doi: 10.17221/8851-CJAS

Effects of retinoic acid on the synthesis of selenoprotein and the antioxidative indices of bovine mammary epithelial cells *in vitro*

L. Jin, S. Yan, B. Shi, R. Sheng, H. Shi, Y. Zhao, J. Li

College of Animal Science, Inner Mongolia Agricultural University, Hohhot, P.R. China

ABSTRACT: The present study was conducted to examine the effects of retinoic acid (RA) on the synthesis of selenoprotein and the antioxidative indices of bovine mammary epithelial cells (BMEC) *in vitro* and to explore the antioxidative mechanisms of RA in the BMEC. The subconfluenced BMEC were divided into six treatments with six replicates per treatment and cultured in a Dulbecco's Modified Eagle's Medium/F12 media (10% fetal bovine serum, 5 μg/ml ovine prolactin, 10 ng/ml epidermal growth factor, 1 g/ml hydrocortisone, 0.5% insulin-transferrin-selenium) containing different levels of RA (0 (control), 0.05, 0.1, 0.2, 1 or 2 μg/ml) for 24 h. Addition of RA promoted the proliferation of BMEC, increased the activities of catalase, superoxide dismutase, total antioxidant capacity, glutathione peroxidase (GPX), thioredoxin reductase (TRXR), and the content of selenoprotein P (SELP) in a dose-dependent manner ($P < 0.05$). The optimal RA dose was 1 μg/ml. However, positive effect of RA tended to be suppressed when RA was increased to 2 μg/ml. The expressions of mRNA and protein of *GPX* in BMEC were up-regulated by RA in a quadratic dose-response relationship ($P < 0.01$), and the addition of 1 μg/ml RA showed the best effect. The mRNA expressions of *TRXR1* and *SELP* as well as the protein expression of TRXR1 were higher at 1–2 μg/ml RA. These results suggested that RA promoted antioxidant function of BMEC by regulating the synthesis of selenoprotein including GPX, TRXR, and SELP *in vitro*.

Keywords: vitamin A; antioxidant function; dairy cows

List of abbreviations: BMEC = bovine mammary epithelial cells, NO = nitric oxide, VA = vitamin A, GPX = glutathione peroxidase, TRXR = thioredoxin reductase, ROS = reactive oxygen species, MDA = malondialdehyde, SELP = selenoprotein P, RA = retinoic acid, ID = iodothyronine deiodinases, BW = body weight, DMEM = Dulbecco's Modified Eagle's Medium, DMSO = dimethyl sulfoxide, MTT = methyl thiazolyl tetrazolium, DTNB = dithio-*bis*-nitrobenzoic acid, GAPDH = glyceraldehyde phosphate dehydrogenase, SOD = superoxide dismutase, CAT = catalase, T-AOC = total antioxidant capacity, iNOS = inducible nitric oxide synthase, MAPK = mitogen-activated protein kinase, RT = reverse transcriptase, RT-PCR = real-time polymerase chain reaction, PBS = phosphate buffered solution

INTRODUCTION

The mammary gland is one of the most metabolically active organs in dairy cows. Under normal physiological conditions, the generated reactive oxygen species (ROS) are neutralized by the antioxidant system and the redox homeostasis is maintained in the body. Due to the high metabolic rate of the mammary epithelial cells during lactation, it can produce large amounts of ROS and lipid peroxides *in vivo*, especially in high-yielding dairy cows. If these excessive oxygen free radicals are

Supported by the National Natural Science Foundation (Project No. 31160466) and the National Key Basic Research Program of China (Project No. 2011CB100800).

The authors have read and approved the final manuscript, and proclaimed no conflicts of interest in this study.

doi: 10.17221/8851-CJAS

not cleared in time, oxidative stress occurs when there is an imbalance between the production of ROS and the host antioxidant capabilities (Basirico et al. 2015), and contributes to compromised immunity and increases incidence of diseases (Sordillo and Aitken 2009). Selenoprotein can scavenge hydrogen peroxide, lipid peroxide radicals and phospholipids, and protect tissues against oxidative damage (Bruzelius et al. 2008). The most extensively studied selenoproteins containing amino acid selenocysteine are the glutathione peroxidases (GPX), the thioredoxin reductases (TRXR), the iodothyronine deiodinases (ID), and selenoprotein P (SELP) (Arner and Holmgren 2000; Burk and Hill 2005; Kohrle 2005). In addition, it has been found that bovine mammary cell line MAC-T can express *GPX1* and *GPX4*, *TRXR1*, and *SELP* (Bruzelius et al. 2010). Therefore to regulate and improve the synthesis of selenoproteins in bovine mammary tissue is an important measure enhancing the antioxidant function, ensuring the health of dairy cows and improving milk quality.

It was found that all-*trans* retinoic acid (RA), a derivative of vitamin A (VA), could affect both mammary cell biology and selenoprotein mRNA expression, and had profound effects on the expression of many genes related to antioxidant functions (Balmer and Blomhoff 2002). Chu et al. (1999) found that the addition of RA increased the expression of *GPX2* mRNA in a human mammary cell line, and other *in vitro* studies have shown that RA increased the activity of iodothyronine deiodinases (ID1 and ID3) in cell systems (Ramauge et al. 1996; Menth et al. 2005). However, very little data is available to explore the effects of RA on the bovine mammary epithelial cells (BMEC). Bruzelius et al. (2010) observed that the addition of RA to BMEC culture resulted in an increase in *GPX1* and *TRXR1* mRNA expressions. Our previous study *in vivo* indicated that the dietary addition of VA in dairy cows could improve SELP concentration and the activities of GPX and TRXR in serum, and enhance antioxidant functions of dairy cows (Jin et al. 2014). However, the mechanism by which RA modulates the antioxidant parameters stays unclear.

In this study, we examined the effects of RA on selenoprotein activity, as well as the expressions of selenoprotein in BMEC *in vitro*, to explore the probable mechanism by which RA regulates antioxidative parameters.

MATERIAL AND METHODS

Preparation of reagents. Retinoic acid (Sigma-Aldrich, St. Louis, USA) was dissolved in dimethyl sulfoxide (DMSO), stored as aliquots of 1000 × stocks at –80°C and parceled in the dark. Immediately before use, the stock solution was diluted to the desired concentration with the Dulbecco's Modified Eagle's Medium/F12 (DMEM/F12) media (Gibco Laboratories, Grand Island, USA). The concentration of DMSO was always less than or equal to 0.5%, which was not toxic (Kurosawa et al. 2009).

Cell culture. The procedures for BMEC culture were according to a modified procedure outlined by Wellnitz and Kerr (2004). Briefly, bovine mammary tissues were collected at slaughterhouse from late lactating Holstein cows (parity = 3, age = 5 years, body weight (BW) = 650 ± 50 kg, milk yield = 20.75 ± 0.50 kg/day). The mammary tissues were minced into small pieces about 1 mm^3 in size and connective tissues were carefully removed. The mammary tissue fragments were digested with 2.5 ml II-type collagenase in a 5 ml centrifuge tube for 60 min at 37°C and gently shaken every 20 min. The digested cells were passed through a 150 μm filter and centrifuged at 800 *g* for 5 min. The supernatant was removed, the pellet resuspended with sterile phosphate buffered solution (PBS), and centrifuged at 800 *g* for 3 min. Cells obtained were cultured in a flask until confluence in DMEM/F12 containing 10% fetal bovine serum (Gibco Laboratories), 5 μg/ml ovine prolactin (Sigma-Aldrich), 10 ng/ml epidermal growth factor (Sigma-Aldrich), 1 mg/ml hydrocortisone (Sigma-Aldrich), 0.5% insulin-transferrin-selenium (Gibco Laboratories), 100 units/ml penicillin, 100 mg/ml streptomycin, and 0.25 mg/ml amphotericin B for 48 h before receiving serum-free medium. The normally growing BMEC showed an oval morphology (Figure 1).

Experimental design. The experiment was conducted to investigate the effects of RA on selenoprotein activity, as well as selenoprotein mRNA expression in BMEC. Serum-free medium was added for 24 h followed by incubation in 0 (control), 0.05, 0.1, 0.2, 1 or 2 mg/ml RA in serum-free medium. Each treatment was made in six duplicates. All cells were harvested after 1 day of treatment.

Cell proliferation assay. Cells were seeded in 96-well culture plates (1×10^4 cells/well) and the cell

doi: 10.17221/8851-CJAS

viability was measured using the methyl thiazolyl tetrazolium (MTT) assay. Briefly, at the indicated time points after the treatment as indicated above, 20 ml of MTT (Sigma-Aldrich) solution (5 mg/ml in 1 × PBS) was added to culture medium at 37°C for another 4 h. Then, 200 ml of medium was aspirated from each well, and 100 ml of DMSO was added to dissolve the formazan crystals. The absorbance of samples was read at 490 nm using a microplate reader (BioTek, Winooski, USA).

Preparation of cell lysates. The culture supernatant was collected for the analysis of SELP concentration and the activities of superoxide dismutase (SOD), catalase (CAT), and of the total antioxidant capacity (T-AOC). Cells were scraped from the plates into ice cold lysis buffer (Tiandz, Beijing, China) followed by centrifugation (1200 *g*, 10 min, 4°C) in order to eliminate cell debris. Lysates were then frozen at –20°C until analysis of the GPX, TRXR activities, and ROS and malondialdehyde (MDA) concentrations.

Measurement of enzyme activity. The activities of GPX, SOD, CAT, T-AOC, and the concentration of MDA were measured using commercial colourimetric assay kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) according to the manufacturer's instructions (Wang et al. 2010). The assay of TRXR activity was based on a method in which the reduction of dithio-*bis*-nitrobenzoic acid (DTNB) was monitored at 412 nm (Hill et al. 1997). The concentration of SELP was measured by radioimmunoassay as described earlier (Hill et al. 1996). The ROS concentration was estimated by the FLx800 fluorescence analyzer (BioTek) using chemical fluorometric enzyme immunoassay described by Kim et al. (2004). Values of GPX, TRXR, and MDA were expressed as IU per mg of protein. Protein concentration was measured with the bicinchoninic acid (BCA) Protein Assay Kit (Pierce, Rockford, USA) and BSA as standards (Miranda et al. 2009).

RNA isolation and reverse transcriptase (RT). Total cellular RNA was extracted using Trizol solution (TaKaRa, Inc., Dalian, China) according to the manufacturer's instructions. Then cDNA was synthesized in a 10 µl reaction system containing 2 µl of 5 × PrimeScript Buffer, 0.5 µl of Prime Script RT Enzyme Mix I, 0.5 µl of Oligo dT Primer (50µM), 0.5 µl of Random 6 mers (100µM), and 6.5 µl of RNA reverse transcriptase (TaKaRa, Inc.) at 37°C for 15 min, then at 85°C for 5 s. The RT products (cDNA) were stored at –20°C for real-time PCR assay.

Real-time polymerase chain reaction (RT-PCR) analysis. All primers (Table S1) were designed using Oligo software (National Biosciences, Plymouth, USA) and custom-synthesized (Sangon Biological Technologies, Shanghai, China). RT-PCR analysis was performed using a Bio-Rad iCycler IQ5 detector system (Bio-Rad Laboratories Inc., Hercules, USA).

Relative levels of specific gene mRNA were quantified using SYBR® Prime Script™ RT-PCR Kit (TaKaRa, Inc.) following manufacturer's instructions. In brief, the PCR reaction system (20 µl) included 10 µl of 2× SYBR® Premix Ex Taq™ and 0.4 µl (10µM) of forward and reverse specific primers, 2 µl of cDNA template, and 7.2 µl of RNA-free H_2O. The reaction mixture was then heated at 95°C for 30 s and subjected to 40 cycles (95°C for 5 s, 60°C for 30 s, and 72°C for 30 s) of PCR. Detection of the fluorescent product was carried out at the end of the melting curve program (70 to 95°C with a heating rate of 0.5°C/s and a continuous fluorescence measurement). The mRNA expression of a target gene was normalized to that of glyceraldehyde phosphate dehydrogenase (GAPDH). Relative expression levels of selenoprotein mRNA were calculated by the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen 2001). The PCR products were analyzed by electrophoresis on 2% agarose gels and stained by nucleic acid dye.

Western blotting. BMEC from experimental condition were collected and homogenized in lysis buffer (Beyotime, Beijing, China). Cell lysates containing 35 µg of protein were boiled in a quarter volume of 5× sample loading buffer (Beyotime) at 100°C for 5 min and separated on 12% SDS-PAGE. Proteins were electrotransferred (Mini Trans-Blot Cell; Bio-Rad Laboratories Inc.) to a polyvinylidene fluoride membrane (Merck Millipore, Billerica, USA) in Tris-glycine-methanol buffer at 100 V for 50 min. Then membranes were blocked with blocking buffer for 1 h. Membranes were then incubated with polyclonal rabbit anti-GPx1 (22 kDa, 1 µg/ml) (Abcam, Cambrige, USA), anti-TrxR1 (55 kDa, 1 : 500) (Sigma-Aldrich), or anti-GAPDH (36 kDa, 1 : 2000) (Proteintech, Chicago, USA) overnight at 4°C. After washing 3 times with PBS/0.1% Tween 20 (PBS-T), membranes were incubated with goat anti-rabbit IgG (1 : 1000) (KPL Laboratories, Gaithersburg, USA) labelled with horseradish peroxidase for 1 h at room temperature. After washing 3 times with PBS-T, proteins were developed by

doi: 10.17221/8851-CJAS

autoradiography using an enhanced chemiluminescence system (Beyotime). Finally, images from radiographic film were scanned and the integrated density was determined by the software Quantity One (Bio-Rad). Relative density was quantified by normalization of the integrated density of each blot to that of the corresponding GAPDH.

Statistical analysis. Statistical analysis was performed using the General Linear Model procedure of SAS software (Statistical Analysis System, Version 9.0, 2003) and used to detect the statistical significance of the treatment groups in regression relation. Regression analysis was conducted to evaluate linear and quadratic effects of RA on the various response criteria. A level of $P < 0.05$ was regarded as significant, while a difference of $0.05 < P < 0.10$ was discussed as a statistical trend.

RESULTS

Effects of RA on cell proliferation. As shown in Table 1, the cell proliferation increased quadratically ($P < 0.0001$) with increasing addition of RA. Cell proliferation reached maximum value when adding 1 μg of RA/ml, and tended to decrease after the addition of RA in the culture medium increased to 2 μg/ml.

Effects of RA on antioxidant parameters. *TRXR* and T-AOC activities improved linearly ($P = 0.033$ and $P = 0.014$, respectively), and the treatments with the addition of 1 and 2 μg of RA/ml had higher TRXR and T-AOC activities compared with control or other treatments, especially the addition of 2 mg of RA/ml had the best effect (Table 2). GPX and CAT activities were enhanced quadratically ($P = 0.020$ and $P = 0.011$, respectively) with increasing addition of RA from 0.05 to 2 mg/ml and the addition of 1–2 mg of RA/ml in culture medium resulted in higher activity, but 1 mg of RA/ml reached the maximum. SOD activity had a linear increase tendency with the increasing addition of RA vs the control ($P = 0.083$). MDA or ROS content was significantly decreased quadratically ($P = 0.047$) or tended to decrease quadratically ($P = 0.085$) with the increasing contents of RA relative to the control.

Effects of RA on selenoproteins mRNA expression. Compared with the control, addition of RA to

Table 1. Effect of RA on BMEC proliferation

Items	Level of RA (mg/ml)						SEM	*P*-value	
	0	0.05	0.1	0.2	1	2		linear	quadratic
Cell proliferation	0.566	0.571	0.592	0.597	0.759	0.604	0.017	0.0184	< .0001

RA = retinoic acid, BMEC= bovine mammary epithelial cells, SEM = standard error of the mean
the number of observations for each mean value was six ($n = 6$)

Table 2. Effect of RA on the antioxidant parameters of BMEC

Items	Level of RA (mg/ml)						SEM	*P*-value	
	0	0.05	0.1	0.2	1	2		linear	quadratic
CAT (IU/ml)	1.13	1.52	1.61	1.57	2.03	1.26	0.17	0.854	0.011
SOD (IU/ml)	19.43	21.6	20.14	21.80	22.86	22.61	2.23	0.083	0.229
T-AOC (IU/ml)	1.44	2.17	2.24	2.49	2.61	3.21	0.43	0.014	0.045
GPX (IU/mg protein)	147	180	188	261	280	237	28	0.103	0.020
TRXR (U/g protein)	2.89	2.92	3.03	3.00	3.39	3.58	0.25	0.033	0.097
SELP (mg/l)	0.19	0.22	0.28	0.25	0.30	0.32	0.06	0.175	0.345
MDA (nmol/mg protein)	3.50	2.50	2.52	3.11	2.06	2.58	0.26	0.140	0.047
ROS (fluorescence intensity/ml)	179	177	158	157	156	159	6	0.139	0.085

RA = retinoic acid, BMEC= bovine mammary epithelial cells, CAT = catalase, SOD = superoxide dismutase, T-AOC = total antioxidant capacity, GPX = glutathione peroxidase, TRXR = thioredoxin reductase, SELP = selenoprotein P, MDA = malondialdehyde, ROS = reactive oxygen species, SEM = standard error of the mean
the number of observations for each mean value was six ($n = 6$)

doi: 10.17221/8851-CJAS

Table 3. Effect of RA on the gene expression of selenoproteins ($2^{-\Delta\Delta Ct}$) in BMEC

Items	Level of RA (mg/ml)						SEM	*P*-value	
	0	0.05	0.1	0.2	1	2		linear	quadratic
GPX1	1.014	1.769	1.903	2.151	2.678	2.175	0.306	0.022	0.0004
GPX4	1.045	1.019	1.080	0.953	1.142	0.823	0.106	0.194	0.123
TRXR1	1.022	1.029	1.105	1.388	1.805	3.221	0.359	< 0.0001	< 0.0001
SELP	1.006	0.956	1.264	1.185	1.276	1.438	0.143	0.020	0.063

RA = retinoic acid, BMEC= bovine mammary epithelial cells, *GPX1* = glutathione peroxidase 1, *GPX4* = glutathione peroxidase 4, *TRXR1* = thioredoxin reductase 1, *SELP* = selenoprotein P, SEM = standard error of the mean
the number of observations for each mean value was six (n = 6)

Table 4. Effect of RA on the protein expression of selenoproteins in BMEC

Items	Level of RA (mg/ml)						SEM	*P*-value	
	0	0.05	0.1	0.2	1	2		linear	quadratic
GPX1	1.028	1.474	2.138	2.469	3.642	2.660	0.114	0.0002	< 0.0001
TRXR1	1.013	1.386	1.534	2.299	2.983	3.108	0.092	< 0.0001	< 0.0001

RA = retinoic acid, BMEC= bovine mammary epithelial cells, GPX1 = glutathione peroxidase 1, TRXR1 = thioredoxin reductase 1, SEM = standard error of the mean
the number of observations for each mean value was six (n = 6)

the culture medium up-regulated *GPX1*, *TRXR1*, and *SELP* mRNA expressions without affecting the *GPX4* mRNA expression (Table 3). With RA increasing from 0.05 to 2 µg/ml, the *GPX1* mRNA expression was significantly enhanced in a quadratic dose-dependent manner (P = 0.0004), and the addition of RA (0.2–2 µg/ml) caused greater *GPX1* mRNA expression than the control and other treatments, especially the addition of 1 µg of RA/ml was more effective, but tended to decrease with the addition of RA at 2 µg/ml. Addition of RA in the culture medium up-regulated *TRXR1* and *SELP* mRNA expressions of BMEC in a linear dose-response relationship (P < .0001 and P = 0.020, respectively), and the treatments with addition of 1–2 µg of RA/ml had higher *TRXR1* and *SELP* mRNA expressions compared with control or other treatments, and the addition of 2 µg of RA/ml was more effective.

Effects of RA on selenoproteins expression. As shown in Table 4 and Figure 2, with increasing RA, the GPX1 expression was enhanced quadrati-

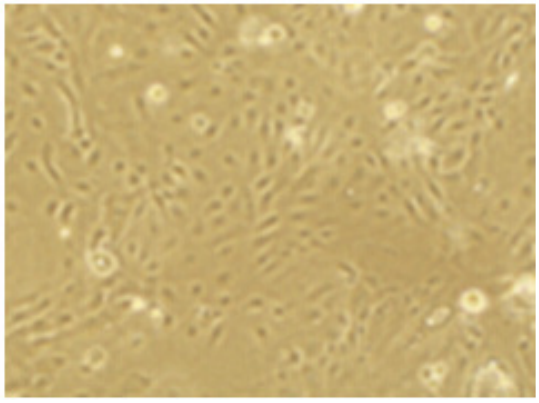

Figure 1. Normal growth of bovine mammary epithelial cells

cells were cultured in Dulbecco's Modified Eagle's Medium/F12 media and showed an oval morphology

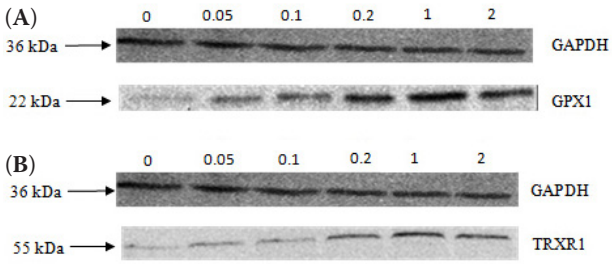

Figure 2. Effect of retinoic acid on glutathione peroxidase 1 (GPX1) expression (**A**) and thioredoxin reductase 1 (TRXR1) expression (**B**) in bovine mammary epithelial cells (BMEC)

BMEC were treated with 0 (control), 0.05, 0.1, 0.2, 1 or 2 µg/ml retinoic acid for 24 h. Expressions of GPX1 and TRXR1 were detected by Western blotting
GAPDH = glyceraldehyde phosphate dehydrogenase

doi: 10.17221/8851-CJAS

cally ($P < .0001$). However, the positive effects of RA tended to be suppressed when the additional dose of RA was increased to 2 μg/kg. RA also increased the expression of TRXR1 in a linear dose-effect relationship ($P < .0001$), and 1–2 μg/kg RA treatments had higher TRXR1 expression.

DISCUSSION

The considerable increase in oxygen requirements during heightened metabolic demands results in augmented rates of ROS production which can induce the process of oxidative stress. Many enzymes, such as CAT, SOD, and T-AOC and selenoproteins of GPX, TRXR, and SELP, act as an important part to control oxidative stress, which protects the cells from oxidative stress-induced damage (Bruzelius et al. 2007; Sordillo et al. 2007). The antioxidant enzymes SOD and CAT play a crucial role as protective enzymes against radicals in tissues and are comprised in the cellular antioxidant defense system (Lima et al. 2015). Although the body has its own enzyme systems that scavenge free radicals, the principle micronutrient antioxidants are vitamins. Some *in vivo* animal trials indicated that VA supplementation could improve the antioxidant functions. Palacios et al. (1996) confirmed that VA could effectively remove free radicals in mouse liver mitochondria and played an important antioxidant function. Jin et al. (2014) showed that addition of 220 IU of VA/kg BW could improve SELP concentration and the activities of GPX and TRXR in serum, and enhance antioxidant functions in dairy cows. Ma et al. (2005) showed that supplementation with 3300–4400 IU of VA/kg in beef cattle diets could significantly improve the serum of activities of SOD and T-AOC. Zhao et al. (2008) reported that supplementation with VA (250 000 IU/day) could slightly increase the activity of SOD and reduce MDA concentration in serum of dairy cows. Qiao (2008) reported that the supplementation of VA (165 IU/kg BW) significantly increased the activity of GPX in serum of dairy cows *in vivo*. However, there are just few studies on the effects of RA on the antioxidant indices of BMEC. Our present study showed that the activities of CAT, SOD, T-AOC were enhanced in a dose-dependent manner with increasing concentration of RA, and reached a maximum level at 1 μg/ml, suggesting that RA could improve antioxidant function of BMEC *in vitro*.

GPX1 is a predominant intracellular form of GPX and is the most extensively studied selenoprotein in dairy cattle (Smith et al. 1997; De Marchi et al. 2015). It converts ROS to less reactive metabolites and thus protects tissues against oxidative damage (Papp et al. 2007). Similar to GPX1, TRXRl is present in the cytosol and is capable of the direct reduction of lipid hydroperoxides and hydrogen peroxide (Papp et al. 2007). SELP is an extracellular glycoprotein that functions both in selenium distribution and has antioxidant activity (Rock and Moos 2010). Bruzelius et al. (2008) indicated that RA affected the pattern of selenoproteins formation in MAC-T cells using an isotope-proteomic approach. The current study indicated that the 0.05–2 mg/ml dose of VA supplementation significantly increased the activities of GPX and TRXR, and decreased the content of MDA in BMEC in a linear or quadratic dose-dependent relationship *in vitro*, which was consistent with several previous studies. All these results suggested that addition of RA could accelerate the activities of selenoprotein enzyme and increase antioxidant function *in vitro*. In addition, the results also indicated that the gene expression of *SELP* was linearly increased with increasing RA, but the SELP concentration was not affected. The probable reason is that RA regulated SELP at the transcription level and not at the translation level, but little data is available on it.

However, the mechanisms by which RA increased the activities of selenoproteins and then improved the antioxidant functions are unclear. Chu et al. (1999) found that 1 μmol/l RA (about 0.3 μg/ml) treatment increased *GPX2* mRNA level 3–11-fold and resulted in a 4-fold increase of GPX activity in human breast cancer cells. Bruzelius et al. (2010) also showed that addition of RA (1 μmol/l) increased *GPX* and *TRXR* mRNA levels in MAC-T cells. In the present experiment, we discussed the regulatory effect of RA on the expression of selenoprotein and its mRNA in BMEC *in vitro*, and the results indicated that *GPX1*, *TRXR1*, and *SELP* mRNA expression as well as the expression of GPX1 and TRXR1 in BMEC were up-regulated at 0.05–2 μg/ml in a linear or quadratic dose-response manner, and that the addition of 1 μg/ml RA was more effective. All of these results suggest that RA was able to improve selenoprotein synthesis and conferred significant antioxidant effects. Taking enzyme activities into consideration, we could infer that the promotion of RA regarding antioxidant

doi: 10.17221/8851-CJAS

capacity in BMEC *in vitro* was associated with the elevating expression of selenoproteins and its mRNA which further led to the increased GPX and TRXR activities.

The mechanism by which RA increased the antioxidative capacity via promoting TRXR synthesis and improving enzyme activity is still unclear. Nitric oxide (NO) is a short-lived signalling molecule that plays an immune regulatory action in the mammalian immune system, which is generated in various tissues from the amino acid L-arginine by inducible nitric oxide synthase (iNOS) (David et al. 2012). NO production is a double edged sword, beneficial as a messenger or modulator for immunologic self-defense, but potentially toxic (Schmidt and Walter 1994). A large number of NO could result in oxidative injury. Tobiume et al. (2002) reported that the activity of TRXR may be involved in signalling pathway of mitogen-activated protein kinase (MAPK). MAPK can be divided into three major subfamilies: extracellular signal-regulated kinases (ERK), c-Jun N-terminal kinases (JNK), and p38 MAPK (Liu et al. 2015). The activation of p38 MAPK resulted in the stimulation of nuclear factor-kappaB (NF-κB)-specific DNA-protein binding and the subsequent expression of iNOS and NO release in murine macrophage cells (Chen and Wang 1999). TRXR could probably inhibit the phosphorylation of p38 MAPK in human pulmonary vascular endothelial cells (Machino et al. 2003) and might have important preventative capacities towards NO-mediated cellular injury in human monocytic macrophage cells (Ferret et al. 2000). Therefore, we speculate the promotion effects of RA on the antioxidant capacity of BMEC are probably associated with the elevated TRXR activity inhibiting the phosphorylation of p38 MAPK and hence decreasing the production of NO. However, little relevant information is avaliable in BMEC, and further investigation is required to examine the exact mechanism.

CONCLUSION

The activity of selenoprotein in BMEC was stimulated in a dose-dependent manner with an incremental addition of RA in culture medium, and the addition of 1 μg of RA/ml had the best effect. Our data also provided a plausible mechanism whereby RA might improve the antioxidative function of BMEC by modulating the mRNA and protein expressions of selenoproteins *in vitro*. However, further investigation is required to examine the exact mechanism by which RA regulates selenoprotein synthesis and modulates the antioxidative function.

Acknowledgement. The authors gratefully thank Sheng Ran, Li Junliang, and Shi Huiyu for their assistance in cell cultures.

REFERENCES

Arner E.S.J., Holmgren A. (2000): Physiological functions of thioredoxin and thioredoxin reductase. European Journal of Biochemistry, 267, 6102–6109.

Balmer J.E., Blomhoff R. (2002): Gene expression regulation by retinoic acid. Journal of Lipid Research, 43, 1773–1808.

Basirico L., Morera P., Dipasquale D., Troscher A., Serra A., Mele M., Bernabucci U. (2015): Conjugated linoleic acid isomers strongly improve the redox status of bovine mammary epithelial cells (BME-UV1). Journal of Dairy Science, 98, 7071–7082.

Bruzelius K., Hoac T., Sundler R., Onning G., Akesson B. (2007): Occurrence of selenoprotein enzyme activities and mRNA in bovine mammary tissue. Journal of Dairy Science, 90, 918–927.

Bruzelius K., Purup S., James P., Onning G., Akesson B. (2008): Biosynthesis of selenoproteins in cultured bovine mammary cells. Journal of Trace Elements in Medicine and Biology, 22, 224–233.

Bruzelius K., Sundler R., Pagmantidis V., Akesson B. (2010): Regulation of selenoprotein mRNA expression by hormones and retinoic acid in bovine mammary cells. Journal of Trace Elements in Medicine and Biology, 24, 251–256.

Burk R.F., Hill K.E. (2005): Selenoprotein P: an extracellular protein with unique physical characteristics and a role in selenium homeostasis. Annual Review of Nutrition, 25, 215–235.

Chen C.C., Wang J.K. (1999): p38 but not p44/42 mitogen-activated protein kinase is required for nitric oxide synthase induction mediated by lipopolysaccharide in RAW 264.7 macrophages. Molecular Pharmacology, 55, 481–488.

Chu F.F., Esworthy R.S., Lee L., Wilczynski S. (1999): Retinoic acid induces GPX2 gene expression in MCF-7 human breast cancer cells. The Journal of Nutrition, 129, 1846–1854.

David O.S., Jason S.C., Joshua D.N., Adam J.F. (2012): The potential of nitric oxide releasing therapies as antimicrobial agents. Landes Bioscience, 33, 271–279.

De Marchi F.E., Palin M.F., dos Santos G.T., Lima L.S., Benchaar C., Petit H.V. (2015): Flax meal supplementation on the activity of antioxidant enzymes and the expression of oxidative stress- and lipogenic-related genes in dairy

doi: 10.17221/8851-CJAS

cows infused with sunflower oil in the abomasum. Animal Feed Science and Technology, 199, 41–50.

Ferret P.J., Soum E., Negre O., Wollman E.E., Fradelizi D. (2000): Protective effect of thioredoxin upon NO-mediated cell injury in THP1 monocytic human cells. Biochemical Journal, 346, 759–765.

Hill K.E., Xia Y., Akesson B., Boeglin M.E., Burk R.F. (1996): Selenoprotein P concentration in plasma is an index of selenium status in selenium-deficient and selenium-supplemented Chinese subjects. The Journal of Nutrition, 126, 138–145.

Hill K.E., McCollum G.M., Burk R.F. (1997): Determination of thioredoxin reductase activity in rat liver supernatant. Analytical Biochemistry, 253, 123–125.

Jin L., Yan S.M., Shi B.L., Bao H.Y., Gong J., Guo X.Y., Li J.L. (2014): Effect of vitamin A on milk performance, antioxidant and immune function of dairy cows. Animal Feed Science and Technology, 192, 15–23.

Kim S.H., Johnson V.J., Sharma R.P. (2004): Selenium attenuates lipopolysaccharide-induced oxidative stress responses through modulation of p38 MAPK and NF-kappaB signaling pathways. Experimental Biology and Medicine, 229, 203–213.

Kohrle J. (2005): Selenium and the control of thyroid hormone metabolism. Thyroid, 15, 841–853.

Kurosawa T., Nakamura H., Yamaura E., Fujino H., Matsuzawa Y., Kawashima T., Murayama T. (2009): Cytotoxicity induced by inhibition of thioredoxin reductases via multiple signaling pathways: role of cytosolic phospholipase $A_{2\alpha}$-dependent and -independent release of arachidonic acid. Journal of Cellular Physiology, 219, 606–616.

Lima L.S., Palin M.F., Santos G.T., Benchaar C., Petit H.V. (2015): Effects of supplementation of flax meal and flax oil on mammary gene expression and activity of antioxidant enzymes in mammary tissue, plasma and erythrocytes of dairy cows. Livestock Science, 176, 196–204.

Liu L., Lin Y., Liu L., Bian Y., Zhang L., Gao X., Li Q. (2015): 14-3-3γ regulates lipopolysaccharide-induced inflammatory responses and lactation in dairy cow mammary epithelial cells by inhibiting NF-κB and MAPKs and up-regulating mTOR signaling. International Journal of Molecular Sciences, 16, 16622–16641.

Livak K.J., Schmittgen T.D. (2001): Analysis of relative gene expression data using real time quantitative PCR and the $2^{-\Delta\Delta Ct}$ method. Methods, 25, 402–408.

Ma X.M., Yang Z.B., Yang W.R., Song M.L. (2005): Effect of different vitamin A levels in diets on antioxidant ability of beef cattle. Chinese Journal of Animal Nutrition, 17, 31–35. (in Chinese)

Machino T., Hashimoto S., Maruoka S., Gon Y., Hayashi S., Mizumura K., Nishitoh H., Ichijo H., Horie T. (2003): Apoptosis signal-regulating kinase 1-mediated signaling pathway regulates hydrogen peroxide-induced apoptosis in human pulmonary vascular endothelial cells. Critical Care Medicine, 31, 2776–2781.

Menth M., Schmutzler C., Mentrup B., Hoang-Vu C., Takahashi K., Honjoh T., Kohrle J. (2005): Selenoprotein expression in Hürthle cell carcinomas and in the human Hürthle cell carcinoma line XTC. UC1. Thyroid, 15, 405–416.

Miranda R., Blanco A., Negro C. (2009): Accumulation of dissolved and colloidal material in papermaking – Application to simulation. Chemical Engineering Journal, 148, 385–393.

Palacios A., Piergiacomi V., Catala A. (1996): Vitamin A supplementation inhibits chemiluminescence and lipid peroxidation in isolated rat liver microsomes and mitochondria. Molecular and Cellular Biology, 154, 77–82.

Papp L.V., Lu J., Holmgren A., Khanna K.K. (2007): From selenium to selenoproteins: synthesis, identity, and their role in human health. Antioxidants and Redox Signalling, 9, 775–806.

Qiao L. (2008): Study on the rule of degradation of vitamin A in rumen and evaluation of effects of ruminal protected vitamin A on dairy cows. Ph.D. Thesis. Hohhot, China: Inner Mongolia Agricultural University. (in Chinese)

Ramauge M., Pallud S., Esfandiari A., Gavaret J., Lennon A., Pierre M., Courtin F. (1996): Evidence that type III iodothyronine deiodinase in rat astrocyte is a selenoprotein. Endocrinology, 137, 3021–3025.

Rock C., Moos P.J. (2010): Selenoprotein P protects cells from lipid hydroperoxides generated by 15-LOX-1. Prostaglandins, Leukotrienes and Essential Fatty Acids, 83, 203–210.

Schmidt H.H.H.W., Walter U. (1994): NO at work. Cell, 78, 919–925.

Smith K.L., Hogan J.S., Weiss W.P. (1997): Dietary vitamin E and selenium affect mastitis and milk quality. Journal of Animal Science, 75, 1659–1665.

Sordillo L.M., Aitken S.L. (2009): Impact of oxidative stress on the health and immune function of dairy cattle. Veterinary Immunology and Immunopathology, 128, 104–109.

Sordillo L.M., Boyle N.O., Gandy J.C., Corl C.M., Hamilton E. (2007): Shifts in thioredoxin reductase activity and oxidant status in mononuclear cells obtained from transition dairy cattle. Journal of Dairy Science, 90, 1186–1192.

Tobiume K., Saitoh M., Ichijo H. (2002): Activation of apoptosis signal-regulating kinase 1 by the stress-induced activating phosphorylation of preformed oligomer. Journal of Cellular Physiology, 191, 95–104.

Wang Y.K., Hong Y.G., Wei M., Wu Y., Huang Z.Q., Chen R.Z., Chen H.Z. (2010): Curculigoside attenuates human

doi: 10.17221/8851-CJAS

umbilical vein endothelial cell injury induced by H_2O_2. Journal of Ethnopharmacology, 132, 233–239.

Wellnitz O., Kerr D.E. (2004): Cryopreserved bovine mammary cells to model epithelial response to infection. Veterinary Immunology and Immunopathology, 101, 191–202.

Zhao G.Q., Guo Y.H., Sun L.S. (2008): Study on vitamin A optimum requirements of lactating Holsteins. Chinese Dairy Cattle, 9, 7–10. (in Chinese)

Received: 2015–01–21
Accepted after corrections: 2015–11–30

Corresponding Author

Prof. Sumei Yan, Inner Mongolia Agricultural University, College of Animal Science, No. 306 Zhaowuda Road, Hohhot, 010018, P.R. China
Phone: +86 4714 303 875, e-mail: yansmimau@163.com