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Vitamin A affects the expression of antioxidant genes in bovine mammary epithelial cells with oxidative stress induced by diethylene triamine-nitric oxide polymer

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ABSTRACT: The considerable increase in oxygen requirements due to the high metabolic rate of the bovine mammary epithelial cells (BMEC) during lactation results in an augmented production of reactive nitrogen species (RNS), such as nitric oxide (NO), which may expose cows to increased oxidative stress. Vitamin A (VA) has been shown in several studies to enhance the antioxidant defence system against oxidative stress, but whether the reason is related to a reduced NO production remains unclear. Diethylene triamine-nitric oxide polymer (NOp) is a type of NO-generating compound, which is safe, efficacious, and releases NO over a long period. The current study was conducted to investigate the effect of VA on the antioxidant function in BMEC and the underlying mechanism by discussing the protection of VA on NO-induced oxidative stress of BMEC. The experiment was conducted using a single-factor completely randomized arrangement. Primary BMEC were isolated from the mammary glands of Holstein dairy cows. The third generation cells were randomly divided into four equal groups with six replicates each. Each group received different combinations of VA and NOp treatment as follows: controls (without VA and NOp), NOp treatment alone, VA treatment alone, and VA and NOp treatment together. The lysates were collected to evaluate the activities of glutathione peroxidase (GPx) and thioredoxin reductase (TrxR) and the contents of reactive oxygen species (ROS) and malondialdehyde (MDA), and the cell-free supernatants were collected to analyze selenoprotein P (SelP) content, inducible nitric oxide synthase (iNOS) activities and nitric oxide (NO), interleukin-1 (IL-1), interleukin-6 (IL-6), tumour necrosis factor-α (TNF-α) contents. The results suggested that compared to the control, the cell proliferation, the activity of the antioxidants GPx and TrxR, the content of SelP and the antioxidant gene expressions of *GPx1*, *GPx4*, and *TrxR1* were significantly decreased ($P < 0.05$), and the contents of ROS and MDA, the activity of iNOS, the contents of NO and IL-1, IL-6, TNF-α, and their mRNA expressions were increased dramatically in the NOp treatment alone group ($P < 0.05$), but the opposite changes were observed in the VA treatment alone group. Compared to the NOp treatment alone, the VA and NOp treatment together significantly improved cell proliferation, the activities of the antioxidants GPx and TrxR, and the gene expressions of *GPx1* and *TrxR1*, and dramatically decreased the contents of ROS and MDA, the activity of iNOS, the contents of NO and IL-1, IL-6, TNF-α and their mRNA expression levels ($P < 0.05$). The present research suggests that VA can improve the antioxidant function of BMEC and protect the cells from experiencing the NOp-induced oxidative stress by regulating antioxidant gene expression. The probable mechanism is that VA can reduce the activity of iNOS and its mRNA expression by down-regulating of the expression of *IL-1*, *IL-6*, and *TNF-α* to reduce NO production. However, the exact mechanism warrants future exploration.

Keywords: nitric oxide; antioxidant enzymes; inflammatory factors

List of abbreviations: BMEC = bovine mammary epithelial cells, RNS = reactive nitrogen species, NO = nitric oxide, VA = vitamin A, NOp = diethylene triamine-nitric oxide polymer, GPx = glutathione peroxidase, TrxR = thioredoxin reductase, ROS = reactive oxygen species, MDA = malondialdehyde, SelP = selenoprotein P, iNOS = nitric oxide synthase, IL-1 = interleukin-1, IL-6 = interleukin-6, TNF-α = tumour necrosis

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factor-α, RA = retinoic acid, LPS = lipopolysaccharide, BW = body weight, DMEM = Dulbecco's Modified Eagle's Medium, FBS = foetal bovine serum, DMSO = dimethyl sulfoxide, MTT = methyl thiazolyl tetrazolium, RGR = relative growth rate, DTNB = dithio-*bis*-nitrobenzoic acid, GAPDH = glyceraldehyde phosphate dehydrogenase, SOD = superoxide dismutase, CAT = catalase, NOS = nitric oxide synthase, eNOS = endothelial nitric oxide synthase, nNOS = neuronal nitric oxide synthase, iNOS = inducible nitric oxide synthase, MAPK = mitogen-activated protein kinase, NF-κB = nuclear factor-kappa B

INTRODUCTION

Dairy cattle experience increased oxidative stress due to the high metabolic rate of the bovine mammary epithelial cells (BMEC) during lactation, which leads to decreased immune function, milk production and quality, and an increased loss of economic revenue (Sordillo and Aitken 2009). Oxidative stress may be defined as an imbalance between pro-oxidant and antioxidant forces resulting in overall pro-oxidant damage (Gitto et al. 2002). The considerable increase in oxygen requirements during times of increased metabolic demands results in the augmented production of reactive oxygen species (ROS) (Sordillo et al. 2009). Normally, a wide range of antioxidant systems working in concert protects the body. Deficiencies of the natural protective substances or an excess exposure to stimulators of ROS production may result in oxidative stress, which occurs when the levels of pro-oxidants exceed the detoxifying capacity of antioxidants (Miller et al. 1993). Therefore, reducing the incidence of oxidative stress is an important factor for improving the immune function of cows and increasing their milk production and quality.

Nitric oxide (NO) is a gas molecule with bioactivity and it is the smallest signalling molecule known. It is synthesized from L-arginine by inducible nitric oxide synthase (iNOS) (MacMicking et al. 1997). However, NO production is a double-edged sword, being beneficial as a messenger or modulator and for immunologic self-defence, but potentially toxic (Schmidt and Walter 1994; Kielbik et al. 2013). High concentrations of NO can damage proteins, lipids membranes, and DNA (Albina and Reichner 1998). Oxidative stress caused by excess NO following the generation of active nitrogen blocks signalling pathways and leads to uncontrollable systemic inflammation (Tsi et al. 2002). Therefore, a complicated system is required to regulate NO production and maintain homeostasis.

Several studies have reported that the production of NO *in vivo* can be inhibited by retinoic acid (RA, a derivative of vitamin A) through down-regulation of the gene expression of interleukin-2 (*IL-2*) in murine T cells (Ertesvag et al. 2009). Hung et al. (2008) noted that RA inhibits the production of iNOS, cyclooxygenase, and cytokines in human chondrocytes induced by interleukin-1 (IL-1), which suppresses the overproduction of NO. Dheen et al. (2005) found that RA suppresses the lipopolysaccharide (LPS)-induced mRNA expression of *iNOS* and tumour necrosis factor-α (*TNF-α*) in dose-dependent manner (0.1–10μM) in rat microglia. These results strongly suggest that RA functions effectively regulate the synthesis of NO and prevent inflammation and oxidative stress. While considerable research has investigated RA activities in humans and other mammals, there is notably little knowledge of the mechanisms by which RA protects BMEC against oxidative injury and affects the oxidative functions of BMEC. Our previous results indicated that a high dose vitamin A (VA) (220 IU/kg body weight (BW)), above the current NRC-recommended level, into the diet improves the antioxidant and immune functions of dairy cows (Jin et al. 2014), but the mechanism of action is unclear. The purpose of the research was to examine the effects of the addition of VA on the antioxidant function of BMEC and to investigate the protective effect of VA on oxidative stress induced by diethylene triamine-nitric oxide polymer (NOp) in BMEC through the regulation of antioxidant genes expression and to elucidate the underlying mechanism of how VA improves the antioxidant function of BMEC.

MATERIAL AND METHODS

The experimental protocol was approved by the Animal Care and Use Committee at the Inner Mongolia Agricultural University, Hohhot, China.

Cell culture and treatments. Primary cells were isolated from the mammary glands of mid-lactation

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Holstein dairy cows at a local abattoir according to a modified procedure described by Wellnitz and Kerr (2004) and Qi et al. (2014). Briefly, after removing several approximately 1 cm^3 pieces of mammary gland tissues aseptically, the tissues were washed with cold phosphate buffered solution (PBS) (HyClone, Logan, USA) containing 100 U/ml penicillin and 100 µg/ml streptomycin (Gibco BRL, USA). The mammary tissue fragments were minced with sterile scissors and then digested by collagenase II (Gibco) at 37°C and 5% CO_2 for 1 h with shaking every 20 min. The digests were filtered through a 200 µm nylon mesh to remove the large tissue fragments, and the filtered liquid was centrifuged at 179 *g* for 5 min, and the supernatant liquid was removed. The cell pellet was resuspended in the culture medium containing Dulbecco's Modified Eagle's Medium/F12 (DMEM/F12) (Gibco) media supplemented with 10% foetal bovine serum (FBS) (Gibco), 0.5% insulin (Gibco), 4 µg/ml prolactin (Sigma-Aldrich, St. Louis, USA), 1 µg/ml hydrocortisone (Sigma-Aldrich), 100 U/ml penicillin, and 100 µg/ml streptomycin under 5% CO_2 and air at 37°C. Cells were passaged twice and subsequently cryopreserved in the DMEM/F12 medium containing 10% FBS and 10% dimethyl sulfoxide (DMSO) (Sigma, Munich, Germany). The cells were randomly divided into four groups with six replicates, and 2 independent experiments were performed. The first group was used as control, group 2 was treated with NOp, group 3 with VA, and group 4 was treated with VA in combination with NOp. The control was treated without VA and NOp for 30 h; the NOp-treated group was treated without VA for 24 h before treatment with NOp for 6 h; the VA-treated group was treated with VA for 30 h. The VA in combination with NOp treatment group was administered NOp for 6 h after VA for 24 h. Cells from the different treatment groups were lysed on ice for 30 min in lysis buffer (Beyotime, Nanjing, China). The lysates were centrifuged at 1200 *g* for 10 min at 4°C to remove cell debris, and the supernatant was used for the analysis of the activities of glutathione peroxidase (GPx) and thioredoxin reductase (TrxR) and the concentrations of malondialdehyde (MDA) and ROS. The cell-free supernatant was collected for analysis of other parameters, including selenoprotein P (SelP) content, iNOS activity, NO, IL-1, interleukin-6 (IL-6), and TNF-α contents. The NOp concentration (1000 µmol/l) and its reaction time (6 h) and the VA concentration (1 µg/ml) and its reaction time (30 h) were performed as a pre-test (data are unpublished).

Working solution preparation. The VA working solution and the NOp working solution were prepared as follows: all-*trans* retinoic acid was dissolved in DMSO to attain the desired concentration of 1 µg/ml and subsequently sterile-filtered; NOp (Sigma-Aldrich) was prepared in ultrapure water at room temperature to reach the concentration of 0.1 mol/l. The resulting solution was added to the cell culture medium to obtain the desired concentration of 1000 µmol/l and then sterile-filtered before the experiments. The two working solutions were stored at –4°C until use.

Cell proliferation assay. Cell proliferation was determined by a methyl thiazolyl tetrazolium (MTT) assay. The data were obtained from three independent experiments of 6 replicates. The cells were distributed into 96-well plates at 1×10^4 cells/well. NOp and VA were added to the cells as in the experimental design. Briefly, after a 30 h incubation, 20 µl of MTT (5 mg/ml in 1× PBS) was added to each well and incubated at 37°C for 4 h. Next, the formazan crystals in each well were dissolved in 100 µl DMSO for 10 min with shaking. The absorbance at 490 nm in each well was recorded immediately using an ELISA microplate reader Synergy H4 (BioTek, Winooski, USA). The higher absorbance meant more cell proliferation. The cell proliferation was expressed as a cell relative growth rate (RGR):

$$RGR = [OD_{490\ nm}\ (\text{treatment group})/OD_{490\ nm}\ (\text{control group})] \times 100\%$$

where:
OD = optical density

Antioxidant parameters and inflammatory factor analysis. The activity of GPx in the cells was measured using a commercial colorimetric assay kit A005 (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) according to the manufacturer's instructions. The absorbance was measured by a spectrophotometer at a wavelength of 412 nm (UN2CO-WFT2100; Aoyi Co. Ltd., Shanghai, China). TrxR activity was assayed using the dithio-*bis*-nitrobenzoic acid (DTNB) method, in which the reduction of DTNB was monitored at 412 nm (Hill et al. 1997). SelP levels were measured by a radioimmunoassay (Hill et al. 1996). The cell

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MDA concentration was measured using a thiobarbituric acid test from a commercial kit (A003-1; Nanjing Jiancheng Bioengineering Institute) following the manufacturer's instructions. The ROS concentration was estimated with an FLx800 fluorescence analyzer Synergy H4 (BioTek) using ELISA as described by Kim et al. (2004).

INOS and NO were measured using ELISA according to a commercial colorimetric assay kit (DRE98359 and DRE98022; Nanjing Jiancheng Bioengineering Institute) following the manufacturer's instructions. The concentrations of IL-1, IL-6, and TNF-α were measured using ELISA by commercial kits according to their respective manufacturer's instructions (KMC0011, EM2IL65, and EBTNF; Pierce, Rockford, USA).

RNA extraction and real-time PCR. Cells were plated in 6-well plates at 2×10^5 cells/well in growth medium and included NOp and VA according to the experimental design and were cultured at 37°C and 5% CO_2 for 30 h. Total RNA was extracted from the cells using an RNAprep pure Cell Kit DP430 (Tiangen, Beijing, China) following the manufacturer's instructions. A 2% agarose gel electrophoresis and microplate reader were used to assess RNA integrity and purity.

cDNA was generated for real-time PCR in a 10-µl volume using PrimeScript RT reagent Kit DRR036A (TaKaRa, Dalian, China). The reaction program of the cDNA synthesis was as follows: 37°C for 15 min and 85°C for 5 s. Real-time PCR reactions were carried out at a final volume of 20-µl reactions containing 10 µl of 2× SYBR Premix Ex Taq™II, 2 µl cDNA, 0.4 µl of each 10µM forward and reverse primers, and 7.2 µl RNase free water. Glyceraldehyde phosphate dehydrogenase (GAPDH) was used as the internal controls. The primers were presented in Table 1. The reactions were performed in a MxPro-Mx3000P real-time PCR machine (Agilent Technologies, Santa Clara, USA) with an initial denaturing step of 95°C for 30 s followed by 40 cycles of 95°C for 30 s (denaturation), 60°C for 30 s (annealing), 72°C for 20 s (extension). The quality and specificity of the PCR products were assessed by the melt curve analysis and subsequent gel agarose electrophoresis. The quantitative real-time PCR data were calculated using the $2^{-\Delta\Delta Ct}$ method.

Table 1. Summary of genes, primer sequences, and amplicon size

Genes	GenBank accession No.	Primer sequences (5'-3')	Length (bp)	Annealing temperature (°C)
GAPDH	XM_001252479	F: GCGCTCTARATGTTCACCTTCC R: AGCATCACCCARACTTTATGTT	314	60
GPx1	NM_174076.3	F: AGTGCGAGGTGARATGGCGAGARA R: TGGGCARAARATCCCTGGAGAGCA	328	60
GPx4	NM_174770.3	F: ATCARAAGAGTTCGCCGCTGGCT R: TCGGARACACAGGCARACAGGCTT	295	60
TrxR1	NM_174625.3	F: AGGAGARAAGCTGTGGAGARAA R: TTATCCCTTGATGGARATCGT	94	60
SelP	NM_174459.3	F: CTTCATCACCACCACCACAG R: GAGGCARAACGTCACTGTCARA	331	60
iNOS	NM_001076799.1	F: TGTCAGCGGCAAGCACCACATT R: CGGCTGGTTGCATGGGAAAACT	289	60
IL-1β	NM_174093.1	F. GCCTTGGGTATCAAGGACAA R: TTTGGGGTCTACTTCCTCCA	90	60
IL-6	NM_173923.2	F: ACAAGCGCCTTCACTCCATTCG R: GCCAGTGTCTCCTTGCTGCTTT	242	60
TNF-α	NM_173966.2	F: TGCTTGTGCCTCAGCCTCTTCT R: ACGAGGGCATTGGCATACGAGT	254	60

F = forward primer, R = reverse primer, *GAPDH* = glyceraldehyde phosphate dehydrogenase, *GPx1* = glutathione peroxidase1, *GPx4* = glutathione peroxidase4, *TrxR1* = thioredoxinreductase1, *SelP* = selenoprotein P, *iNOS* = inducible nitric oxide synthase, *IL-1β* = interleukin-1β, *IL-6* = interleukin-6, *TNF-α* = tumour necrosis factor-alpha

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Table 2. Effects of cellular damage induced by NOp on cell proliferation, antioxidant indicators, and mRNA expression

Parameters		Control	NOp	VA	VA + NOp	SEM	*P*-value
Absorbance values		0.567^{b}	0.441^{c}	0.713^{a}	0.652^{ab}	0.0313	0.0003
Antioxidant indicators	GPx (IU/mg protein)	146.94^{b}	96.01^{c}	189.27^{a}	167.34^{ab}	8.711	< 0.0001
	SelP (mg/l)	0.55^{b}	0.43^{c}	0.67^{a}	0.47^{bc}	0.029	0.0002
	TrxR (U/g protein)	2.67^{b}	2.15^{c}	3.26^{a}	2.89^{ab}	0.145	0.0022
	ROS (fluorescence intensity/ml)	138.97^{b}	172.26^{a}	124.45^{b}	145.68^{b}	6.304	0.0017
	MDA (nmol/mg Pr)	1.63^{c}	4.63^{a}	1.29^{c}	3.68^{b}	0.232	< 0.0001
Gene expression	*GPx1*	1.041^{b}	0.555^{c}	2.136^{a}	1.215^{b}	0.138	< 0.0001
	GPx4	1.014^{a}	0.755^{b}	0.999^{a}	0.849^{ab}	0.063	0.0296
	TrxR1	1.009^{b}	0.65^{c}	1.561^{a}	1.196^{b}	0.103	0.0004
	SelP	1.026^{a}	0.895^{a}	1.26^{a}	1.193^{a}	0.123	0.2799

NOp = diethylene triamine-nitric oxide polymer, VA = vitamin A, GPx = glutathione peroxidase, SelpP = selenoprotein P, TrxR = thioredoxin reductase, ROS = reactive oxygen species, MDA = malondialdehyde, SEM = standard error of the mean
means in the same row not followed by the same letter differ significantly ($P < 0.05$)

Statistical analysis. Data were analyzed using the General Linear Model procedure of SAS software (Statistical Analysis System, Version 9.0, 2004) to test the significance and multiple comparisons. A value of $P < 0.05$ was regarded as significant, whereas the differences were considered to be a statistical trend when $0.05 < P < 0.10$.

RESULTS

Cell proliferation, antioxidant indicators, and mRNA expression. The results indicated that compared with the control group, the absorbance was lower for the NOp treatment ($P < 0.05$) but greater ($P < 0.05$) for the VA treatment (Table 2); the absorbance of the VA + NOp-treated groups was significantly greater ($P < 0.05$) than that of the NOp-treated group. There was no significant difference in the absorbance between the VA + NOp-treated group and the VA-treated group ($P > 0.05$). As shown in Table 2, the NOp-treated group displayed a significant reduction in the activities of GPx and TrxR, as well as the concentration of SelP, compared with the control group, but the VA-treated group showed the opposite trend. The VA + NOp-treated group showed a signifi-

Table 3. Effects of cellular damage induced by NOp on the production of NO, concentration of cytokines, and mRNA expression

Parameters		Control	NOp	VA	VA + NOp	SEM	*P*-value
Cytokines	NO (Umol/l)	18.6^{c}	96.56^{a}	20.94^{c}	78.2^{b}	2.937	< 0.0001
	iNOS (IU/ml)	14.93^{c}	22.29^{a}	15.75^{c}	18.93^{b}	0.413	< 0.0001
	IL-1 (ng/ml)	12.0^{c}	34.97^{a}	12.42^{c}	25.06^{b}	0.547	< 0.0001
	IL-6 (pg/ml)	17.22^{c}	30.88^{a}	17.63^{c}	23.9^{b}	0.826	< 0.0001
	TNF-α (ng/ml)	36.5^{c}	99.5^{a}	44.2^{c}	79.9^{b}	0.011	< 0.0001
Gene expression	*IL-1β*	1.036^{c}	2.253^{a}	1.41b^{c}	1.636^{b}	0.169	0.0013
	IL-6	1.091^{b}	3.177^{a}	1.395^{b}	1.906^{b}	0.297	0.0007
	TNF-α	1.033^{c}	2.924^{a}	1.402^{c}	2.172^{b}	0.237	0.0002
	iNOS	1.056^{b}	2.108^{a}	1.545^{b}	1.468^{b}	0.158	0.0071

NOp = diethylene triamine-nitric oxide polymer, VA = vitamin A, NO = nitric oxide, iNOS = nitric oxide synthase, IL-1 = interleukin-1, IL-6 = interleukin-6, TNF-α = tumour necrosis factor-α, SEM = standard error of the mean
means in the same row not followed by the same letter differ significantly ($P < 0.05$)

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cant increase in the activities of GPx and TrxR ($P < 0.05$) compared to the NOp-treated group. The activities of GPx and TrxR in the VA-treated group were not significantly higher than in the VA + NOp-treated group, but SelP concentration was. Compared with the control group, the NOp-treated group exhibited a significant increase in the contents of ROS and MDA ($P < 0.05$). The VA-treated group and the VA + NOp-treated group showed dramatically decreased contents of ROS and MDA compared to the NOp-treated group ($P < 0.05$). The MDA content in the VA-treated group was markedly lower than that in the VA + NOp-treated group, but no significant differences existed between the two groups.

The NOp-treated group exhibited down-regulated ($P < 0.05$) mRNA expression for *GPx1*, *GPx4*, and *TrxR1* in comparison with the control group, and the VA + NOp-treated group showed a significantly up-regulated ($P < 0.05$) mRNA expression for *GPx1* and *TrxR1* compared to the NOp-treated group ($P < 0.05$), and similar results were found for the gene expressions of *SelP* and *GPx4* but the difference was not significant ($P > 0.05$).

Production of NO, concentration of cytokines, and mRNA expression. As shown in Table 3, compared to the control group, the NOp-treated group significantly increased ($P < 0.05$) the production of iNOS, the contents of NO, and the cytokines of IL-1, IL-6, and TNF-α and their mRNA expression, but the increases observed in the VA-treated group were not significant ($P > 0.05$). Compared with the NOp-treated group, the VA + NOp-treated group exhibited a significant decrease in the production of iNOS, the contents of NO, and the cytokines of IL-1, IL-6, and TNF-α and their mRNA expression. However, the VA-treated group was significantly lower than the VA+NOp-treated group in the above parameters with the exception of the iNOS and IL-6 mRNA expressions.

DISCUSSION

In general, the natural antioxidative defence system protects against free radicals (Gitto et al. 2002). However, overproduction of free radicals causes cell injury. Therefore, the balance between the generation of free radicals and antioxidant defences is critical. In RAW 264.7 macrophages, a high level of NO production is accompanied by cell apoptosis (Tsi et al. 2002). Jin et al. (2014) reported that supplementation with a high dose of vitamin A (220 IU/kg BW), which is above the current NRC-recommended level in the diet, can improve the antioxidant function. Thus, we postulate that the positive effects of VA on the antioxidant function in the cells were at least partially achieved by the decreased production of NO. In the current study, we determined the promotion of VA on the antioxidant function and examined the possible antioxidative mechanism by investigating the protective effect of VA on oxidative stress in BMEC induced by NOp.

Zhi et al. (2000) showed that newborn rat calvarial osteoblastic cells cultured in all-*trans* retinoic acid for 24 h up-regulate the proliferation of osteoblasts. Rock et al. (1995) noted that carotenoids promote human mammary epithelial cell proliferation and differentiation. In the current study, cell proliferation was significantly improved when BMEC were treated with VA, which is consistent with previous results. However, Cheli et al. (2003) indicated that RA inhibits the proliferation of BMEC lines. The explanation for this inconsistent result is unclear, and it can most likely be attributed to the effect of time and dose-dependent effects of RA on cell proliferation and may also depend on the original state of the tissues when they were necropsied as noted above or the passage number used. In the present study, the NOp-administered group showed a significantly reduced cell proliferation and an increased NO production compared with the control group, indicating that adding exogenous NO damages BMEC. Cell proliferation in the VA + NOp-treated group was significantly higher than in the NOp-treated group, indicating that the addition of VA in advance can protect cells from NOp-induced damage.

The enzymatic antioxidant defence system primarily includes superoxide dismutase (SOD), catalase (CAT), GPx, and TrxR, and this system protects cells against ROS toxicity and lipid peroxidation. These protein markers, plus selenoproteins like SelP, can be used to reflect antioxidant function. SOD converts the superoxide anion radical to hydrogen peroxide (H_2O_2) and CAT cleaves this H_2O_2 into water (H_2O) and oxygen (Husain et al. 1987). GPx is the earliest discovered selenoprotein (Brigelius 1999) to maintain low levels of intracellular H_2O_2 by degrading it to H_2O to reduce the damage by free radicals (Rotruck et al. 1973). GPx is also a selenoenzyme that catalyzes the oxida-

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tion of glutathione (GSH) to oxidized glutathione (GSSG) and thereby scavenges to H_2O_2 (Rana et al. 2002). TrxR reduces the process of the redox reaction excessively (Hawkes and Alkan 2010) and the scavenge oxidants such as H_2O_2 or alkyl hydroperoxide (Smith et al. 1999), preventing cells from oxidative damage. SelP has a relationship with the transportation of selenium and antioxidant function, having the activity of lipid peroxidase, which prevents damage from lipid metabolites (Rock and Moos 2010). In other words, the selenoproteins GPx1, GPx4, TrxR1, and SelP play a vital role in controlling the process of cellular oxidative stress and protect the cells from oxidative stress damage (Bruzelius et al. 2008). The antioxidant enzyme system in cells is the main indicator of cellular antioxidant levels, it can scavenge ROS and maintain the balance between oxidants and antioxidants. MDA is an important indicator, which reflects the extent of the attack by free radical and its content can reflect the degree of lipid peroxidation in the cell (Sehirli et al. 2008). Fraile-Bermudez et al. (2015) found that the GPx antioxidant emzyme activity was positively correlated with CAT activity and that the antioxidant enzymes SOD and GPx negatively correlated with MDA (Mladenov et al. 2015). The current experiment mainly determined the MDA and ROS contents and a degree of antioxidant enzyme activity, and our results showed that the contents of MDA and ROS in the VA-treated group were significantly lower than in the control, indicating that VA enhanced cellular antioxidant function, which is consistent with the results of previous studies. The contents of MDA and ROS in the VA + NOp-treated group were significantly lower than in the NOp-treated group, which indicates that VA is vital in attenuating oxidative damage caused by NO and plays a protective role. Jin et al. (2014) reported that supplementation with 220 IU of VA/kg of BW can improve the ability of antioxidant function by increasing the activities of SOD, CAT, T-AOC, GPx, and TrxR and the concentration of SelP *in vivo*. Han (2003) found that the content of MDA decreased along with increasing doses of VA after rats had been perfused with different doses of VA. Huang et al. (2008) suggested that the level of VA has a negative relation with the content of MDA in the serum. Vaskova et al. (2014) found that a high dose of VA significantly enhanced the activity of GPx in rat liver. All these results indicate that VA plays a positive role in improving the antioxidant function of animals. However, the data on the effects of VA on GPx is relatively abundant but minimal for TrxR and SelP. In the current research, we demonstrated that the addition of VA in advance can lead to increases in the activities of GPx and TrxR as well as in the SelP concentration compared to the NOp-treated group and similar results were detected for the mRNA expression of *GPx1*, *GPx4*, and *TrxR1*. All of these results suggest that VA improves the antioxidant function of BMEC and protects BMEC from the oxidative injury induced by NOp. However, the exact mechanism is unclear. The results also indicate that the mRNA expression of *SelP* was not significantly different among the groups, which is inconsistent with the SelP content results with the exception of the VA + NOp group compared with VA alone. The reason for this finding is that the SelP concentration may be regulated at the translational level, rather than the transcription level, but little has been reported in this area to verify this phenomenon. Therefore, the exact interpretation needs to be further explored.

Nitric oxide (NO) is a gas molecule with the properties of free radicals which are involved in a variety of physiological functions in cells and tissues (Moncada and Higgs 2006), such as vascular regulation, homeostasis, bone formation and resorption, neurotransmission, and immune function (Ugar-Cankal and Ozmeric 2006). Schmidt and Walter (1994) confirmed that L-arginine and oxygen molecules continuously generate endogenous low concentrations of NO through a multistep redox reaction catalyzed by nitric oxide synthase (NOS). There are three isoforms of NOS, namely endothelial nitric oxide synthase (eNOS), neuronal nitric oxide synthase (nNOS), and inducible nitric oxide synthase (iNOS). eNOS and nNOS maintain normal physiological functions in low level expression continuously, but iNOS is in a silent state and is not endogenously expressed *in vivo*. When macrophages and lymphocytes are stimulated with inflammatory cytokines or endotoxin, iNOS is activated, resulting in tissue injury and accelerated inflammation due to the production of NO (Pan et al. 2010). NO has the double-dose effect because small amounts of NO have a direct bactericidal effect (Boqdan et al. 2000) and accelerate blood circulation, whereas an excess of NO can damage healthy cells, resulting in the oxidative

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stress of tissue (Banan et al. 2000). Many of the toxic effects of NO overproduction are mediated by the peroxynitrite ($ONOO^-$), a product of the reaction of NO with superoxide anions. Therefore, a high concentration of NO has an amplification effect on oxygen-free radicals damage, promoting inflammation and tissue damage and increasing levels of oxidative stress (Zhang et al. 2013). The current results indicate that the NOp-treated group significantly increases the concentration of NO, the activity of iNOS and its mRNA expression, but the addition of VA in advance elicited the opposite changes, indicating that VA can down-regulate the activity of iNOS and its gene expression to reduce NO production and protect cells from NOp-induced injury.

However, its specific protective mechanism is unclear. Hung et al. (2008) and Ertesvag et al. (2009) identified that RA strongly suppressed production of NO by down-regulating the gene expression of *IL* in human chondrocytes or in mice. Balato et al. (2013) found that the pro-inflammatory factor TNF-α enhanced the gene expression of *IL-1* in human skin tissue *in vivo* and it was inhibited by RA. Wahab et al. (2015) found that the interleukin-1 receptor antagonist could also decrease the NO concentration in cerebrospinal fluid and lower iNOS activities in the septic rat. Dheen et al. (2005) and Hong et al. (2014) indicated that RA inhibited the production of TNF-α and decreased the gene expression of *iNOS* in murine RAW264.7 cells and in activated rat microglia induced by LPS to reduce NO production. All of these results suggest that VA may inhibit the activity of iNOS through IL and TNF-α to reduce oxidative stress. However, data available regarding the effects of RA on BMEC is scarce. The present study examined the effects of the addition of VA to the BMEC before induction by NOp on NO production, iNOS activity, cytokines content, and their gene expression and the results show that BMEC treated with NOp alone significantly improved NO production and the concentration and gene expression of the cytokines *IL-1*, *IL-6*, and *TNF-α*, as well as the activity and the expression of *iNOS*, inferring that VA is protective against NOp-induced oxidative damage in BMEC. We postulated the idea that VA reduces the activity of iNOS and its mRNA expression by down-regulating of the expression of *IL-1*, *IL-6*, and *TNF-α* to reduce NO production. However, there are few reports in this field and even fewer data points concerning the effects of the interleukin receptor on NO production in BMEC. Therefore, more research is required to explain the exact mechanism.

Hawkes and Alkan (2010) reported that S-glutathionylation regulates signalling pathways and transcription factors, including P38 mitogen-activated protein kinase (MAPK) and nuclear factor-kappa B (NF-κB). Hsieh and Papaconstantinou (2006) and Yoo et al. (2013) found that the decreased activity of TrxR in mice and EMT6 in murine breast cancer cells, which closely relates to the activation of c-Jun NH_2-terminal kinase (JNK) and p38 of the MAPK signalling pathways, promotes the binding activity of NF-κB and DNA to thereby induce the expression of *iNOS* and the production of NO. Bruzelius et al. (2010) have shown that RA up-regulates the gene expression of *TrxR1* in the bovine mammary cell line MAC-T. Chu et al. (1999) found that medium supplemented with RA can strongly induce the expression of the *GPx2* gene and result in the increase of GPx activity in MCF-7 cells. Therefore, the regulation of VA on NO formation is probably related to the increased activity of TrxR inhibiting *iNOS* mRNA expression and NO production ultimately through the MAPK pathway. However, the exact mechanism regarding the Trx-mediated regulation of the P38 MAPK pathway in BMEC needs to be explored in the future.

CONCLUSION

The results from this study demonstrated that VA plays a critical role in protecting against NOp-induced oxidative stress by regulating the antioxidant gene expression. We propose that VA pre-protects the BMEC from NO-induced oxidative stress and damage, probably due to the reduced activity of iNOS and its mRNA expression by the down-regulation of the expression of *IL-1*, *IL-6*, and *TNF-α* to reduce NO production. However, the exact mechanism of how NO production is decreased in BMEC and how it is regulated by Trx-mediated regulation of the MAPK pathway warrants further investigation.

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