



# Mechanism Underlying the Protective Effect of Selenium on NO-Induced Oxidative Damage in Bovine Mammary Epithelial Cells

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## Abstract

This experiment was conducted to investigate the effects and mechanism of selenium (Se) on antioxidant and immune function of bovine mammary epithelial cells (BMEC) damaged by nitric oxide (NO). The third-generation BMEC was randomly divided into eight treatments with six replicates. The BMEC in the control group was cultured in the medium without Se and diethylenetriamine/NO (DETA/NO) for 30 h. For the DETA/NO group and Se protection group BMEC were exposed to different concentrations of Se (0, 10, 20, 50, 100, 150, and 200 nmol/L) for 24 h, followed by treatment with DETA/NO (1000  $\mu$ mol/L) for 6 h. Compared with the control group, DETA/NO decreased proliferation rate and activity of thioredoxin reductase (TrxR;  $P < 0.05$ ). Additionally, DETA/NO decreased the gene expression of both nuclear factor-E2-related factor 2 (Nrf2) and TrxR, as well as the protein expression level of TrxR. However, the activity, and expression levels of inducible nitric oxide synthase (iNOS), as well as the concentration and gene expression level of interleukin-1 $\beta$  (IL-1 $\beta$ ) and the concentration of NO significantly increased ( $P < 0.05$ ). The gene expression levels of indexes related to the mitogen-activated protein kinase (MAPK) signaling pathway showed similar changes. Treatment of BMEC with Se significantly reversed DETA/NO-induced changes in a linear or quadratic dose-dependent manner ( $P < 0.05$ ), with greatest benefit at 50 nmol/L. These data suggests that Se improves the antioxidant function of BMEC, and protects cells from DETA/NO-induced oxidative damage, primarily by enhancing the activity of TrxR and decreasing the concentration of NO through modulation of Nrf2 and MAPK signaling pathways.

**Keywords** Selenium · Nitric oxide · Bovine mammary epithelial cells · Oxidative damage · Protective effect

## Introductions

Dairy cattle experience oxidative stress due to the high metabolic rate of bovine mammary epithelial cell (BMEC) during lactation. This oxidative stress leads to decreased immune function, milk production, and milk quality, and ultimately results in economic losses [1]. Our previous studies indicated that selenium (Se) treatment protects dairy cows and BMEC from oxidative damage [2, 3]; however, the exact mechanism by which Se maintains the redox state is still unclear. Nitric oxide (NO), one of the smallest known bioactive products of mammalian cells, can be produced by almost all cells and mediates several biological effects. At some concentrations, NO can have a positive physiological function; however,

excessive NO formation is thought to contribute to oxidative stress and blocks antioxidant signaling pathways. Due to high metabolic requirements, dairy cows produce a great deal of NO during lactation, leading to oxidative stress and resultant cell toxicity [4]. Obata et al. indicated that activation of p38 mitogen-activated protein kinase (p38MAPK) can promote the production of tumor necrosis factor-alpha (TNF- $\alpha$ ), interleukin-1 $\beta$  (IL-1 $\beta$ ), and interleukin-6 (IL-6) in RAW 264.7 cells, resulting in the production of NO [5]. Studies of human chondrocytes indicated that selenomethionine (SeMet) inhibited gene expression of IL-1 $\beta$ -induced inducible nitric oxide synthase (iNOS) with corresponding reductions in NO production [6]. Ferret et al. observed an inverse correlation between cell susceptibility to NO damaging effects and thioredoxin (Trx) and Trx reductase (TrxR) expression. The correlating relation suggests that the Trx system may prevent NO-mediated cellular injury in monocytic macrophage cells [7]. Changes in the redox status of Trx activates apoptosis signal-regulating kinase-1 (ASK-1) and the MAPK pathway [8]. Previous studies from our research group demonstrated that Se can effectively improve antioxidant status and increase

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TrxR activity [2]. Nuclear factor-E2-related factor 2 (Nrf2) plays an important role in the regulation of the cellular Trx system, which consists of Trx and TrxR [9]. Choi et al. found that baicalein effectively enhances cellular antioxidant defense capacity through the inhibition of ROS generation, the activation of the Nrf2 signaling pathway and the increase in TrxR1 expression, which protected C6 cells from oxidative stress [10]. Therefore, our hypothesis is that Se protects BMEC from oxidative stress through upregulation of Nrf2, resulting in increased TrxR activity, which inhibits MAPK signaling pathway and prevents overproduction of NO.

An ideal exogenous NO donor to induce cell injury is diethylenetriamine (DETA)/NO [11]. Previous studies from our research group investigated the suitable condition for oxidative damage model of BMEC induced by DETA/NO [4]. The present study was carried out to investigate the protective effect of varying concentrations of Se on oxidative stress in BMEC caused by DETA/NO. Additionally, the study was designed to elucidate the underlying mechanism of the anti-oxidative effects of Se, and whether it was due to increased TrxR activity, and modulation to the MAPK signaling pathway resulting in downregulation of NO production.

## Materials and Methods

All experiments for the projects using mammary tissues were reviewed and approved by the Animal Care and Use Committee at the Inner Mongolia Agricultural University, Hohhot, China.

### BMEC Isolation and Primary Cell Culture

Mammary tissue was collected from healthy lactating Holstein cows. Samples of mammary tissue had outer layers removed and the inner tissue was placed in phosphate-buffered saline (PBS) supplemented with 300 U/mL of antibiotic-antimycotic solution. The tissues were sequentially washed three times with PBS + 300 U/mL of antibiotic-antimycotic solution, then tissues were transferred to 75% ethanol for 30 s, followed by washing three times with PBS + 100 U/mL of antibiotic-antimycotic solution. The tissues were transferred into a culture dish, trimmed, and cut into pieces the size of rice grains. Pieces of tissue that were rich with acini were transferred into a 5-mL centrifuge tube containing 0.05% collagenase type II (0.5%, Gibco BRL, Grand Island, NY) and the tissues were minced thoroughly, allowing separation of BMEC from erythrocytes. The cell suspension was incubated for 1 h at 37 °C in a CO<sub>2</sub> constant temperature incubator (Heal Force Bio-Meditech Holdings Ltd., China) and the suspension was shaken every 20 min. The digested suspension was filtered through an 80-

mesh sieve, and the dispersed cells were collected by centrifugation at 400×g for 5 min. The precipitate was resuspended in PBS and then centrifuged at 400×g for 3 min. After centrifugation, the cell pellet was resuspended in 5 mL of growth DMEM/F12 medium containing 10% fetal bovine serum (FBS) (Sigma Chemical Co., St. Louis, MO, USA); 5 µg/mL insulin transferrin Se (Sigma Chemical Co., St. Louis, MO, USA); 10 ng/mL epidermal growth factor (Sigma Chemical Co., St. Louis, MO, USA); 1 µg/mL hydrocortisone (Sigma Chemical Co., St. Louis, MO, USA); 5 µg/mL prolactin (Sigma Chemical Co., St. Louis, MO, USA); 2.5 µg/mL amphotericin B (Sigma Chemical Co., St. Louis, MO, USA); 100 IU/mL penicillin (Sigma Chemical Co., St. Louis, MO, USA), 100 µg/mL streptomycin (Sigma Chemical Co., St. Louis, MO, USA); and basal medium (15.6 g DMEM/F12 powder, 1000 mL ultra-pure water and 2.4 g sodium bicarbonate). Cell counts were determined by cytometry (ECI Inc., Yokohama, Japan). The BMEC was seeded into 25-cm<sup>2</sup> cell culture flasks at an approximate density. The cells were then incubated in an atmosphere of 5% CO<sub>2</sub> at 37 °C. When the confluence of BMECs reached 80 to 90%, the BMECs were subcultured twice. Third-generation BMECs were seeded at approximate densities of 1 × 10<sup>4</sup>, 1 × 10<sup>5</sup>, 3 × 10<sup>5</sup>, and 5 × 10<sup>5</sup> cells/well into 96-well plates (200 µL per well), 6-well plates (2.5 mL per well), 60-mm culture dishes (3.5 mL per well), and 25-cm<sup>2</sup> culture flasks (3.5 mL per flask). Cells were incubated with DMEM/F12 growth medium supplemented with 10% FBS in an atmosphere of 5% CO<sub>2</sub> at 37 °C for 24 h. The medium was decanted when the confluence of BMECs reached 80 to 90%, and then the cells were serum starved for 24 h.

### Experimental Design

After cells were serum starved for 24 h, BMECs were randomly divided into eight treatments, each containing six replicates. Group 1 was the control group (CON, cultured in medium without SeMet and DETA/NO for 30 h); group 2 was the DETA/NO treatment group (DETA/NO, pretreated with the medium without SeMet for 24 h followed by treatment with 1000 µmol/L DETA/NO for 6 h); and groups 3–8 were the Se prevention group (NSe10, NSe20, NSe50, NSe100, NSe150, NSe200), pretreated with 10, 20, 50, 100, 150, and 200 nmol/L SeMet for 24 h followed by treated with 1000 µmol/L DETA/NO for 6 h). The concentration of Met in culture medium was 115,704.69 nmol/L, and the added concentrations of Met in the SeMet-treatment groups were relatively low and same with corresponding concentrations of Se (10, 20, 50, 100, 150, and 200 nmol/L); thus, Met has little effect on this trial. The dose and processing time of DETA/NO were determined based on previous research [4].

## Cell Viability Assay

Cell viability was assessed by the mitochondrion-dependent reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide salt (MTT) to formazan. After the various incubation times indicated, cells in 96-well plates were exposed to 20  $\mu$ L of 5 mg/mL MTT and incubated at 37 °C for 4 h. The medium was removed from each well, and 100  $\mu$ L dimethyl sulfoxide (DMSO; Sigma Chemical Co., St. Louis, MO, USA) was added to lyse the cells. The 96-well plate was shaken for 10 min then analyzed with an automatic microplate reader (Synergy H4, BioTek Co., Japan) at 490 nm. Cell viability of the control group was expressed as 100%, while cell viability of the treatment group was expressed as percent (%) viability = OD treatment group/OD control group  $\times$  100.

## Measurement of Antioxidant and Immune Parameters

The cell culture medium was collected in 1.5-mL centrifuge tubes and centrifuged at 10,000 $\times$ g for 5 min at 4 °C. The supernatant was transferred into new tubes and stored at -80 °C until subsequent analysis. The activities of superoxide dismutase (SOD), catalase (CAT), and total antioxidant capacity (T-AOC) were measured using commercial colorimetric assay kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China). The activities of iNOS, selenoprotein P (SeIP), and the concentrations of NO, TNF- $\alpha$ , IL-1 $\beta$ , and IL-6 were analyzed using commercial ELISA kits (Neobioscience Co., Beijing, China). The absorbance was measured using a spectrophotometer at the appropriate wavelengths.

After the medium were collected, the cells in 60-mm culture dishes were scraped and lysed for 30 min in 600  $\mu$ L of lysis buffer followed by centrifugation (10,000 $\times$ g, 10 min, 4 °C). The supernatant was collected into 1.5-mL Eppendorf tubes and stored at -80 °C for analysis of TrxR and glutathione peroxidases (GPx) activities and reactive oxygen species (ROS) and malondialdehyde (MDA) concentrations. Activity of GPx was measured using commercial colorimetric assay kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) according to the manufacturer's instructions. The TrxR activity and ROS levels were analyzed using commercial ELISA kits (Neobioscience Co., Beijing, China). The concentration of MDA was measured using thiobarbituric acid positive reactant (TBAR) assay. The absorbance was measured using a spectrophotometer at the appropriate wavelengths.

## Relative Expression of Selenoprotein mRNA in BMECs

Total RNA extraction was carried out according to the manufacturer's instructions for the RNAiso™ Plus extraction kit (Takara Bio. Inc., Dalian, China). After the cells were cultured

for 26 h in 6-well plates, the medium was decanted, plates were washed twice with PBS, and 1-mL Trizol Plus lysate was added to each well and repeatedly pipetted to ensure thorough lysis of the cells. The cell suspension was collected in 1.5-mL Eppendorf tubes. The cDNA probes were created using a PrimeScript™ reverse transcription kit (Takara Bio. Inc., Dalian, China) according to the manufacturer's instructions. The PCR primers were designed using Oligo and Premier software using data obtained from GenBank. Relative mRNA expression of GPx1, GPx4, SeIP, TrxR1, Nrf2, iNOS, IL-1 $\beta$ , IL-6, TNF- $\alpha$ , p38MAPK, c-Jun N-terminal kinase (JNK), and extracellular signal-regulated kinase (ERK1/2) in BMECs were determined by fluorescence-based real-time PCR (SYBR Green I) using a SYBR Premix Ex Taq™ kit (Takara Bio. Inc., Dalian, China), according to the manufacturer's instructions. Housekeeping genes used were  $\beta$ -actin, UXT, and 18SrRNA, and the qPCR data were normalized using the geometric mean of the three internal control genes. The  $2^{-\Delta\Delta C_t}$  method was used to analyze the data. Information on primers used in the real-time quantitative PCR is described in Table 1. The PCR products were electrophoresed on 2% agarose gels (100 V, 20 min), photographed under UV light, and compared with a DNA Marker. Sequence analysis was carried out by Shanghai Sangon Biotech.

## Western Blots

After the cells were cultured for 24 h in 25-cm<sup>2</sup> culture flasks, the medium was decanted, and the culture flasks were washed twice with PBS. Cells were scraped, lysed for 5 min in RIPA lysis at 4 °C, and centrifuged (10,000 $\times$ g, 10 min, 4 °C). The cell suspension was collected in 1.5-mL Eppendorf tubes and used for protein expression of related indices in cells. Protein expressions of TrxR1, GPx1, and iNOS in cells were determined using Western blotting with the internal reference gene  $\beta$ -actin. Cell lysates containing 30  $\mu$ g of protein were boiled in 1/4 volume of 4 $\times$  sample loading buffer at 95 °C for 5 min and separated on 12% SDS-PAGE with a 5% stacking gel in electrophoretic buffer. Proteins were electro transferred to a polyvinylidene fluoride membrane in electrophoresis buffer at 100 V for 50 min. The membrane was then incubated with blocking buffer for 1 h at room temperature, followed by washing three times for 2 min with PBST. The blocked membrane was then incubated with diluted polyclonal rabbit anti- $\beta$ -actin (1:2000; Proteintech Co. Ltd. Shanghai, China); polyclonal rabbit anti-iNOS (1:500; Proteintech Co., Chicago, China); polyclonal rabbit anti-TrxR1 (1:500; Abcam Co., Cambridge, MA, USA); and polyclonal rabbit anti-GPx1 (1:1000; Abcam Co., Cambridge, MA, USA) overnight at 4 °C. Following overnight incubation, the membrane was washed three times for 5 min with PBST, followed by incubation with goat anti-rabbit IgG (1:1000; KPL Co., Gaithersburg, MD, USA) for 1 h at room temperature. The membrane was then washed three times for

**Table 1** Sequences of PCR primers for genes

Genes	Accession no. <sup>a</sup>	Primer sequences (5'–3') <sup>b</sup>	Length (bp)	Annealing temperature (°C)
GPx1	NM_174076.3	F: AGTGCAGGTTGARATGGCGA GARA R: TGGGCARAARATCCCTGGAGAGCA	328	60
GPx4	NM_174770.3	F: ATCARAAGAGTTCCGCCGCTGGCT R: TCGGARACACAGGCARACAGGCTT	295	60
TrxR1	NM_174625.3	F: AGGAGARAAGCTGTGGAGARAA R: TTATCCCTTGATGGARATCGT	94	60
SelP	NM_174459.3	F: CTTTCATCACACCACCACAG R: GAGGCARAACGTCACCTGCARA	331	60
Nrf2	NM_0010111678.2	F: GCAGAGACATTCCCGTTTGT R: CCTGAGGAGGAGCAGTGAAG	115	60
iNOS	NM_001076799.1	F: TGTACAGCGCAAGCACACATT R: CGGCTGGTTGCATGGGAAAAGT	289	60
IL-1 $\beta$	NM_174093.1	F: GCCTGGGTATCAAGGACAA R: TTTGGGGTCTACTTCCTCCA	90	60
IL-6	NM_173923.2	F: ACAAGCGCCTTCACTCCATTTCG R: GCCAGTGTCTCCTTGCTGCTTT	242	60
TNF- $\alpha$	NM_173966.2	F: TGCTTGTGCCTCAGCCTCTTCT R: ACGAGGGCATTGGCATACGAGT	254	60
p38-MAPK	NM_001102174	F: CCAGCTCAGCAGATTATGCG R: CGGCATCTGGGTCAAAGACT	109	60
ERK1/2	NM_001110018	F: ATATCCACTCCGCCAACGTG R: AAAGCCAGTGTGGTCATGCT	134	60
JNK1	NM_001192974	F: AGCTTGGAAACACCATGTCCT R: CCGAGTCAGCTGGGAAAAGT	135	60
$\beta$ -actin	NM_173979.3	F: AACTCCATCATGAAGTGTGACG R: GATCCACATCTGCTGGAAGG	234	60
UTX	NM_001037471	F: CATTGAGCGACTCCAGGAAG R: GGCCACATAGATCCGTGAAG	112	60
18S rRNA	Robinson et al. 2007	F: GTAACCCGTTGAACCCCAT R: CCATCCAATCGGTAGTAGCG	152	60

GPx glutathione peroxidase, TrxR1 thioredoxin reductase 1, SelP selenoprotein P, Nrf2 nuclear factor-E2-related factor 2, iNOS nitric oxide synthase, IL-1 $\beta$  interleukin-1 $\beta$ , IL-6 interleukin-6, TNF- $\alpha$  tumor necrosis factor-alpha, p38MAPK p38 mitogen-activated protein kinase, JNK c-Jun N terminal kinase, ERK1/2 extracellular signal-regulated kinase

<sup>a</sup> 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>)

<sup>b</sup> F forward primer, R reverse primer

8 min with PBST. Color development was carried out with the ECL ultra-sensitive luminescence kit according to manufacturer's instructions. The membrane was kept in the dark for 2 min and then photographed with an ImagQuant (GE Healthcare Co., Fairfield, Connecticut, USA). The image intensity was analyzed with Quantity One software (Bio-Rad Laboratories Inc., Hercules, CA, USA), and the relative expression of the target protein was calculated (relative expression of target protein = objective protein gray value/reference protein gray value).

### Statistical Analysis

Data were analyzed using the general linear model procedure of SAS software (Statistical Analysis System, Version 9.0) to test the significance of the treatment groups by regression analysis. Regression analysis was conducted to evaluate linear and quadratic effects of Se on all response

criteria except for negative controls. Differences between means were determined using Duncan's multiple comparisons to test for significance. Differences were considered to be significant when  $P < 0.05$ , and a statistical trend was indicated when  $0.05 < P < 0.1$ .

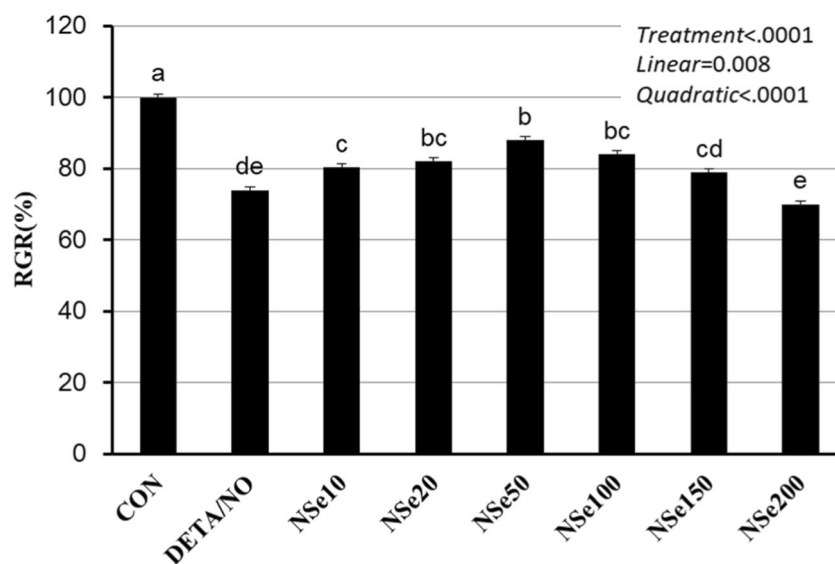
## Results

### Effect of Se on the Proliferation of Cells Damaged by DETA/NO

Treatment of BMEC with 1000  $\mu\text{mol/L}$  DETA/NO for 6 h resulted in a loss of cellular viability (26.10%) compared with the control cells (Fig. 1). However, the cytotoxic effect of DETA/NO was blocked by pretreatment with Se in a quadratic dose-dependent manner ( $P < 0.01$ ). Cell proliferation in the NSe20, NSe50, and NSe100 group was



**Fig. 1** Effects of Se on viability, antioxidant indexes, and inflammatory cytokine concentrations in NO-induced BMEC. RGR = relative growth rate. Data are expressed as the mean  $\pm$  SEM. a–e Means in the treatment groups not followed by the same letter differ significantly ( $P < 0.05$ ) whereas the differences were considered to be a statistical trend when  $0.05 < P < 0.10$



significantly greater than that in the DETA/NO group, especially in the NSe50 group ( $P < 0.01$ ).

#### Effect of Se on the Antioxidative and Immune Parameters of Cells Damaged by DETA/NO

The activities of T-SOD, T-AOC, CAT, GPx, and TrxR, as well as the concentration of SelP significantly decreased in the DETA/NO group ( $P < 0.05$ ) compared with the control group. Compared to the DETA/NO treatment group, Se treatment significantly reversed DETA/NO induced changes in a quadratic dose-dependent manner ( $P < 0.01$ ). The activities of T-AOC, GPx, and TrxR in the NSe50 and NSe100 groups were significantly greater than the DETA/NO group ( $P < 0.05$ ); however, there was no difference between the DETA/NO, NSe10, and NSe200 groups ( $P > 0.05$ ). The activities of CAT in the NSe10, NSe20, and NSe50 groups were significantly greater than the DETA/NO group, particularly in the NSe50 group ( $P < 0.01$ ). T-SOD activities in all the prevention groups were higher than that in the DETA/NO group, especially in the NSe50 group ( $P < 0.05$ ). As expected, increased ROS, MDA, NO, TNF- $\alpha$ , IL-1 $\beta$ , and IL-6 generation was detected in BMECs after stimulation with DETA/NO alone ( $P < 0.05$ ). Change in iNOS activity exhibited the similar pattern. However, pretreatment with 50 nmol/L Se significantly reduced DETA/NO-induced ROS and MDA production ( $P < 0.05$ ). There was a significant decrease in NO concentrations and iNOS activities in the NSe10, NSe20, and NSe50 groups as compared with those in the DETA/NO group ( $P < 0.05$ ), and the minimum concentration was observed at 50 nmol/L Se. TNF- $\alpha$  and IL-1 $\beta$  concentrations in the NSe20 and NSe50 groups and IL-6 concentration in the NSe100 group were lower than in the DETA/NO group;

however, there was no significant change in the other Se prevention groups compared with the control group ( $P > 0.05$ ) (Table 2).

#### Effect of Se on Gene and Protein Expressions in BMEC Damaged by DETA/NO

A significant decrease in the gene expression of *Nrf2* and *SelP* and the gene/protein expression of *GPx1* and *TrxR1* was observed after 6 h of incubation with 1000  $\mu$ mol/L DETA/NO compared to the control ( $P < 0.05$ ). However, gene expression levels of *TNF- $\alpha$* , *IL-1 $\beta$* , and *IL-6* as well as the gene and protein expression levels of iNOS increased dramatically in response to DETA/NO injury ( $P < 0.05$ ). A similar augment was also observed in the gene expression of three mitogen-activated protein kinases (*p38MAPK*, *JNK*, *ERK1/2*) in DETA/NO group when compared with the control ( $P < 0.05$ ). However, Se treatment significantly upregulated the gene expression of *Nrf2*, *TrxR1*, *SelP*, and *GPx1* relative to those in DETA/NO group in a quadratic dose-dependent manner ( $P < 0.01$ ), and the greatest increase in those gene expressions were in BMEC treated with 50 nmol/L Se compared with the other Se prevention groups. Changes in GPx1 and TrxR1 protein expressions exhibited the similar patterns. There was a significant decrease in *IL-1 $\beta$*  and *iNOS* gene expressions in the NSe20, NSe50 group as compared with those in the DETA/NO group ( $P < 0.05$ ), and the minimum concentration was observed at 50 nmol/L Se. *TNF- $\alpha$*  and *IL-6* gene expressions and iNOS protein expression in all the Se prevention group were significantly lower than those in the DETA/NO group ( $P < 0.05$ ), especially in the NSe50 group. As we have noticed, the gene expression levels of *p38MAPK* (NSe10, NSe20, NSe50, NSe100, NSe150 groups) and *JNK* (NSe20, NSe50, NSe100, NSe150 groups) in Se-treated cells were significantly elevated with respect to those of DETA/NO

**Table 2** Effects of Se on antioxidant indexes and inflammatory cytokine in BMEC damaged by DETA/NO

Items	CON	DETA/ NO	NSe10	NSe20	NSe50	NSe100	NSe150	NSe200	SEM	P value	Treatment	
											Linear	Quadratic
T-SOD (IU/mL)	20.13a	13.96e	15.91d	17.82c	20.41a	18.78bc	17.52c	17.37c	0.49	< .0001	0.0036	< .0001
T-AOC (IU/mL)	2.57a	1.27c	1.79bc	1.97abc	2.24ab	2.28ab	1.88abc	1.56bc	0.20	0.0153	0.8804	0.0048
CAT (IU/mL)	2.72a	1.60de	2.18c	2.21bc	2.55ab	1.96cd	1.72de	1.42e	0.11	< .0001	0.0003	0.0003
GPx (IU/mg Pr)	34.70a	17.38c	21.93bc	21.18bc	38.71a	36.29a	29.97ab	23.27bc	2.41	< .0001	0.0881	< .0001
TrxR (pg/mL)	55.40ab	27.94e	29.05e	47.26abc	59.37a	43.81bcd	41.11cde	31.79de	4.00	< .0001	0.8909	< .0001
SeIP (ng/mL)	10.33ab	9.28c	9.75bc	9.77bc	10.83a	9.98abc	10.09abc	10.49ab	0.27	0.0211	0.0010	0.0572
MDA (nmol/mg Pr)	2.66c	3.53ab	3.49ab	3.17bc	2.60c	2.99bc	3.29ab	3.84a	0.16	0.0007	0.1645	0.0002
ROS (IU/mL)	119.63c	160.66a	146.84ab	154.19ab	133.01c	143.53abc	168.16a	157.13ab	7.30	0.0079	0.2571	0.1288
iNOS (IU/mL)	5.66c	14.87a	9.23bc	8.05c	7.33c	7.32c	9.47bc	12.61ab	1.06	0.0004	0.7285	0.0002
NO (umol/L)	186.17b	217.06a	192.63b	188.35b	181.35b	206.24a	215.41a	218.74a	3.62	< .0001	< .0001	0.0031
TNF- $\alpha$ (pg/mL)	172.56c	210.69a	199.91ab	190.06b	188.75b	201.42ab	208.50a	209.91a	3.92	0.0097	0.0378	0.0491
IL-1 $\beta$ (pg/mL)	116.84d	151.18a	130.88b	129.16bc	121.37cd	136.00b	146.24a	148.02a	2.44	< .0001	0.0002	< .0001
IL-6 (pg/mL)	287.07d	320.93abc	308.53cd	314.40bc	317.60abc	285.60d	338.27ab	343.10a	7.70	0.0004	0.0178	0.0114

SEM means standard error of the mean; The values in the same row with the same letters mean no significant difference ( $P > 0.05$ ), while with different letters mean significant difference ( $P < 0.05$ )

in a quadratic dose-dependent manner ( $P < 0.05$ ), the minimum level was observed at 50 nmol/L Se. The gene expression of ERK1/2 did not differ significantly among groups ( $P > 0.05$ ) (Table 3 and Figs 2, 3, 4, 5).

## Discussion

Changes in cell viability, antioxidant indices, and proinflammatory cytokine are the primary indicators of cell antioxidant and immune status. Under physiological conditions, free radicals participate in signal transduction, while excess free radicals are removed by the antioxidant defense system. Exposure to exogenous stimulus leads to the release and accumulation of large amounts of free radicals. Excessive generation of free radicals promotes DNA, protein, as well as organelle damage and triggers apoptosis as well as cytokine release [12, 13]. Cellular events occurring during oxidative stress are always associated with inflammatory responses. At the site of an acute inflammatory reaction, pro-inflammatory substances and inflammatory mediators can also induce the synthesis and release of NO [14]. Excessive production of NO leads to the formation of reactive and toxic intermediates generically termed reactive nitrogen species (RNS) [15], the blocking of signaling pathway in cells and the occurrence of uncontrolled systemic inflammation, resulting in further tissue damage. Jin et al. found that Se supplementation protected against cadmium-induced apoptosis and reduced the production of NO as well as the activity and expression of iNOS in chicken pancreas [16]. On the contrary, Se deficiency induced higher inflammatory damage in chicken intestinal tissues, which were accompanied by higher levels of iNOS and NO

[17], and even led to hepatocyte apoptosis in broiler by over-expressing micro-RNA-193b-3p and increasing apoptosis-related gene expression [18], indicating that Se played protective effects against tissue damage in chickens. The study of Hua et al. showed that IL-1 $\beta$  induced pronounced expression of iNOS, and stimulated production of NO, leading to more aggravated inflammation [19]. The related study of murine macrophages suggested that Se decreased the expression of genes and proteins of inflammatory factors [20], which reduced the release of inflammatory factors, playing a protective role in improving the anti-oxidation and anti-inflammatory function of cells. The current results indicated that the presence of DETA/NO significantly inhibited the cell proliferation and activities of antioxidant enzymes (TrxR, GPx, SelP, SOD, CAT, T-AOC) in BMEC, but increased the release of MDA; free radicals (ROS, NO); cytokines (TNF- $\alpha$ , IL-1 $\beta$ , IL-6); and expression of iNOS, suggesting that DETA/NO induced cell oxidative damage and cytokines produced further exacerbated oxidative stress. However, the Se prevention group exhibited changes that were reversed compared to the changes associated with the parameters described above for the DETA treatment group, indicating that Se protected BMEC from DETA/NO-induced oxidative damage, but little data on the protection mechanism is available.

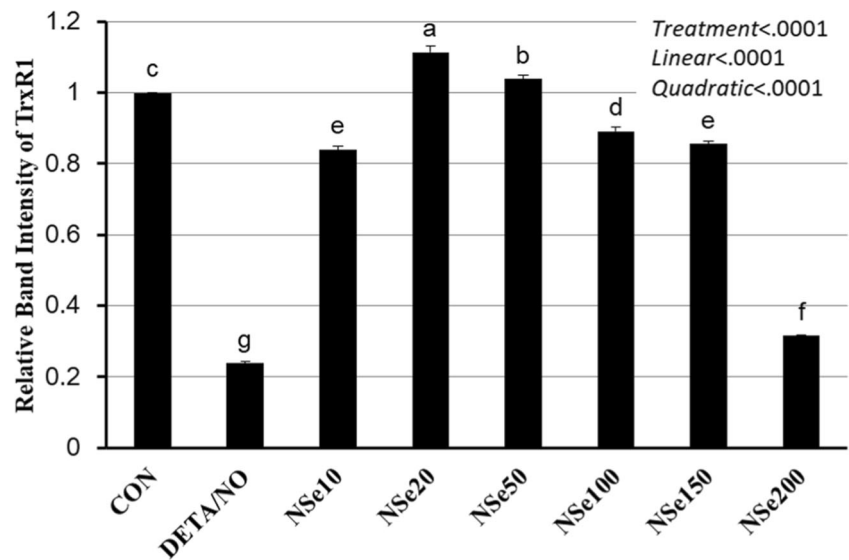
MAPK family consists of three types of protein kinase: p38MAPK, JNK and ERK; they can be activated by the diverse upstream kinases. An upstream kinase of the MAPK signaling pathway is apoptosis signal-regulating kinase-1 (ASK-1); therefore, a reduction in ASK-1 activity due to TrxR activity will result in the subsequent inhibition of MAPK signaling pathway [8]. Under normal circumstances, the reduced form of Trx binds to the N-terminal region of

**Table 3** Effects of Se on expression of mRNA involved in antioxidant indexes and inflammatory cytokine in BMEC damaged by DETA/NO

Items	CON	DETA/ NO	NSe10	NSe20	NSe50	NSe100	NSe150	NSe200	SEM	P value		
										Treatment	Linear	Quadratic
GPx1	1.00bc	0.37e	0.61de	0.79cd	1.48a	1.29ab	1.28ab	0.80cd	0.09	<.0001	0.0035	<.0001
GPx4	1.00b	0.89b	1.05ab	1.03ab	1.35a	0.94b	0.95b	0.84b	0.09	0.0608	0.1912	0.1561
TrxR1	1.00b	0.61d	0.75c	0.81c	1.19a	1.14a	0.96b	0.72c	0.05	<.0001	0.2647	<.0001
Nrf2	1.00b	0.52d	0.63d	0.92b	1.45a	1.08b	0.87bc	0.70cd	0.06	<.0001	0.5746	<.0001
SelP	1.00c	0.62d	1.16bc	1.53a	1.71a	1.55a	1.41ab	0.99c	0.08	<.0001	0.5365	<.0001
IL-1 $\beta$	1.00d	3.03a	1.86bcd	1.25cd	1.95bc	2.21ab	2.39ab	2.59ab	0.24	0.0002	0.2187	0.1784
IL-6	1.00d	6.26a	2.96bc	2.18cde	1.26de	2.05cde	2.56bcd	3.71b	0.37	<.0001	0.2087	<.0001
TNF- $\alpha$	1.00d	3.03a	1.92b	1.09d	0.98d	1.18cd	1.84bc	2.15b	0.20	<.0001	0.8693	<.0001
iNOS	1.00d	3.29ab	2.66abc	2.13c	1.81cd	2.46bc	3.13ab	3.54a	0.27	<.0001	0.0352	0.0065
P38MAPK	1.00c	2.21a	1.50bc	1.27bc	1.08c	1.49bc	1.45bc	1.87ab	0.17	0.0053	0.7725	0.0144
JNK	1.00c	1.89a	1.56ab	1.37bc	1.06c	1.21bc	1.35bc	1.67ab	0.13	0.0011	0.4897	0.0601
ERK1/2	1.00b	1.77a	1.65ab	1.34ab	1.38ab	1.45ab	1.40ab	1.97a	0.19	0.0783	0.6950	0.0018

SEM means standard error of the mean; The values in the same row with the same letters mean no significant difference ( $P > 0.05$ ), while with different letters mean significant difference ( $P < 0.05$ )

**Fig. 2** Effects of Se on protein expression of TrxR1 in BMEC damaged by DETA/NO

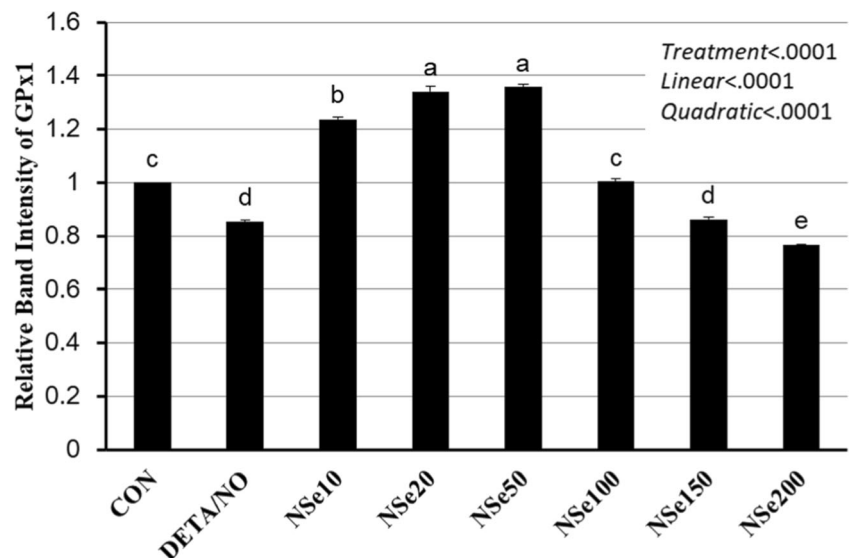


ASK-1 and inhibits kinase activity. Change in the redox status of Trx induces ASK-1 activation, and subsequent activation of the MAPK pathway. The MAPK cascade is one of the important signaling pathways in oxidative stress responses and responds to various types of stress [21]. It is said that activation of MAPK signaling pathway can induce the expression of a multitude of genes related to pro-inflammatory mediators such as chemokines, adhesion molecules, and cytokines. These pro-inflammatory mediators can regulate the expression of iNOS, which results in the release of large amounts of NO [22, 23]. The excessive release of NO activates MAPK, resulting in a positive feedback loop leading to an inflammatory response, and damage of articular chondrocytes in rabbits [24]. The addition of Se may inhibit MAPK signaling pathway by upregulating the expression of selenoprotein, resulting in downregulation of the expression of inflammatory factors. This downregulation of inflammatory factors promotes the

restoration of immune homeostasis and restoration of bovine health [25]. According to the present results, DETA/NO treatment significantly decreased the activities, gene and protein expression of TrxR and GPx, while activating the p38MAPK and JNK pathways. It is important to note that the ERK1/2 pathway was not activated by the DETA/NO treatment. Most of the DETA/NO-induced changes were significantly reversed in the Se prevention group. Therefore, we inferred that the ability of Se to protect BMEC from NO-induced oxidative damage and apoptosis was due to inhibition of the MAPK signaling pathway by increased TrxR activity. The inhibition of the MAPK pathway results in decreased IL-1 $\beta$ , IL-6, and TNF- $\alpha$  concentrations, which reduced the production of NO, and the activity and expression of iNOS.

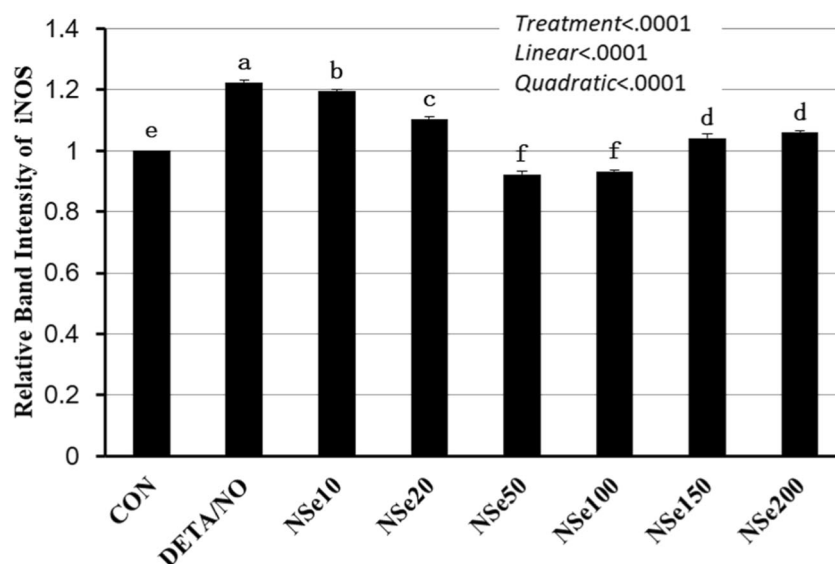
The Nrf2 pathway reduces inflammation by activating antioxidant cascades. Under homeostatic conditions the transcriptional activity of Nrf2 is suppressed by

**Fig. 3** Effects of Se on protein expression of GPx1 in BMEC damaged by DETA/NO





**Fig. 4** Effects of Se on protein expression of iNOS in BMEC damaged by DETA/NO

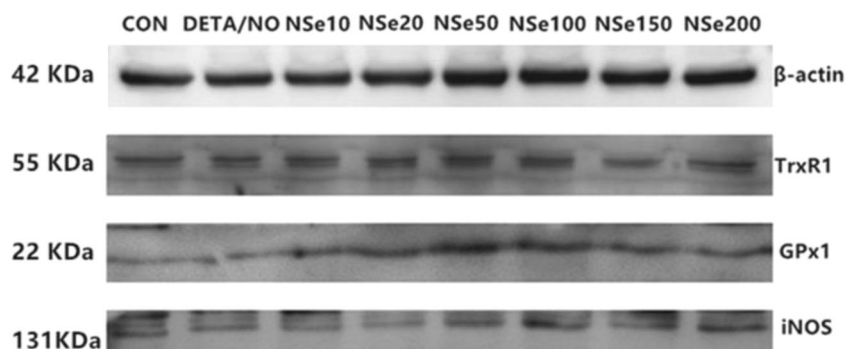


association with Kelch-like ECH-associated protein 1 (Keap1). However, under oxidative stress conditions, Nrf2 undergoes a rapid nuclear translocation, binding to electrophile response element (EpRE) and antioxidant response element (ARE). The ARE locates in the promoter regions of target antioxidant genes, which include heme oxygenase-1 (HO-1), NAD(P)H/quinone oxidoreductase 1 (NQO1), glutamate cysteine ligase catalytic subunit (GCLC), and TrxR1. Therefore, activation of the Nrf2 pathway induces the transcription of the above antioxidant genes [26]. Our results indicate that Se treatment can protect BMEC through upregulation of the Nrf2 pathway. Upregulation of the pathway resulted in increased expression and activity of TrxR, which promoted the antioxidant function of BMEC. In the current study, we did not silence the TrxR gene; therefore, the exact mechanism by which Se acts needs to be explored further.

At nutritional concentrations, Se can promote cell proliferation and enhance antioxidant ability and immune function. However, excessive Se results in decreased immunity, increased oxidative damage, and a series of clinical pathology changes [27]. Based on our results, the protective effect of Se is related to dose; where

10 nmol/L Se had no significant protective effects, while 20–100 nmol/L Se had some protective effects. The greatest antioxidant effect was observed when Se was included at 50 nmol/L. At Se concentrations greater than 50 nmol/L, the antioxidant effect appeared to plateau or decrease; particularly at 200 nmol/L, where no protective effect was observed, and cell damage was occurring. The cell damage was likely caused by Se toxicity, which can damage the cellular membrane and proteins due to Se-generated ROS [28, 29]. One of the main mechanisms of Se toxicity that has been hypothesized is that sulfur components of proteins are being substituted with Se, which disrupts trace mineral balance [28]. Still another route to SeMet toxicity could be the replacement of Met by SeMet which may influence the activity of enzymes if SeMet replaces Met in the vicinity of the active site [30]. This substitution may interrupt metabolic pathways and cascades in the organism, leading to metal disruption and increased oxidative stress [31]. Moderate concentrations of selenium can be stored in the organism and are used to maintain homeostatic functions. Due to the narrow concentration range between essentiality and toxicity, Se represents an important toxicological hazard [32].

**Fig. 5** Effects of Se on protein expression in BMEC damaged by DETA/NO



Potentially toxic dietary concentrations in ruminant feeds are considered to be 2 to 5 ppm, which is about 10- to 20-fold greater than dietary levels considered to be adequate [33]. Therefore, appropriate addition of Se is the basic prerequisite in order to obtain antioxidant and anti-inflammatory effects of Se, without toxicity occurring.

The addition of Se improves the antioxidant function of BMEC and protects cells from oxidative damage induced by DETA/NO. This is the result of upregulation of Nrf2, enhancing the activity of TrxR, which inhibiting the activation of MAPK signaling pathway. The inhibition of MAPK signaling pathway results in decreased concentrations of NO. Optimal Se dose is at 50 nmol/L, but antioxidant effects are observed when Se is included at 20 to 100 nmol/L. Greater concentrations of Se can cause damage to cells due to Se toxicity.

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