and thoroughly minced with scissors. Minced tissues were washed 3 times in PBS to remove milk and blood and digested with collagenase II (0.5%) (Gibco BRL, USA) for 1 h. The digesta was filtrated and the filtrate was centrifuged to obtain the BMECs.

The isolated BMECs were grown in Dulbecco's modified Eagle medium/F12 (DMEM/F12) (Gibco) supplemented with 10% (vol/vol) fetal bovine serum (FBS) and containing 5 µg ovine prolactin, 10 ng epidermal growth factor, 1 µg hydrocortisone, 100 U penicillin, 100 µg streptomycin, and 0.25 µg amphotericin B (Sigma-Aldrich, USA) per ml. The cells were seeded in 25 cm^2 culture flasks and cultivated at 37°C in 5% CO_2 to about 90% confluency.

Experimental design. The BMECs were seeded on 96-well plates (200 µl/well) at a cell density of 1×10^4/ml and 6-well plates (2.5 ml/well) at a cell density of 1×10^5/ml. After 24 h of incubation in DMEM/F12 medium containing 10% FBS, the supernatant was removed and new serum-free medium was added to starved incubate for 24 h. The starved incubated cells were then randomly divided into four groups with six duplicates. Cells in the first group were incubated for 30 h in serum-free medium without Se and H_2O_2 addition (Control). Cells in the second group were incubated for 30 h in serum-free medium supplemented with 50 nM selenomethionine (SeMet) (Sigma-Aldrich) (Se treatment). In the third group, cells were incubated for 24 h in serum-free medium without Se addition, and then treated with 600 µM H_2O_2 for 6 h (H_2O_2 damage). Cells in the fourth group were incubated for 24 h in serum-free medium containing 50 nM SeMet, and then treated with 600 µM H_2O_2 for 6 h (Se prevention).

Cell viability assay. The cells incubated in 96-well plates were used for cell viability assay. Cell viability was determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (Sigma-Aldrich) assay as described elsewhere (Kim et al. 2002). After the incubation according to the experimental design described above, 20 µl MTT solution (0.5 mg/ml) were added into each well and incubated for another 4 h at 37°C. The supernatant was removed and formazan formation was resolved with 150 µl dimethyl sulfoxide (DMSO) (Sigma-Aldrich). The absorbance was measured at 490 nm using a microplate reader Synergy H4 (BioTek, Japan).

Measurement of antioxidative parameters. The cells incubated in 6-well plates were lysed by scraping cells into ice-cold lysis buffer. GPX activity and malondialdehyde (MDA) content in the lysate and the activities of superoxide dismutase (SOD) and catalase (CAT) in the culture medium were assayed using commercial colorimetric assay kits (Nanjing Jiancheng Bioengineering Institute, China) according to the manufacturer's instructions. Intracellular TrxR activity was measured using the 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB) reduction assay as described previously (Hill et al. 1997). SelP content in the culture medium was measured by radioimmunoassay method described previously (Hill et al. 1996). Intracellular ROS level was measured by detecting the fluorescence intensity of oxidant-sensitive 2'7'-dichlorofluorescein, as described previously (Kim et al. 2004).

Determination of parameters related to ARA release and metabolism. The activities of cPLA2 and 5-LOX (both Mybiosource, USA) in the lysate and the concentrations of ARA (R&D systems, USA) and 15-HPETE (Cayman, USA) in the culture medium were assayed using the commercial ELISA kits according to the manufacturer's instructions.

RNA isolation, cDNA synthesis, and gene expression assay. The cells incubated in 6-well plates were harvested and total cellular RNA was isolated using a RNAiso^{TM}Plus kit (TaKaRa, Inc., China) according to the manufacturer's instructions. First-strand cDNA was synthesized from 500 ng of total RNA using a PrimeScript^{TM} reverse transcript kit (TaKaRa, Inc.) according to the manufacturer's instructions. Synthesized cDNA was diluted 10 times with sterile water and stored at –80°C. Primer Premier & Oligo Software (PREMIER Biosoft International, USA) were used to design specific primers for *GPX1, TrxR1, SelP, cPLA2,* and *β-actin* based on the known bovine mRNA sequences of these genes (Table 1). General reverse transcription-polymerase chain reactions (RT-PCRs) were first performed to confirm the specificity of the primers.

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Table 1. Sequences of reverse transcription-polymerase chain reaction primers for bovine *β-actin, GPX1, TrxR1, SelP,* and *cPLA2*

Genes	Primer sequences(5'-3')	Accession No.[1]	Length (bp)	Annealing temperature (°C)
β-actin	F: TCCTGCGGCATTCACGAAACT R: AGAAGCATTTGCGGTGGACGA	NM_173979	316	60
GPX1	F: AGTGCGAGGTGAATGGCGAGAA R: TGGGCAAAATCCCTGGAGAGCA	NM_174076.3	328	60
TrxR1	F: AGGAGAAAGCTGTGGAGAAA R: TTATCCCTTGATGGAATCGT	NM_174625.3	94	60
SelP	F: CTTCATCACCACCACCACAG R: GAGGCAAACGTCACTGTCAA	NM_174459.3	331	60
cPLA2	F: ATTGCCCGACTATCATTCAC R: ATGCTGTGGGTTTGCTTAG	NM_001075864	351	60
5-LOX	F: ACTGGCAGGARAGACCGCATGTT R: ACCTGGTTGAGCTGGATGGCARA	NM_001192792.1	316	60

GPX1 = cytosolic glutathione peroxidase, *TrxR1* = thioredoxin reductase 1, *SelP* = selenoprotein P, *cPLA2* = cytosolic phospholipase A2, 5-LOX = 5-lipoxygenase

[1]the whole mRNA sequences of the related genes are obtained from NCBI Entrez Nucleotide (http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=Nucleotide&itool=toolbar)

The mRNA expression of *GPX1, TrxR1, SelP, cPLA2, 5-LOX,* and *β-actin* (internal control) were analyzed using a SYBR PrimeScript^{TM} RT-PCR Kit (TaKaRa, Inc.) according to the manufacturer's instructions. The PCR reaction system (20 µl) contained 10 µl 2×SYBR Premix Ex *Taq*^{TM} (TaKaRa, Inc.), 0.4 µl (10 mM) each of forward and reverse primers, 2 µl cDNA templates, and 7.2 µl RNA-free H_2O. RT-PCR was performed under the following conditions: 95°C for 30 s, followed by 40 cycles of 95°C for 5 s, 60°C for 30 s, and 72°C for 30 s. Following PCR, melting curves were generated by stepwise increases in temperature from 70 to 95°C with a heating rate of 0.5°C/s. The specificity of PCR products of the genes were confirmed by gel electrophoresis and sequencing, and the relative expressions of mRNA were calculated by the $2^{-\Delta\Delta Ct}$ method using *β-actin* as the control gene.

Western blot analysis. The cells incubated in 6-well plates were washed twice with ice-cold PBS and lysed in modified RIPA buffer (Beyotime, China). The lysates were centrifuged at 12 000 *g* for 10 min at 4°C and the supernatants were collected. After total protein determination using bovine serum albumin as standard, the proteins (50 µg) were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDA-PAGE) and transblotted onto polyvinylidene fluoride membranes. The membranes were incubated with specific primary antibody (anti-GPX1 and anti-TrxR1: Abcam, UK; anti-β-actin: Proteintech, USA) and then with an appropriate secondary antibody coupled to horseradish peroxidase.

Phosphorylation levels of proteins in MAPK pathway. Phosphorylated extracellular signal-regulated kinase (ERK), c-Jun N-terminal kinase (JNK), and p38 MAPK were measured using an InstantOne ELISA Kit (eBioscience, USA). In brief, the cells cultured in 6-well plates were washed with PBS and lysed with lysis buffer. The lysates were centrifuged and the supernatants were added to the microplate assay wells. Antibody cocktail mix (capture antibody and detection antibody) was added to each well. The microplate was sealed and incubated for 1 h at room temperature on a microplate shaker (300 rpm). The wells were washed 3 times with wash buffer and detection reagent was added to each well. After 30 min the reaction was stopped by adding a stop solution. The absorbance was measured at 450 nm using a microplate reader Synergy H4 (BioTek).

Statistical analysis. The data were analyzed by one-way ANOVA procedure of the SAS software (Statistical Analysis System, Version 9.0, 2003), followed by Duncan's test. All variables were expressed as the means and standard errors of the mean. Differences at $P < 0.05$ were considered as significant.

doi: 10.17221/76/2017-CJAS

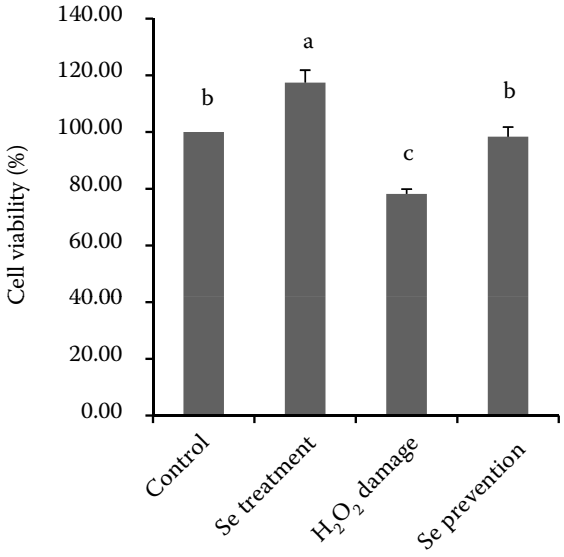

Figure 1. Effect of selenium on cell viability of bovine mammary epithelial cells under H_2O_2 stress
data are expressed as means ± SEM of 3 independent experiments, different letters above histograms show significant difference between treatments ($P < 0.05$)

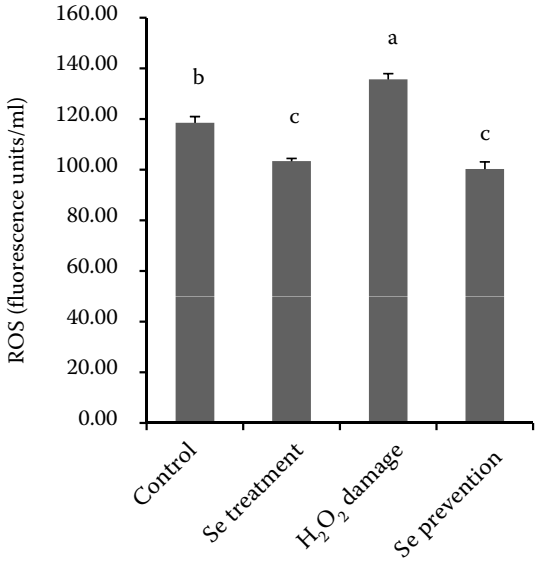

Figure 2. Effect of selenium on reactive oxygen species (ROS) level in bovine mammary epithelial cells under H_2O_2 stress
data are expressed as means ± SEM of 3 independent experiments, different letters above histograms show significant difference between treatments ($P < 0.05$)

RESULTS

Se attenuated H_2O_2-induced ROS production and decrease of cell viability. Figures 1 and 2 show that exposure of cells to H_2O_2 induced cytotoxicity as demonstrated by the loss of cell viability and increased intracellular ROS level compared with the control group, and Se pretreatment markedly attenuated H_2O_2-induced ROS production and decrease of cell viability.

Se suppressed the decline of antioxidative enzyme activities induced by H_2O_2. H_2O_2 stress significantly decreased SelP concentration in the culture medium and intracellular activities of GPX, TrxR, SOD, and CAT compared with the control group. Se pretreatment markedly inhibited H_2O_2-induced decline of the above parameters (Table 2).

Effect of Se on mRNA and protein expressions of selenoproteins in BMECs damaged by H_2O_2. H_2O_2 treatment had no effects on mRNA levels of *GPX1,* but significantly downregulated mRNA expressions of *TrxR1* and *SelP* compared with the control group. Se pretreatment markedly suppressed the decrease of *TrxR1* and *SelP* mRNA expressions induced by H_2O_2 (Table 3).

Stimulation of cells with H_2O_2 resulted in a significant decrease of GPX1 and TrxR1 protein expressions compared with the control group, while

Table 2. Effect of Se on antioxidative parameters in cells under oxidative stress

Items	Control	Se treatment	H_2O_2 damage	Se prevention	*P*-value	SEM
GPX (U/mg protein)	149.43^{b}	264.99^{a}	70.91^{c}	161.71^{b}	< 0.0001	17.96
TrxR (U/g protein)	2.60^{b}	5.33^{a}	1.10^{c}	4.19^{a}	< 0.0001	0.42
SelP (mg/l)	0.24^{b}	0.54^{a}	0.12^{c}	0.30^{b}	< 0.0001	0.02
SOD (U/ml)	12.40^{b}	15.92^{a}	7.78^{c}	11.44^{b}	< 0.0001	0.52
CAT (U/ml)	2.74^{b}	3.62^{a}	1.66^{c}	2.83^{b}	0.0003	0.24
MDA (nmol/mg protein)	3.48^{a}	1.93^{b}	3.52^{a}	2.38^{ab}	0.0100	0.34

GPX = glutathione peroxidase, TrxR = thioredoxin reductase, SelP = selenoprotein P, SOD = superoxide dismutase, CAT = catalase, MDA = malondialdehyde, SEM = standard error of the mean

$^{a–c}$means within a row with different superscripts differ ($P < 0.05$)

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Table 3. Effects of Se on the mRNA expressions of selenoproteins in cells under oxidative stress

Items	Control	Se treatment	H_2O_2 damage	Se prevention	*P*-value	SEM
GPX1	0.018[b]	0.079[a]	0.018[b]	0.065[a]	< 0.0001	0.007
TrxR1	0.004[b]	0.007[a]	0.002[c]	0.007[a]	< 0.0001	0.001
SelP	0.018[b]	0.033[a]	0.004[c]	0.018[b]	0.0012	0.003

GPX1 = cytosolic glutathione peroxidase, *TrxR1* = thioredoxin reductase 1, *SelP* = selenoprotein P, SEM = standard error of the mean

[a–c]means within a row with different superscripts differ ($P < 0.05$)

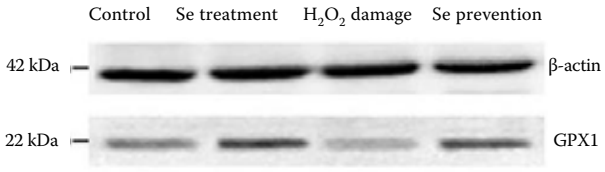

Figure 3. Effect of selenium on cytosolic glutathione peroxidase (GPX1) protein expression in bovine mammary epithelial cells under H_2O_2 stress

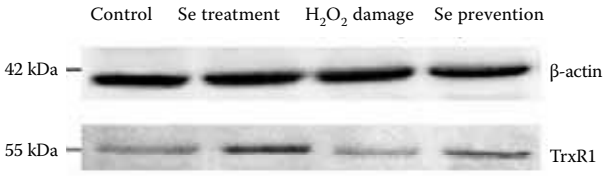

Figure 4. Effect of selenium on thioredoxin reductase 1 (TrxR1) protein expression in bovine mammary epithelial cells under H_2O_2 stress

Se pretreatment markedly inhibited H_2O_2-induced decline of GPX1 and TrxR1 protein expressions (Table 6, Figures 3 and 4).

Se inhibited H_2O_2-induced ARA release and metabolism by LOX pathway. Treatment of cells with H_2O_2 caused a significant increase of cPLA2 activity and mRNA expression in cells as well as ARA content in the culture medium. Se itself had no effect on the mRNA expression and activity of cPLA2 and the release of ARA, but Se inhibited H_2O_2-induced increase of these parameters (Table 4). H_2O_2 treatment also increased 5-LOX activity and mRNA expression in cells as well as 15-HPETE production in the culture medium compared with the control group. Similarly, Se itself did not alter the mRNA expression and activity of 5-LOX and 15-HPETE production, but Se suppressed H_2O_2-induced increase of these indexes (Table 4).

Effect of Se on the activation of MAPK signaling pathway induced by H_2O_2. Stimulation of cells with H_2O_2 increased the phosphorylation of p38 MAPK and JNK, but not that of ERK1/2. Se alone had no effect on any of the three proteins; however, Se attenuated H_2O_2-induced activation of p38 MAPK and JNK (Table 5).

Table 4. Effects of Se on the parameters related to arachidonic acid (ARA) release and metabolism in cells under oxidative stress

Items	Control	Se treatment	H_2O_2 damage	Se prevention	*P*-value	SEM
cPLA2 mRNA expression	0.0045[b]	0.0046[b]	0.0115[a]	0.0062[b]	0.0004	0.0008
cPLA2 activity (U/ml)	53.12[b]	52.32[b]	64.16[a]	56.53[b]	0.0004	1.31
ARA content (pmol/l)	2.13[b]	2.12[b]	2.84[a]	2.40[b]	< 0.0001	0.08
5-LOX mRNA expression	0.0015[b]	0.0013[b]	0.0039[a]	0.0013[b]	0.0076	0.0004
5-LOX activity (pg/ml)	204.57[b]	78.38[c]	233.14[a]	96.00[c]	< 0.0001	6.98
15-HPETE content (ng/ml)	90.63[b]	56.38[c]	110.38[a]	71.88[c]	< 0.0001	5.21

cPLA2 = cytosolic phospholipase A2, 5-LOX = 5-lipoxygenase, 15-HPETE = 15-hydroperoxyeicosatetraenoic acid, SEM = standard error of the mean

[a–c]means within a row with different superscripts differ ($P < 0.05$)

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Table 5. Effects of Se on phosphorylation levels of extracellular signal-regulated kinase (ERK), c-Jun N-terminal kinase (JNK), and p38 MAPK in cells under oxidative stress (optical density value)

Items	Control	Se treatment	H_2O_2 damage	Se prevention	*P*-value	SEM
JNK	0.41[b]	0.47[b]	0.95[a]	0.61[b]	0.0002	0.04
p38	0.37[b]	0.33[b]	0.64[a]	0.48[b]	0.0038	0.04
ERK1/2	0.32[a]	0.29[a]	0.34[a]	0.29[a]	0.2200	0.02

SEM = standard error of the mean

[a,b]means within a row with different superscripts differ ($P < 0.05$)

Table 6. Effect of Se on protein expression of selenoproteins in cells damaged by H_2O_2

Items	Control	Se treatment	H_2O_2 damage	Se prevention	*P*-value	SEM
GPX1	0.93[c]	1.61[a]	0.51[d]	1.40[b]	< 0.0001	0.01
TrxR1	0.94[c]	1.91[a]	0.65[d]	1.38[b]	< 0.0001	0.01

GPX1 = cytosolic glutathione peroxidase, TrxR1 = thioredoxin reductase 1, SEM = standard error of the mean

[a–d]means within a row without the same superscripts significantly differ ($P < 0.05$)

DISCUSSION

Oxidative stress, associated with the formation of ROS, is believed to be a primary contributing factor to the incidence of various cattle diseases including mastitis. Cell viability and some antioxidant substances like GPX, TrxR, SelP, SOD, and CAT are useful markers for oxidative stress in cells. The changes of cell viability and these antioxidant substances induced by H_2O_2 in cultured cells have been reported previously. For example, H_2O_2 treatment significantly decreased the viability of human umbilical vein endothelial cells (Yang et al. 2010). Under the condition of oxidative stress, GPX activity in bovine mammary epithelial cells (Miranda et al. 2011), TrxR activity in bovine peripheral blood mononuclear cells (Sordillo et al 2007), and SOD activity in N9 murine microglia cells (Chen and Chen 2011) were also decreased markedly. Consistent with these earlier reports, the present study showed that stimulation of cells with 600 μM H_2O_2 for 6 h resulted in the significant decrease of cell viability and antioxidant enzyme activities, such as GPX, TrxR, SelP, SOD and CAT, which is accompanied by an increase of intracellular ROS level. Based on these results, we investigated further the mRNA and protein expression of antioxidative seleproteins in BMECs induced by H_2O_2, and found that stimulation of cells with 600 μM H_2O_2 for 6 h significantly downregulated the mRNA levels of *GPX1, TrxR1,* and *SelP* as well as the protein expression levels of GPX1 and TrxR1. The results indicated that 600 μM H_2O_2 induced the decrease of antioxidant function and cell damage, which is likely to be due to decreased selenoprotein expression and antioxidant enzyme activities.

Se is an essential component of several enzymes such as GPX, TrxR, and SelP, which play a key role in removing potentially damaging lipid hydroperoxides and hydrogen peroxides and protecting cell from oxidative damage. Se supplementation has been shown to upregulate the mRNA expression of *GPX1*, *TrxR1,* and *SelP*, and increase GPX and TrxR activities in bovine and human endothelial cells (Hara et al. 2001; Miller et al. 2001). In this experiment, pretreatment of cells with Se decreased ROS production and prevented H_2O_2-induced decrease of cell viability and antioxidant enzyme activities (GPX, TrxR, SelP, SOD, and CAT). Se also inhibited H_2O_2-induced decrease of *GPX1*, *TrxR1,* and *SelP* mRNA expressions as well as GPX1 and TrxR1 protein expressions, and all these variables returned at least to control levels. The results indicated that the protection of Se against oxidative damage induced by H_2O_2 in BMECs appears to be related to the increased expressions and activities of selenoproteins. Similarly, Se supplementation by SelP and selenomethionine prevented oxidative damage of human endothelial cells and bovine mammary epithelial cells by restoring the expression and enzymatic activity of GPX (Miranda et al. 2011). The protection of Se against H_2O_2-induced

doi: 10.17221/76/2017-CJAS

cell oxidative damage by the stimulation of TrxR expression and activity was also recorded in human artery endothelial cells (Miller et al. 2001).

The profound mechanism is not clear, although increased selenoprotein expression and activity are necessary for Se to protect cells from oxidative damage. ARA can be released from phospholipids by the activation of cPLA2 (Piotrowska-Tomala et al. 2012). The uncontrolled ARA release can induce intracellular ROS production by activating NADPH oxidase or inhibiting the activities of complexes I and III in mitochondrial respiratory chain (Schonfeld and Wojtczak 2007). The metabolism of ARA by LOX pathway can produce lipid hydroperoxides, such as 15-HPETE (Sordillo et al. 2008). The modification of ARA release and metabolism in response to selenoprotein activity in cultured cells has been reported previously. For example, TrxR reduced cPLA2 activity and ARA release in mouse fibrosarcoma cells (Kurosawa et al. 2009) and GPX inhibited effectively 5-LOX activity and reduced the oxidation of 5-HETE to 5-HPETE in human peripheral blood monocytes (Straif et al. 2000). In this study, we found that pretreatment of BMECs with Se markedly inhibited H_2O_2-induced increase of cPLA2 expression and activity and ARA release. Se pretreatment also blocked the H_2O_2-induced increase of 5-LOX expression and activity and 15-HPETE production. The results indicated that the protection of Se against oxidative damage induced by H_2O_2 in BMECs may be related to the decreased ARA release and its metabolism by LOX pathway.

The MAPK cascade is one of the important signaling pathways in oxidative stress responses and responds to various types of stress, such as ROS, tumor necrosis factor-α, and LPS (Korbecki et al. 2013). The release and metabolism of ARA can be modulated directly by MAPK pathway because cPLA2 is a downstream substrate of this pathway (Camps et al. 2000). MAPK family consists of three types of protein kinase: p38 MAPK, JNK, and ERK. According to the present results, Se pretreatment significantly inhibited H_2O_2-induced activation of p38 MAPK and JNK but not ERK in BMECs. Obviously, p38 MAPK and JNK are involved in inhibitory response of Se on H_2O_2-induced cell oxidative damage. Similar studies reported that Se decreased *Staphylococcus aureus*-induced activation of p38 MAPK and JNK in RAW264.7 macrophages (Bi et al. 2016) and LPS-induced activation of p38 MAPK, JNK, and ERK in mouse mammary epithelial cells (Zhang et al. 2014). Further additional molecular studies on gene transcription and protein expression level are required to show the roles of MAPK pathway on ARA release and its metabolism.

This study provided evidence that Se has a beneficial effect for BMECs on the antioxidant system through the modulation of ARA metabolism, the protection of Se against oxidative damage may be related to the up-regulation of selenoprotein TrxR activity. Some results indicated that TrxR reduced cPLA2 activity and ARA release in mouse fibrosarcoma cells (Kurosawa et al. 2009). However, the certain mechanism has not been well understood in BMECs. Further studies are warranted to elucidate the molecular mechanism underlying the modulation of TrxR on ARA metabolism in BMECs by inhibition or gene silencing of TrxR.

CONCLUSION

The profound mechanisms of Se function in the antioxidant system are not fully understood. Our study demonstrated that Se prevented H_2O_2-induced oxidative damage by increasing selenoproteins synthesis, inhibiting MAPK signaling pathway, and then decreasing ARA release and its metabolism by LOX pathway. However, these observations only illustrate an example of an *in vitro* experiment and further study *in vivo* is needed.

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doi: 10.17221/76/2017-CJAS

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Received: 2017–07–17
Accepted after corrections: 2017–12–12