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Isoflavone Ameliorates H_2O_2 Induced Injury by Activating the Antioxidant System of Sow Mammary Gland Cell

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Abstract

The antioxidant and protective properties of a synthetic soybean isoflavone (ISO) were investigated using sow mammary gland cell. Cells were cultured with 10, 20 and 30 uM ISO, respectively, under 80 uM $FeSO_4 \cdot 7H_2O/H_2O_2$ conditions. After 48 h of incubation, the cells in the presence of ISO were lost less compared with that of control under oxidative damage by $H_2O_2/FeSO_4$; ISO decreased the cell number at G1 and G2 stages, increased the cell number at S stage (all $P < 0.05$), it also reduced apoptosis of the cells ($P < 0.01$, $P < 0.01$, $P < 0.05$). The addition of ISO significantly promoted cell proliferation ($P < 0.05$) from 3rd to 6th days. Upon these, the activities of total superoxide dismutase (SOD), glutathione peroxidase (GPX), total antioxidant ability (T-AOC) also were increased and the activities of NADPH oxidase (NOX) decreased by ISO treatment (all $P < 0.05$). ISO decreased the concentration of ROS ($P < 0.05$) and MDA ($P < 0.05$). 10 uM, 20 uM, 30 uM ISO increased the relative mRNA abundance of SOD1 (all $P < 0.05$) and SOD2 ($P < 0.05$, $P < 0.01$, $P < 0.05$) and SOD3 (all $P < 0.05$); ISO significantly increased the relative mRNA abundance of GPX4 (all $P < 0.01$) and NOX4 (all $P < 0.05$), 20 uM ISO also increased the relative mRNA abundance of NOX2 ($P < 0.05$). It was concluded that supplementation of ISO enhanced the anti-oxidative function and prevented lipid peroxidation, possibly through the activation of the antioxidant enzymes and inhibition of cell apoptosis.

Keywords

Isoflavone, Antioxidant System, Sow Mammary Gland Cell

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1. Introduction

Recently, more and more researches showed that soybean isoflavones (ISO) have been shown to possess antioxidant activity [1] [2], which might be related to their anticancer, anti-inflammatory, and cardio-protective function. Some of the biological actions of ISO were partly attributed to their antioxidant properties, either through their reducing capacities or through their influence on intracellular redox status [3]. The phenolic compounds of ISO can be direct, stemming from their activity as free radical scavengers, or indirect, as modulators of intracellular pro- and anti-oxidant enzymes [4]. In research on human subjects, dietary ISO decreased risks of cardiovascular disease, osteoporosis, breast and prostate cancer, and menopausal symptoms [5] [6]. In animal models, dietary isoflavone enhanced the antioxidant function and prevented lipid oxidation of chicken [7]. Yousef *et al.* [8] found that ISO decreased the production of free radicals in plasma, liver, brain, testes, and kidney of male rabbits. Liu *et al.* [9] found that diets containing ISO (150 and 250 mg/kg) obviously elevated antioxidant enzymatic levels such as superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPX) in various organs of rats fed diets containing partially oxidized oil. Our previous study [10] showed that dietary ISO improved the activities of antioxidant indices and productive performance of sows. Based on the results, we further investigated the mechanism of ISO playing their antioxidant function through the mammary cell culture experiment. Chen *et al.* [11] used MCF-7 human breast cancer cells as model and found that ISO induced apoptosis of this cell. Peng & Kuo [12] used Caco-2 intestinal cells as model to research the antioxidant function and related mechanism of ISO. However, very few studies have looked at their antioxidant effects in domestic animal cells. This is the first time we investigate the antioxidant function and related mechanism of ISO on normal sow mammary cell, which will provide an important basis for the research of human breast health.

2 Materials and Methods

2.1. Cell and Chemicals

Sow mammary cell line was obtained from Department of Animal Science, Texas A&M University, USA, and were maintained according to their recommendation. The soybean isoflavone (ISO) was from Guangdong New Land Co., and its purity was >98%. Dimethylsulfoxide (DMSO) and 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazoliumbromide (MTT) were purchased from Sigma Chemical Co. (St. Louis, MO, USA).

2.2. Cell Culture and Treatment

Cells were cultured with an initial concentration of 5×10^5 cells/ml, and cultured in 96-well microtiter plates for both morphology observation and cell proliferation assay. Others chemical indexes were tested using cells cultured in 6-well microtiter plates. The ISO was diluted in DMSO and added into the serum-free media to reach final concentrations of 10, 20 and 30 uM. The control cells were similarly treated with the same amount of DMSO (0.025% (v/v)). Oxidative damage was introduced by adding 80 uM of ferrous ions (as $FeSO_4 \cdot 7H_2O$) and H_2O_2 to each well for 2 hours.

2.3. Observation of Cell Morphology

Cells were incubated for 48 h in the presence or absence of ISO (0, 10, 20 and 30 uM). The morphological changes of the cells were observed using phase-contrast microscopy (Axiovert 25 HBO 50/AC, Carl Zeiss, Jena, Gemany).

2.4. Cell Proliferation Assay

The colorimetric MTT metabolic activity assay [13] was used to determine relative numbers of living cells. Mammary cells (1×10^5 cells/well), from above, were cultured in 96-well plates at 37˚C, and exposed to varying concentrations (0 - 30 uM, n = 12, each) of ISO for 1 - 6 d. After incubation with 80 uM of ferrous ions, the medium was removed and the cells were washed twice with PBS, 20 uL of MTT solution (5 mg/mL in PBS) and 100 uL of fresh medium were added and incubation continued for another 4 h. The resultant formazan crystals were dissolved in DMSO (100 uL) and the absorbance was measured (Spectra Max M5, USA) at 490 nm.

2.5. Cell Cycle and Cell Apoptosis Identification

Cells were incubated with ISO for 48 h in 6-well plates according to the condition from above. Cell cycle distributions were determined by measuring the celluar DNA content using flow cytometry. Cells were washed with 700 mL/L ethanol for 20 min and stored at 4˚C overnight, then washed with PBS, and stained with 100 uL of 50 mg/L PI at 4˚C for 30 min. The cells were then subjected to flow cytometric analysis on a FACS cancytofluorimeter (BD Co., USA). Cell apoptosis were analyzed using cell apoptosis testing kits (BD Co., USA) according to manufacturer's instructions.

2.6. Biochemical Determinations

For enzymes and malondialdehyde (MDA), triphosphopyridine nucleotide (NADPH) measurement, the cells were harvested by scraping in a buffer containing 80 mmol/L potassium phosphate and 5 mmol/L EDTA, pH 7.6. The activities of total SOD (T-SOD), GPX, CAT and the total antioxidant ability (T-AOC) was measured using colorimetric methods with a spectrophotometer (Biomate 5, Thermo Electron Corporation, Rochester, NY, USA) and the assay kits purchased from Nanjing Jiancheng Institute of Bioengineering (Nanjing, Jiangsu, PR China) according to the appropriate procedures. Cellular content of MDA and NO was determined with commercial test kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China).

2.7. Measurement of ROS Level in the Cells and Medium

Intracellular levels of ROS were measured as described by Sen *et al.* [14]. Cells were washed and re-suspended in 200 uL of DMEM/F19 and were loaded with the cell-permeate probe 20, 70-dichlorodihydro-fluorescein diacetate (H_2DCFDA), at a final concentration of 40 uM. After incubation for 30 min at 37˚C in the dark, fluorescence was measured with the spectrofluorimeter using 507 nm as excitation and 530 nm as emission wavelengths. Fluorescence intensities were expressed on the basis of mg protein in each culturedish.

2.8. Isolation of RNA and Real-Time PCR

At the end of the experiment, the cells were harvested for RNA extraction by first removing the medium and washing the cells briefly in PBS and then scraped in the lysis buffer from the RNeasy Mini Kit (Qiagen GmbH, Hilden, Germany). Total RNA was extracted from cell samples using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. The quality and quantity of RNA was assessed by OD_{260}/OD_{280} and gel electrophoresis. The cDNA was produced using a commercial kit containing Reverse Transcriptase XL (AMV) and RNA sin (Invitrogen Co., USA). Real-time PCR was performed using with qPCR Mix (TaKaRa, BIOINC, Japan) according to the manufacturers' instructions. The DNA was sequenced by Sangon Technical Co. Ltd. (Shanghai, China). Primers of the genes were designed and listed in **Table 1**. The real-time PCR cycling conditions used were 95˚C for 40 s, followed by 40 cycles at 95˚C for 5 s and 58˚C for 20 s, then onecycle at 72˚C for 30 s. The Ct (threshold cycle) value for each reaction was used to calculate gene expression, relative to β-actin mRNA, expressed as $2^{-\triangle\triangle Ct}$.

2.9. Statistical Analysis

Data were expressed as means ± SE. The effects of ISO at the various concentrations were compared with that of control by one-way analysis of variance using computing software SAS (v6.12, SAS Institute, USA). *P*-values < 0.05 were considered to be significant and *P*-values < 0.01 were considered to be extremely significant.

3. Results

3.1. Cells Proliferation Analysis

Cell proliferation rate of mammary gland cell was tested from d 1 to day 6 by MTT method (**Figure 1**). MTT assay indicated that ISO treatment did not affect the proliferation rate of mammary gland cells at the first 2 days compared with the control ($P > 0.05$); 20 uM ISO increased the proliferation rate of mammary gland cells at the 3rd and 4th days ($P < 0.05$); 10 uM, 20 uM ISO increased the proliferation rate of cells at the 5th and 6th

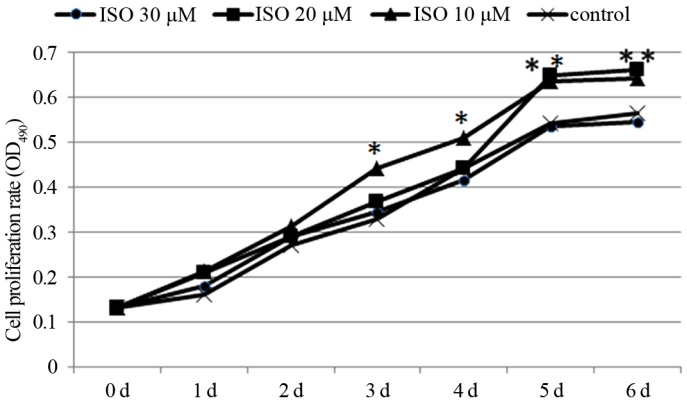

Figure 1. Effects of ISO on cell proliferation from 0 d to 6 d. "*" indicates significantly different from control ($P < 0.05$); "**" indicates extremelysignificantly different from control ($P < 0.01$).

Table 1. The primer sequence of selected genes.

Gene name	Primer sequence
CAT	Up 5'-CCTCAAGTACGTCCGACCAG-3' Down 5'-TTCCATGCGATGTCATTGCG-3'
SOD1	Up 5'-GGTCCTCACTYCAATCCTG-3' Down 5'-GGTCCTAACTTCAATCCTG-3'
SOD2	Up 5'- GATCACCATG TTGTGCAGG-3' Down 5'-GTTGTTCACGTAGGCCGCGT-3'
SOD3	Up- 5'- GAGCCCAGCC CCGACCTGAT-3' Down 5'-CAGCGTGGCGGACGGCTGCA-3'
GPX1	UP 5'-CCCACGCTCGGTGTATGCCT-3' DOWN 5'-AGAAAGCGACGGCTGTAC-3'
GPX4	Up 5'-TGTGGTTTACGGATTCTGG-3' DOWN 5'- CCTTGGGCTGGACTTTCA-3'
NOX2	Up 5'- ACCCTTTCACCCTGACCTCT-3' Down 5'-AATCCCTGCTCCCACTAACA-3'
NOX4	Up 5'-TGGAACGCACTACCAGGATG-3' Down 5'-TTCGGCACAATACAGGCACA
NOX5	Up 5'-GCCTGGCGACTACTTGTATCTG-3' Down 5'-CTTCCTCTGACTCCTTCTCATTTTC-3'
Beta-actin	Up 5'- GACCTGACCGACTACCTCAT -3' Down 5'- GCCGATGGTGATGACCTGGC -3'

days ($P < 0.05$).

3.2. Cell Cycle and Cell Apoptosis Analysis

Under oxidative damage by $H_2O_2/FeSO_4$, the cells in the presence of ISO were lost less compared with that of control, and the cells cultured with 10 uM or 20 uM ISO grew obviously better (**Figure 2**). In the analysis of apoptosis, 10 uM, 20 uM and 30 uM ISO treatment reduced apoptosis of the mammary gland cells compared to the control ($P < 0.01$, $P < 0.01$, $P < 0.05$) respectively (**Figure 2**). In the analysis of the cell cycle, ISO (10 - 30 uM) decreased the cell number at G1 and G2 stages, increased the cell number at S stage ($P < 0.05$) (**Figure 3**).

3.3. Biochemical Analyses

The activities of antioxidant or pro-oxidant enzymes were listed in **Table 2**. Compared with the control, 10 uM, 20 uM, 30 uM ISO increased the activities of SOD by 23.98%, 33.69%, 17.89% and decreased the activities of

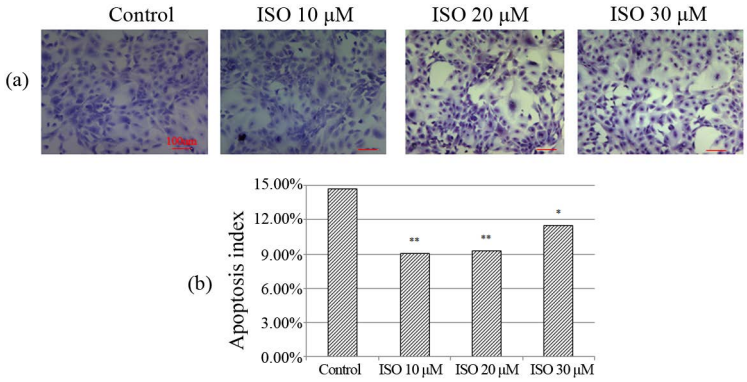

Figure 2. Effects of ISO on the morphology and apoptosis index of sow mammary gland cells. (a) Themorphology of the cell under different concentration of ISO; (b) The apoptosis index analysis of the cells. "*" indicates significantly different from control ($P < 0.05$); "**" indicates extremely significantly different from control ($P < 0.01$).

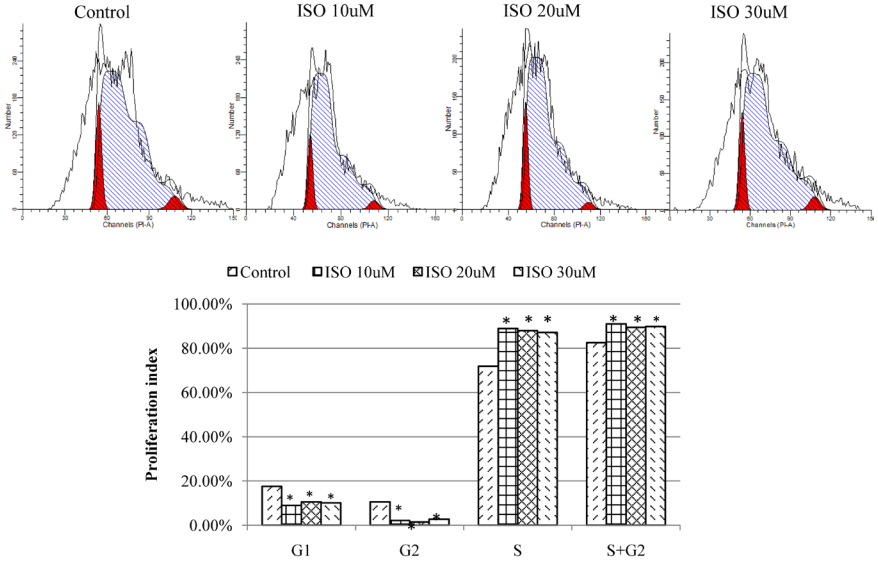

Figure 3. Effects of ISO on cell cycle of sow mammary gland cells. Cellcycle was analyzed by using proliferation index. "*" indicates significantly different from control ($P < 0.05$).

Table 2. Effects of ISO on the activities of antioxidant or pro-oxidant enzymes in cells (n = 16).

Treatment	Control	ISO 10 uM	ISO 20 uM	ISO 30 uM
SOD (U/mgprot)	15.76 ± 1.14	19.54 ± 1.59*	21.07 ± 1.22*	18.58 ± 1.26*
CAT (U/mgprot)	0.89 ± 0.06	1.03 ± 0.08*	1.12 ± 0.10*	1.04 ± 0.14
GSH-Px (U/mgprot)	51.85 ± 6.33	84.33 ± 4.55**	89.12 ± 6.19**	69.24 ± 5.22*
T-AOC (U/mgprot)	2.81 ± 0.15	4.60 ± 0.24**	3.76 ± 0.15**	3.39 ± 0.23*
NOX (ng/ml)	0.54 ± 0.03	0.42 ± 0.03*	0.40 ± 0.02*	0.45 ± 0.03*
ODC (ng/ml)	1.15 ± 0.12	1.48 ± 0.20	1.27 ± 0.11	1.29 ± 0.17

"*" indicates significantly different from control (P < 0.05); "**" indicates extremely significantly different from control (P < 0.01).

NADPH oxidase (NOX) by 22.22%, 25.93%, 16.67% (all $P < 0.05$); 10 uM, 20 uM ISO significantly increased the activities of GPX and T-AOC ($P < 0.01$), and 30 uM ISO increased the activities of GPX and T-AOC ($P < 0.05$); 10 uM and 20 uM ISO increased the activities of CAT ($P < 0.05$). ISO treatment did not affect the activity

of ornithine decarboxylase (ODC) ($P > 0.05$).

3.4. ROS, MDA and NO Analysis

From **Table 3**, compared with the control, 10 uM, 20 uM, and 30 uM ISO decreased the concentration of ROS ($P < 0.05$); 10 uM, 20 uM ISO decreased the concentration of MDA in the cells ($P < 0.05$); 10 uM, 20 uM, and 30 uM ISO did not affect the concentration of NO in the cells ($P > 0.05$).

3.5. Relative mRNA Expression in Mammary Gland Cells

The result from **Table 4** showed that ISO did not affect the relative mRNA abundance of CAT compared with the control; 10 uM, 20 uM, 30 uM ISO increased the relative mRNA abundance of SOD1 (all $P < 0.05$) and SOD2 ($P < 0.05$, $P < 0.01$, $P < 0.05$) and SOD3 ($P < 0.05$, $P < 0.05$, $P > 0.05$); ISO significantly increased the relative mRNA abundance of GPX4 (all $P < 0.01$), but did not affect relative mRNA abundance of GPX1except for 20 uM ISO; 10 uM, 20 uM, 30 uM ISO increased the relative mRNA abundance of NOX4 (all $P < 0.05$), 20 uM ISO also increased the relative mRNA abundance of NOX2 ($P < 0.05$), but did not affect the relative mRNA abundance of NOX5 (all $P > 0.05$).

4. Discussion

Our results showed that ISO stimulated the mammary gland cells proliferation by inducing cell accumulation in M or S phases and decreasing the cells number at G1 and G2 phases. Matsukawa *et al.* [15] found that the proliferation of human gastric cancer cells (HGC-27) was inhibited by ISO and most of the cells were arrested in the M phase. Cell cycle arrest is a characteristic of cells and the cell cycle progresses through different phases commonly referred to as checkpoints [16]. Cell cycle checkpoints are transient delays in S or M phase to response to DNA damage by cellular stressors, including ROS, and allow time for the activation of repair mechanisms [17]. Following repair of damaged DNA, cells resume cell cycle progression. However, if the damage is too severe, the cells may undergo apoptosis or irreversible senescence [18]. ROS caused DNA damage and induced M phase arrest and apoptosis [19] and genistein inhibited it [20] [21]. Other reports [22] [23] showed that genistein (one kind of ISO) induced cell cycle arrest and cell apoptosis because of its anti-angiogenesis and

Table 3. Effects of ISO on the content of oxidant products in cells (n = 16).

Treatment	Control	ISO 10 uM	ISO 20 uM	ISO 30 uM
ROS (photons/min)	63.11 ± 9.12	39.44 ± 4.87*	43.92 ± 4.59*	51.04 ± 6.33*
MDA (nmol/mL)	1.95 ± 0.24	1.47 ± 0.11*	1.62 ± 0.23*	1.71 ± 0.12
NO (nmol/mL)	9.52 ± 0.78	8.19 ± 0.97	8.37 ± 1.11	8.87 ± 1.02

"*" indicates significantly different from control ($P < 0.05$).

Table 4. Effects of ISO on the genes expression related with antioxidant enzymes in cells (n = 16).

Treatment	Control	ISO 10 uM	ISO 20 uM	ISO 30 uM
CAT ($\times 10^{-2}$)	1.16 ± 0.22	1.26 ± 0.37	1.01 ± 0.46	1.23 ± 0.33
SOD1 ($\times 10^{-2}$)	0.75 ± 0.08	1.94 ± 0.21*	1.83 ± 0.34*	1.34 ± 0.24*
SOD2 ($\times 10^{-3}$)	2.56 ± 0.33	3.48 ± 1.09*	4.17 ± 1.10**	3.14 ± 0.54*
SOD3 ($\times 10^{-3}$)	0.94 ± 0.08	1.45 ± 0.13*	1.38 ± 0.15*	1.26 ± 0.26
GPX1 ($\times 10^{-3}$)	2.13 ± 0.44	2.56 ± 0.34	3.49 ± 0.54*	3.15 ± 0.46
GPX4 ($\times 10^{-2}$)	0.69 ± 0.10	2.88 ± 0.34**	3.16 ± 0.39**	2.40 ± 0.45**
NOX2 ($\times 10^{-3}$)	5.66 ± 0.54	7.04 ± 0.88	8.12 ± 1.03*	6.12 ± 1.01
NOX4 ($\times 10^{-2}$)	0.79 ± 0.11	1.24 ± 0.08*	1.31 ± 0.12*	1.18 ± 0.20*
NOX5 ($\times 10^{-3}$)	1.02 ± 0.23	1.89 ± 0.21	1.67 ± 0.27	1.87 ± 0.32

"*" indicates significantly different from control ($P < 0.05$); "**" indicates extremely significantly different from control ($P < 0.01$).

antioxidant activities. In the present research, it also implied that ISO increasing the cell proliferation and decreasing the cell apoptosis by affecting cell cycle checkpoint were related with its antioxidant function. In recent years, ROS and antioxidant usage have received significant attention because aberrant production or regulation of ROS activity has been shown to contribute to development of some prevalent diseases and conditions [24]. Dietary flavonoids appeared to play a role in prevention of a number of chronic diseases, such as cancer and cardiovascular disease, with a particular focus on isoflavones [25].

Several previous studies revealed that genistein prevented apoptotic cell death via its antioxidant properties [26] [27]. The present study demonstrated an important role of ISO in decreasing ROS generation and thereby exerting an antioxidant effect. It is known that intracellular ROS are produced by mitochondria as well as by a number of ROS-generating plasmamembrane and cytosolic enzymes [28]. Among the ROS-producing enzymes, the NADPH oxidase (NOX) family was the major source of non-mitochondrial ROS in many cells, including phagocytes and non-phagocytes [29]-[31]. The present study indicated that ISO decreased the activities of NOX. Consistent with the present results, the inhibitory role of genistein in regulating NOX also was demonstrated in human oral squamous carcinoma cells [32] and in a diabetic mouse model subjected to chronic i.p. treatment with genistein [33]. ROS attacked the membrane polyunsaturated fatty acids, leding to lipid peroxidation and cell membrane function lost, so detecting the content of ROS and MDA can indirectly reflect the cells by free radicals attack severity. Our research showed that 10 – 30 uM ISO decreased the content of ROS and MDA in mammary cell and increased the activities of SOD, GPX and T-AOC *et al.*, especially the activity of GPX. In this present study, the GPX activity was found to be around 70 U/g of protein using ISO treatment, which was higher than the mean activity found in bovine mammarytissue (mean 33 U/g, range 4 - 62 U/g) [34]. SOD, GPX and CAT were the three main enzymes of the antioxidant system, and our result showed that ISO protected the antioxidant system of the sow mammary cells. Choi *et al.* [35] reported that genistein increased the activities of SOD, decreasing the cell apoptosis, which implied that the increase of SOD activity was related with the decrease of cell apoptosis.

We determined the mRNA level of SOD1, SOD2, and SOD3 present in sow mammary gland cells because these three enzymes acted spatially to maintain steady-state concentrations of $O2^{-}$ through conversion to H_2O_2. In the present research, ISO increased the mRNA level of SOD1, SOD2 and SOD3. GPX was taken as the main enzymes removing the peroxide from cytoplasm. It also was the main selenoprotein in mammary gland cells. GPX4 could savage the ROS like other members of GPX family [36], it also has special antioxidant function，playing very important function in regulating cell apoptosis. Only GPX4 could transform the PLOOH located in the cell membrane to corresponding hydroxyl compound, terminating the peroxidation and protecting the membrane from oxidative damage [36]-[38]. GPX 3 was the only selenoprotein thus far detected in milk [39] [40] together with an observation of SeP in mouse milk [41], and its expression has been detected in bovine, human and mouse mammary tissue [34] [42], the mRNA abundance of GPX1 and GPX4 also were detected in bovine mammary gland cells [43]. In the present study, the gene expression of GPX4 and GPX1 was increased by ISO. These results showed that GPX in sow mammary gland cells respond to ISO supplementation, and that the changes in their mRNA level are similar to the one found in bovine mammary tissue and other systems. Sow mammary gland cell is therefore as a useful model system for studying the GPX or selenoprotein biosynthesis. As NOX family was the major source of non-mitochondrial ROS in many cells [30] [31]. We also identified the gene expression of NOX (NOX2, NOX4 and NOX5) in sow mammary cells treated by ISO. The result indicated that ISO decreased the gene expression of NOX2 and NOX4, which consistent with the activity of NOX enzyme results. Taken together, ISO increased the genes expression of SOD and GPX with concomitant decreases in NOX, indicative of decreased oxidative stress.

5. Conclusion

The results presented here suggested that supplementation of ISO enhanced the anti-oxidative function and prevented lipid peroxidation, possibly through the activation of the antioxidant enzymes and inhibition of cell apoptosis.

Acknowledgements

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