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Effect of Low-Selenium/High-Fat Diet on Pig Peripheral Blood Lymphocytes: Perspectives from Selenoproteins, Heat Shock Proteins, and Cytokines

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Abstract The aim of the present study was to clarify the effect of low selenium (Se)/high fat on the mRNA expression of selenoproteins, heat shock proteins (HSPs) and cytokines in pig peripheral blood lymphocytes. Forty crossbred boar piglets with healthy lean body weights of 10 kg were randomly divided into four treatment groups (group C, group L-Se, group H-fat, and group L-Se-H-fat) (n = 10/group) and fed with the corresponding diet for 16 weeks. The pig peripheral blood lymphocytes were extracted, and the mRNA expression of selenoproteins, HSPs, and cytokines was measured. Most mRNA levels for selenoproteins decreased in group L-Se, group H-fat, and group L-Se-H-fat, except Gpx1, Gpx2, Selt, and Selm, which were elevated in group H-fat. At the same time, low-Se/high-fat diet increased the expression of HSPs (HSP40, HSP60, HSP70, and HSP90) and inflammatory cytokines (IL-1α, IL-1β, IL-6, IL-8, IL-9, iNOS, COX-2, NF-κB, and TNF-α) in group L-Se, group H-fat, and group L-Se-H-fat, and genes in group L-Se-H-fat showed greater increases. Also, low-Se/high-fat diet inhibits the expression of TGF-β1 and IFN-γ. In summary, a low-Se/high-fat diet can cause relevant selenoprotein expression changes and promote the expression of pro-inflammatory factors and HSPs, and low Se enhances the expression of HSPs and inflammation factors induced by high fat. This information is helpful for understanding the effects of low-Se and high-fat diet on pig peripheral blood lymphocytes.

All authors have read the manuscript and have agreed to submit the manuscript in its current form for consideration for publication in this journal.

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Keywords Low selenium · High fat · Lymphocyte · Selenoprotein · Heat shock protein · Inflammatory cytokine

Introduction

Se is a trace element that plays an important role in animal and human immune function. Long-term Se deficiency decreases animal serum antibody concentrations, reduces disease resistance, and significantly suppresses the immune response [1]. The content of Se in the diet regulates the activity of phagocytic cells to kill microorganisms [2]. Animals fed with Se-deficient diets have weakened organismal resistance, a reduced B lymphocyte population, and a decreased response of lymphocyte blastogenesis to mitogens [3, 4]. Fat is a necessary substance to maintain human and animal health, and it plays a role in maintaining cell structure and function, protecting the body's tissues, providing energy, and other functions. However, studies indicate that excessive intake of fat not only causes obesity but also promotes the production of inflammatory factors [5, 6]. High-fat diet can cause changes in the expression of systemic inflammatory factors, and inflammatory factors such as IFN-γ can regulate adaptive immunity in obesity [7, 8]. Long-term high-fat diet also interferes with insulin signaling pathways, leading to peripheral insulin resistance and immune disorders [9]. Blastogenesis of spleen lymphocytes was markedly inhibited when rats were fed with a high-fat diet [10]. A high-fat diet also significantly decreased the number of regulatory T cells in obese adipose tissue and led to an inflammatory state with insulin resistance [11].

HSPs and cytokines are reliable indicators of changes in immune status [12]. It has been demonstrated that Se deficiency causes higher expression of HSPs and leads to oxidative stress [13]. Immune function damage also significantly changes HSP expression [14, 15]. Also, lack of Se

induced the production of immune-suppressive factors and promoted the release of oxygen metabolites [16]. Se deficiency can cause inflammation, thus increasing the expression of pro-inflammatory factors (IL-1γ, IL-6, and IL-7) and damaging the immune system [17]. The same phenomenon occurred in rats, for example, levels of IL-6, IL-1β, and TNF-α increased when rats were fed with a Se-deficient diet [18]. Studies have shown that selenium can regulate the changes in animals [19]. Selw served as an antioxidant in chicken myoblasts [20]. Selk knockout exhibited specific immune cell defects in mice [21]. In terms of immune cell function, selenoprotein deficiency leads to oxidant hypertonicity in T cells and thereby suppresses T cell proliferation in response to T cell receptor stimulation [22]. Thus, Se and the regulation of selenoproteins by Se play important roles in immune function. The levels of inflammation-related cytokines (IL-1, IL-6, and IFN-γ), which are synthesized and secreted by immune cells in obese individuals, were boosted tremendously [8]. Stephen E et al. have reported that a high-fat diet increases tissue expression of TNF-α [23]. HSPs have been identified as adipokines; among them, HSP60 has been well described as the most closely related to metabolic and immune disorders caused by obesity [24].

The above studies suggested that low-Se and high-fat diet could cause immune disorders. When tissue or cell functions are disrupted, the expression of cytokines and HSPs shows abnormalities. Selenoproteins, as the principal bearers of the biological function of Se, also play an important role in the immune system. To our knowledge, the effects of low Se and high fat on the expression of cytokines, HSPs, and selenoproteins in pig peripheral blood lymphocytes are not reported. In order to explore the effects of low Se and high fat on the pig's immune cells, we established low Se, high fat, and low-Se/high-fat pig models and detected the expression of selenoproteins, HSPs, and cytokine genes in pig peripheral blood lymphocytes, with further analysis of the effects of low Se and high fat on the expression of selenoprotein, HSP, and cytokine mRNA.

Materials and Methods

Materials or Chemicals

Lymphoprep was purchased from Tianjin Haoyang Biological Products Co., Ltd. TRIzol reagent was purchased from Invitrogen, America. The mRNA reverse transcription kit was provided by Thermo Fisher Scientific Inc. Chloroform, isopropanol, and ethanol were purchased from Tianjin Yonglargest chemical reagents Limited. All other chemicals were obtained from commercial sources at the highest quality available.

Treatment of Experimental Animals

All procedures used in the present study were approved by the Institutional Animal Care and Use Committee of Northeast Agricultural University. All experimental piglets were provided by normal pig farms and bred in the Northeast Agricultural University facility. Forty crossbred boar (Duroc × Landrace × Yorkshire, without castration) piglets with healthy lean body weights of 10 kg were randomly divided into four treatment groups of 10 piglets per treatment group. The study used a 2 × 2 factorial design, where the control group is group C (DE > 3290 kcal/kg, Se = 0.3 mg/kg), and the experimental groups are group L-Se (DE > 3290 kcal/kg, Se = 0 mg/kg), group H-fat (DE > 3490 kcal/kg, Se = 0.3 mg/kg), and group L-Se-H-fat (DE > 3490 kcal/kg, Se = 0 mg/kg). The diets were fed according to the experimental design and were prepared based on the normal fat diet with addition of 0 or 0.03 g/kg sodium selenite (1%, Se = 10,000 mg/kg). A complementary granulated diet including corn, soybean meal, and wheat bran, was obtained from Longjiang County, a typical Se-deficient region of Heilongjiang Province in China, which blends material without supplemental Se. Experimental animals were fed for 16 weeks.

Preparation of the Pig Peripheral Blood Lymphocytes for Analysis

Fresh porcine venous blood (30 mL) was mixed with sodium citrate tribasic in a 7:1 ratio. The lymphocyte preparations were enriched by centrifugation (2000 rpm) for 20 min. The upper layer was removed after centrifugation, and 18 ml of PBS was added to the remaining part, followed by mixing. The mixture was added to the cell separation medium at a ratio of 2:1. Then, the mixture was centrifuged (2000 rpm) for 15 min. The second layer was collected in tubes containing 30 mL of PBS, mixed well, and then centrifuged (1800 rpm) for 20 min. The cells were recovered from the tube, resuspended, and washed two times in 10 mL of PBS. Pig peripheral blood lymphocytes were prepared for further experiments.

Design of Primers and Quantitative PCR

The total RNA was isolated from pig peripheral blood lymphocyte samples using Trizol reagent according to the manufacturer's instructions (Invitrogen, China). The concentration and purity of the total RNA were determined at 260 nm/280 nm using a spectrophotometer (Gene Quant 1300/100, General Electric Company, USA). First-strand cDNA was synthesized from 5 μg of total RNA using oligo dT primers and SuperScript II reverse transcriptase according to the manufacturer's instructions (Roche, USA). The synthesized

cDNA was diluted five times with sterile water and stored at − 80 °C before using.

The primers used for selenoprotein, HSP, cytokine, and inflammatory cytokine assays were designed by Premier Software (PREMIER Biosoft International, USA) and are summarized in Table 1 for quantitative PCR (qPCR). The β-actin gene was used as an internal reference. Gene expression levels were detected via qPCR using a Light Cycler® 480 System (Roche, Basel, Switzerland) and fast Universal SYBR Green Master Mix (Roche, Basel, Switzerland). The reactions were performed in a 20 μL reaction mixture containing 10 μL of 2 × SYBR Green I PCR Master Mix (TaKaRa, China), 2 μL of diluted cDNA, 0.4 μL of each primer (10 μM), 0.4 μL of 50 × ROX reference Dye II, and 6.8 μL of PCR-grade water. The PCR procedure for all of them was 95 °C for 30 s, followed by 40 cycles of 95 °C for 15 s, 60 °C for 30 s, and 60 °C for 30 s. The melting curve analysis showed only one peak for each PCR product. Electrophoresis was performed with the PCR products to verify primer specificity and product purity. The relative mRNA abundance was calculated according to the method of $2^{-\Delta\Delta CT}$, which accounts for gene-specific efficiencies and is normalized to the mean expression of the above-mentioned index.

Statistical Analysis

Statistical analyses of all data were performed using GraphPad Prism (version 5.0, GraphPad Software Inc., San Diego, CA, USA). All datasets showed normal distributions and passed equal variance testing. Quantitative data are shown as the mean ± SD, and $P < 0.05$ was considered statistically significant. Bars with different superscript letters represent statistically significant differences ($P < 0.05$). We used two-way ANOVA to investigate the relationship between low Se and high fat.

Results

MRNA Levels of Selenoprotein in Pig Peripheral Blood Lymphocytes

To determine the effects of low Se, high fat, and their combination on the mRNA level of selenoprotein in pig peripheral blood lymphocytes, we used qPCR to examine 23 selenoproteins (Gpx1, Gpx2, Gpx3, Gpx4, Dio1, Dio2, Dio3, TrxR1, TrxR2, TrxR3, Sephs, Sep, Selh, Seli, Selm, Sepp, Selt, Selx, SelK, Sepn, Sels, Selo, and Sepw). However, we did not detect certain transcripts, such as Dio1, Dio2, Dio3, TrxR2, TrxR3, Sels, and Selo; this result might be explained by tissue differences, with several selenoproteins failing to express significant levels in the pig peripheral blood lymphocytes.

Table 1 Gene-specific primers for qPCR

Gene	Primer sequence (5′ → 3′)
ACT	Forward: CCCAAAGCCAACCGTGAGAA
	Reverse: CCACGTACATGGCTGGGGTG
Gpx1	Forward: GATGCCACTGCCCTCATGA
	Reverse: TCGAAGTTCCATGCGATGTC
Gpx2	Forward: AGAATGTGGCCTCGCTCTGA
	Reverse: GGCATTGCAGCTCGTTGAG
Gpx3	Forward: TGCACTGCAGGAAGAGTTTGAA
	Reverse: CCGGTTCCTGTTTTCCAAATT
Gpx4	Forward: TGAGGCAAGACGGAGGTAAACT
	Reverse: TCCGTAAACCACACTCAGCATATC
TrxR1	Forward: GATTTAACAAGCGGGTCATGGT
	Reverse: CAACCTACATTCACACACGTTCCT
Sephs	Forward: TGGCTTGATGCACACGTTTAA
	Reverse: TGCGAGTGTCCCAGAATGC
Sep	Forward: ACAGCCCTGCCAAGCAGAT
	Reverse: AACAGGGAGGCTGGGTAACAC
Selh	Forward: TGGTGGAGGAGCTGAAGAAGTAC
	Reverse: CGTCATAAATGCTCCAACATCAC
Seli	Forward: GATGGTGTGGATGGAAAGCAA
	Reverse: GCCATGGTCAAAGAGTTCTCCTA
Selm	Forward: CAGCTGAATCGCCTCAAAGAG
	Reverse: GAGATGTTTCATGACCAGGTTGTG
Sepp	Forward: AACCAGAAGCGCCAGACACT
	Reverse: TGCTGGCATATCTCAGTTCTCAGA
Selt	Forward: GGCTTAATAATCGTTGGCAAAGA
	Reverse: TGGCCCCATTGCCAGATA
Selx	Forward: ATCCCTAAAGGCCAAGAATCATC
	Reverse: GGCCACCAAGCAGTGTTCA
Selk	Forward: CAGGAAACCCCCCTAGAAGAA
	Reverse: CTCATCCACCGGCCATTG
Sepn	Forward: ACCTGGTCCCTGGTGAAAGAG
	Reverse: AGGCCAGCCAGCTTCTTGT
Sepw	Forward: CACCCCTATCTCCCTGCAT
	Reverse: GAGCAGGATCACCCCAAACA
HSP40	Forward: AGACCTCCAACAACATTCCAG
	Reverse: TAATCCTGGCTGGGTAAATGA
HSP60	Forward: CTCATCTCACTCGGGCTTATG
	Reverse: TATCACCGTCCTTCCCTTTG
HSP70	Forward: CAAAGCAGACCCAGACTTTCA
	Reverse: AGAAGGTTGTTGTCCCTGGTC
HSP90	Forward: AAATCCAGACCATTCCATCATC
	Reverse: TGAAGCCAGAAGACAGCAGAG
IL-1α	Forward: ACCCGACTGTTTGTGAGTGC
	Reverse: TTCCCAGAAGAAGAGGAGACTG
IL-1β	Forward: TCTCCAGCCAGTCTTCATTGT
	Reverse: GCCATCAGCCTCAAATAACAG
IL-2	Forward: ATTGCACTAACCCTTGCACTC
	Reverse: CAACTGTAAATCCAGCAGCAA
IL-4	Forward: CGGCACATCTACAGACACCAC

Table 1 (continued)

Gene	Primer sequence (5′ → 3′)
	Reverse: CTTCATGCACAGAACAGGTCA
IL-6	Forward: GCTATGAACTCCCTCTCCACA
	Reverse: ACCTTTGGCATCTTCTTCCAG
IL-8	Forward: TGAGAAGCAACAACAACAGCA
	Reverse: AGCACAGGAATGAGGCATAGA
IL-10	Forward: AGTGGGCTATTTGTCCTGACTG
	Reverse: GGGCTCCCTAGTTTCTCTTCC
IL-12p40	Forward: CTCTTCACGGACCAAATCTCA
	Reverse: GACACAGATGCCCATTCACTC
IFN-γ	Forward: AGCTTTGCGTGACTTTGTGTT
	Reverse: GGTCCACCATTAGGTACATCTG
TGF-β1	Forward: TGTCCACCATTCATTTGTTCC
	Reverse: AGGCATTCAGGATAAGGTCCA
NF-κB p50	Forward: CCATGCTGGAACCACTAAATC
	Reverse: TACGGCCTCTCTGTCATCACT
NF-κB p65	Forward: GTGTGTAAAGAAGCGGGACCT
	Reverse: CACTGTCACCTGGAAGCAGA
TNF-α	Forward: ACCAGCCAGGAGAGAGACAAG
	Reverse: AGCGTGTGAGAGGGAGAGAGT
iNOS	Forward: ACCACGGAACCTAATGATGG
	Reverse: GAGTTGGAGAGGGAGGGAGAT
COX-2	Forward: TCCAAATACAACCCTGTCCTG
	Reverse: CCACATCTTACCGCCTGATTA
PTGES	Forward: CACTCCCTCCTCCGTCCTAA
	Reverse: ATGGCACCTGAATCCTCAAG

Consequently, we present the 16 selenoprotein genes that showed significant changes in expression levels. Data analysis was performed from three perspectives. First, Fig. 1 shows the effects of four diets on selenoprotein mRNA levels. Group C was the control group and was compared to the normal Se and normal fat content diet group. Group L-Se was the normal fat/low-Se group, group H-fat was fed a diet with normal Se/high-fat content, and group L-Se-H-fat was the high-fat content/low-Se content group. Compared with group C, the mRNA level of selenoprotein was lowest in the pig peripheral blood lymphocytes from group L-Se. Among these selenoprotein genes, we noted that Se deficiency had the most pronounced effects on the mRNA expression of Gpx2, Selx, and Sep in the pig peripheral blood lymphocytes. Compared with group C, the mRNA levels of Gpx2, Selx, and Sep in group L-Se were reduced by 73–80%. In addition, it was noted that in group L-Se-H-fat, the mRNA level of selenoprotein was lower than in group C. The effect on the mRNA expression of Selk, Sepn, and Sep in the pig peripheral blood lymphocytes in group L-Se-H-fat was 82–84% lower compared with group C. Second, the levels of selenoprotein for further study are shown in Fig. 2. The upper part of the chart shows Se = 0.3 mg/kg, and the lower part of the chart shows Se < 0.03 mg/kg. Our results show that the effect of fat content on selenoprotein mRNA levels follows a similar trend. For most selenoprotein genes, the changes for each selenoprotein gene follow a similar pattern across the groups. Third, to summarize the patterns of Se content and effects of fat content on selenoprotein in pig peripheral blood lymphocytes, a two-way ANOVA was conducted. The results are presented in Table 2. The primary source of variation in parameters of selenoprotein in pig peripheral blood lymphocytes was the concentration of dietary Se. The concentration of dietary fat was a minor factor in most cases. Among these selenoprotein genes, Selx is the exception in that the minor factor is the interaction between the concentration of Se and fat.

MRNA Levels of HSPs in Pig Peripheral Blood Lymphocytes

To examine whether low Se, high fat, and their combination could cause the synthesis of stress proteins, we detected the mRNA expression of HSP40, HSP60, HSP70, and HSP90. Up-regulation of the mRNA for HSP40, HSP60, HSP70, and HSP90 in pig peripheral blood lymphocytes was observed after pigs were fed with the low-Se/high-fat diet (Fig. 3). Compared with group C, the mRNA expression of HSP40, HSP60, HSP70, and HSP90 significantly increased due to Se deficiency and high fat ($P < 0.05$). Se deficiency and high fat induced the sharpest up-regulation of HSP90. Compared with group C, group H-fat, group L-Se, and group L-Se-H-fat were 5.28-, 52.08-, and 62.5-fold higher, respectively. Also, two-way ANOVA was conducted to summarize the patterns of Se content and fat content effects on HSP40, HSP60, HSP70, and HSP90. The results are shown in Table 3. The primary source of variation in the parameters in pig peripheral blood lymphocytes was the concentration of fat. A secondary source of variation in the parameters was the concentration of Se. In addition, our results in Table 3 show a 13.16–16.31% total variation caused by the interaction of low Se and high fat for HSP40, HSP60, and HSP70. Among them, the most obvious effects of fat content are on HSP90, which showed 97.84% of the total variation.

MRNA Levels of Cytokines (IL-1α, IL-1β, IL-6, IL-8, IL-9, TGF-β1, IFN-γ, iNOS, COX-2, NF-κB, and TNF-α) in Pig Peripheral Blood Lymphocytes

Figure 4 shows the mRNA levels of cytokines (IL-1α, IL-1β, IL-6, IL-8, IL-9, TGF-β1, and IFN-γ). Compared with group C, the mRNA levels of the cytokines IL-1α, IL-1β, IL-6, IL-8, and IL-9 are higher, and the others are lower. Group L-Se, group H-fat, and group L-Se-H-fat were 1.26- to 49.44-fold higher than group C. In this dataset, IL-1α and IL-6 in group L-Se-H-fat showed obvious changes. IL-1α was 49.44 times higher and IL-6 48 times higher than the control group.

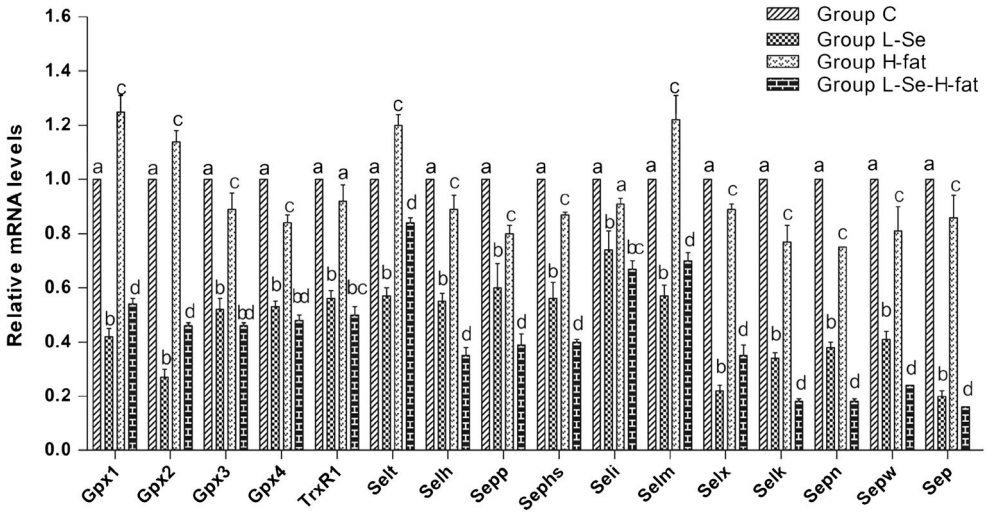

Fig. 1 The selenoproteins mRNA levels. Analysis of the mRNA levels of selenoproteins from pig peripheral blood lymphocytes. The results are from at least four independent experiments. Data are represented as the mean ± SD ($n = 4$). Bars with different superscript letters represent statistically significant differences ($P < 0.05$) among group C, group L-Se, group H-fat, and group L-Se-H-fat

Figure 5 shows the mRNA levels of inflammatory cytokines (iNOS, COX-2, NF-κB, and TNF-α). Compared with group C, the mRNA expression of inflammatory factors in group L-Se, group H-fat, and group L-Se-H-fat all increased. In addition, the increased expression of TNF-α was the most dramatic; group L-Se-H-fat was 39.44 times higher than group C.

To examine the patterns of Se content and fat content effects on cytokines (IL-1α, IL-1β, IL-6, IL-8, IL-9, TGF-β1, and IFN-γ) and inflammatory cytokines (iNOS, COX-2, NF-κB, and TNF-α), we conducted a two-way ANOVA. The results are shown in Table 3. The primary source of variation in the parameters (including cytokines and inflammatory cytokines) in pig peripheral blood lymphocytes was the concentration of fat. The exceptions were COX-2 and IL-1α in which the primary source of variation was the concentration of Se. Secondary sources of variation in the parameters included the concentration of Se. Also, the interaction between Se content and fat content on NF-κB p50 and IL-6 was similar to the effect of only Se. The interaction between Se content and fat content on IL-6 is 29.01% of the total variation, while the Se content is 30.53% of the total variation.

Discussion

Lymphocytes are important components of the immune response, which is produced by lymphoid organs and mediated by the organism's cellular immunity, humoral immunity, and other immunological functions. Cytokine is a general term that refers to biologically active small proteins secreted by cells, which mediate inflammation and the immune response, regulating cell physiology and immune system function, and are important cellular signal molecules. The changes in cytokine expression in lymphocytes represent immunological damage of low Se and high fat. In order to further detect Se-deficient and high-fat-induced lymphocyte damage and host

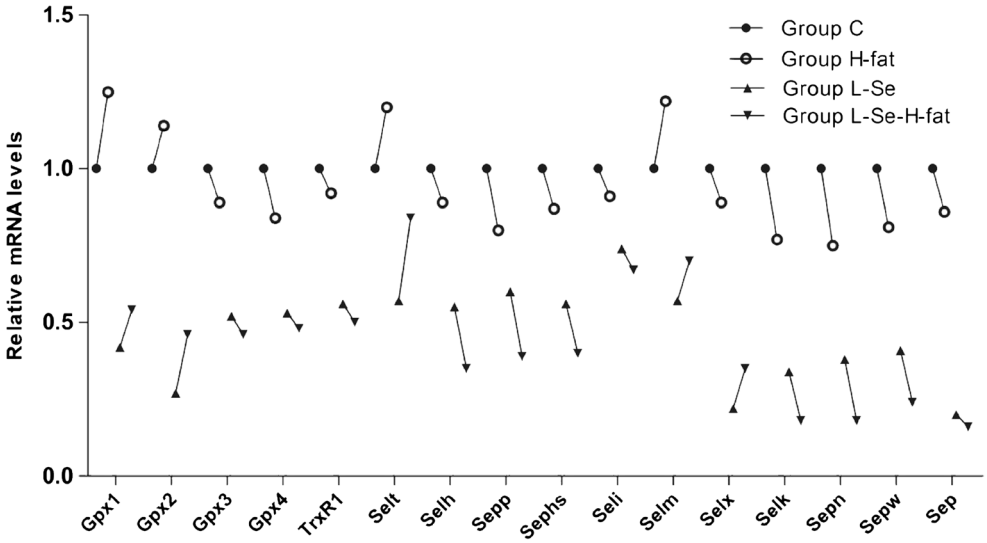

Fig. 2 The mRNA levels of selenoproteins. Effects of selenoproteins in the different dietary content groups in pig peripheral blood lymphocytes

Table 2 Results of a repeated measure two-way ANOVA performed to test the effects of selenium content and fat content on selenoprotein levels in pigs

Variable	Source of variation	% of total variation	*df*	Mean square	*F*	*P* value	
Gpx1	Selenium content × fat content	0.91	1	0.01268	12.37	0.0245	*
	Selenium content	90.94	1	1.261	809.2	< 0.0001	***
	Fat content	7.4	1	0.1027	100.2	0.0006	***
	Error	0.4495	4	0.001558			
Gpx2	Selenium content × fat content	0.12	1	0.001875	3.75	0.1249	ns
	Selenium content	94.42	1	1.491	2294	< 0.0001	***
	Fat content	5.17	1	0.08167	163.4	0.0002	***
	Error	0.1646	4	0.00065			
Gpx3	Selenium content × fat content	0.18	1	0.0012	1.125	0.3486	ns
	Selenium content	94.94	1	0.6348	432.8	< 0.0001	***
	Fat content	3.37	1	0.02253	21.12	0.0101	*
	Error	0.8774	4	0.001467			
Gpx4	Selenium content × fat content	1.4	1	0.008008	23.44	0.0084	**
	Selenium content	92.04	1	0.525	707.9	< 0.0001	***
	Fat content	5.8	1	0.03308	96.8	0.0006	***
	Error	0.5201	4	0.0007417			
Selh	Selenium content × fat content	0.74	1	0.006075	5.695	0.0755	ns
	Selenium content	89.83	1	0.7351	760.4	< 0.0001	***
	Fat content	8.43	1	0.06901	64.7	0.0013	**
	Error	0.4725	4	0.0009667			
Seli	Selenium content × fat content	0.13	1	0.0003	0.375	0.5734	ns
	Selenium content	85.13	1	0.1925	70.44	0.0011	**
	Fat content	8.49	1	0.0192	24	0.008	**
	Error	4.8342	4	0.002733			
Selk	Selenium content × fat content	0.37	1	0.0048	5.236	0.084	ns
	Selenium content	90.35	1	1.166	1185	< 0.0001	***
	Fat content	8.69	1	0.1121	122.3	0.0004	***
	Error	0.3049	4	0.0009833			
Selm	Selenium content × fat content	0.65	1	0.005208	1.611	0.2732	ns
	Selenium content	84.82	1	0.6769	356.3	< 0.0001	***
	Fat content	11.96	1	0.09541	29.51	0.0056	**
	Error	0.9524	4	0.0019			
Selt	Selenium content × fat content	0.71	1	0.004408	9.618	0.0362	*
	Selenium content	72.54	1	0.4524	456.2	< 0.0001	***
	Fat content	25.82	1	0.161	351.3	< 0.0001	***
	Error	0.6361	4	0.0009917			
Selx	Selenium content × fat content	3.35	1	0.04563	59.52	0.0015	**
	Selenium content	96.01	1	1.307	992.5	< 0.0001	***
	Fat content	0.02	1	0.0003	0.3913	0.5655	ns
	Error	0.387	4	0.001317			
Sep	Selenium content × fat content	0.43	1	0.0075	4.639	0.0976	ns
	Selenium content	97.53	1	1.688	1077	< 0.0001	***
	Fat content	1.3	1	0.02253	13.94	0.0202	*
	Error	0.3622	4	0.001567			
Sephs	Selenium content × fat content	0.12	1	0.0008333	1.136	0.3465	ns
	Selenium content	89.95	1	0.6165	711.4	< 0.0001	***
	Fat content	8.99	1	0.06163	84.05	0.0008	***
	Error	0.5058	4	0.0008667			

Table 2 (continued)

Variable	Source of variation	% of total variation	*df*	Mean square	*F*	*P* value	
Sepn	Selenium content × fat content	0.1	1	0.0012	2.4	0.1963	ns
	Selenium content	86.77	1	1.068	4577	< 0.0001	***
	Fat content	12.89	1	0.1587	317.4	< 0.0001	***
	Error	0.0758	4	0.0002333			
Sepp	Selenium content × fat content	0.01	1	0.00003333	0.009112	0.9285	ns
	Selenium content	77.59	1	0.4961	428.3	< 0.0001	***
	Fat content	19.4	1	0.124	33.9	0.0043	**
	Error	0.7246	4	0.001158			
Sepw	Selenium content × fat content	0.03	1	0.0003	0.1452	0.7226	ns
	Selenium content	89.51	1	1.009	455.3	< 0.0001	***
	Fat content	8.94	1	0.1008	48.79	0.0022	**
	Error	0.7864	4	0.002217			
TrxR1	Selenium content × fat content	0.04	1	0.0002083	0.1761	0.6963	Ns
	Selenium content	95.71	1	0.5677	351.1	< 0.0001	***
	Fat content	2.36	1	0.01401	11.84	0.0263	*
	Error	1.0903	4	0.001617			

defenses, such as inflammatory reactions and immune functions, the mRNA expression levels of IL-1α, IL-1β, IL-6, IL-8, IL-9, TGF-β1, IFN-γ, iNOS, COX-2, NF-κB, and TNF-α were examined by qPCR. IL-1α, IL-1β, IL-6, IL-8, and IL-9 are pro-inflammatory factors that are important indicators of inflammation. Increases in their levels represent the generation of inflammation in the organism and can cause other organism-wide pathological damage. IL-8 is produced by various types of inflammatory stimuli and then exerts a variety of functions on leukocytes. IL-9 is a key molecule that affects differentiation of T(reg) function and T(H)17 cells, promoting the differentiation of IL-6 [25]. TNF-α and IL-1β can be used as indirect indexes for detecting immune function because they inhibit activated immune cells in inflammation [26]. TGF-β1 plays a predominant role in immunological activation and suppression [27]. The NF-κB pathway is an important pathway for the regulation of inflammatory factors, and Se can regulate IL-1, IL-6, and TNF-a through the NF-κB pathway [28]. Two target genes of NF-κB are the inflammatory enzymes COX-2 and iNOS [29, 30]. IFN-γ can rapidly activate host macrophages and enhance the immune responses of organisms [31]. TGF-β1 and IFN-γ are anti-inflammatory

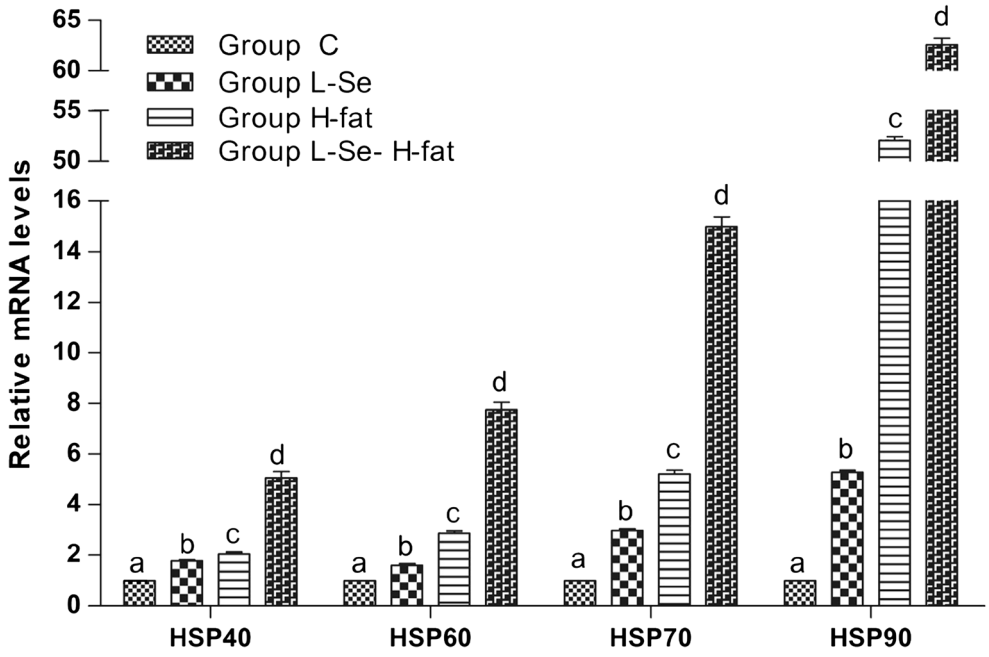

Fig. 3 The mRNA levels of HSPs. Analysis of the mRNA levels of HSPs in pig peripheral blood lymphocytes. The results are taken from at least four independent experiments. Data are presented as the mean ± SD ($n = 4$). Bars with different superscript letters represent statistically significant differences ($P < 0.05$) among group C, group L-Se, group H-fat, and group L-Se-H-fat

Table 3 Results of repeated measures two-way ANOVA performed to test the effects of selenium content and fat content on the levels of HSPs, cytokines, and inflammatory cytokines in pigs

Variable	Source of variation	% of total variation	*df*	Mean square	*F*	*P* value	
HSP40	Selenium content × fat content	13.16	1	5.018	362.3	< 0.0001	***
	Selenium content	37.36	1	14.25	670.9	< 0.0001	***
	Fat content	48.93	1	18.66	1347	< 0.0001	***
	Error	0.3342	6	0.02124			
HSP60	Selenium content × fat content	16.31	1	18.49	789	< 0.0001	***
	Selenium content	26.44	1	29.98	1357	< 0.0001	***
	Fat content	57.01	1	64.64	2759	< 0.0001	***
	Error	0.1169	6	0.02209			
HSP70	Selenium content × fat content	13.16	1	60.84	1656	< 0.0001	***
	Selenium content	29.91	1	138.3	2810	< 0.0001	***
	Fat content	56.82	1	262.8	7150	< 0.0001	***
	Error	0.0639	6	0.04922			
HSP90	Selenium content × fat content	0.32	1	38.72	311.3	< 0.0001	***
	Selenium content	1.82	1	218.4	1457	< 0.0001	***
	Fat content	97.84	1	11,750	94,440	< 0.0001	***
	Error	0.0075	6	0.1499			
NF-κB p50	Selenium content × fat content	22.75	1	605.5	9083	< 0.0001	***
	Selenium content	23.97	1	637.9	8964	< 0.0001	***
	Fat content	53.24	1	1417	21,260	< 0.0001	***
	Error	0.016	6	0.07116			
NF-κB p65	Selenium content × fat content	4.48	1	0.1024	4.659	0.0742	ns
	Selenium content	16.8	1	0.3844	33.49	0.0012	**
	Fat content	69.95	1	1.6	72.81	0.0001	***
	Error	3.0106	6	0.01148			
TNF-α	Selenium content × fat content	17.44	1	559.1	3277	< 0.0001	***
	Selenium content	37.94	1	1216	6578	< 0.0001	***
	Fat content	44.56	1	1428	8374	< 0.0001	***
	Error	0.0346	6	0.1849			
iNOS	Selenium content × fat content	11.48	1	21.81	233.9	< 0.0001	***
	Selenium content	17.19	1	32.66	355.5	< 0.0001	***
	Fat content	70.75	1	134.4	1442	< 0.0001	***
	Error	0.2901	6	0.09188			
COX-2	Selenium content × fat content	22.24	1	43.53	1804	< 0.0001	***
	Selenium content	52.35	1	102.5	1773	< 0.0001	***
	Fat content	25.16	1	49.25	2041	< 0.0001	***
	Error	0.1772	6	0.05779			
IFN-γ	Selenium content × fat content	0	1	0.0001563	0.4491	0.5277	ns
	Selenium content	0.93	1	0.02976	94.59	< 0.0001	***
	Fat content	98.94	1	3.177	9132	< 0.0001	***
	Error	0.0588	6	0.0003146			
IL-1α	Selenium content × fat content	26	1	1655	17,760	< 0.0001	***
	Selenium content	41.29	1	2627	89,860	< 0.0001	***
	Fat content	32.7	1	2081	22,330	< 0.0001	***
	Error	0.0028	6	0.02924			
IL-1β	Selenium content × fat content	8.37	1	0.2627	36.03	0.001	***
	Selenium content	33.33	1	1.046	100.1	< 0.0001	***
	Fat content	54.91	1	1.723	236.3	< 0.0001	***
	Error	1.9966	6	0.01044			

Table 3 (continued)

Variable	Source of variation	% of total variation	*df*	Mean square	*F*	*P* value	
IL-6	Selenium content × fat content	29.01	1	1815	104,500	< 0.0001	***
	Selenium content	30.53	1	1910	177,300	< 0.0001	***
	Fat content	40.45	1	2531	145,600	< 0.0001	***
	Error	0.001	6	0.01077			
IL-8	Selenium content × fat content	0.79	1	20.12	112.9	< 0.0001	***
	Selenium content	2.98	1	75.52	283	< 0.0001	***
	Fat content	96.12	1	2435	13,660	< 0.0001	***
	Error	0.0632	6	0.2668			
IL-9	Selenium content × fat content	5.91	1	79.25	1743	< 0.0001	***
	Selenium content	20.4	1	273.5	2541	< 0.0001	***
	Fat content	73.62	1	987.1	21,710	< 0.0001	***
	Error	0.0482	6	0.1076			
TGF-β1	Selenium content × fat content	0	1	0.0001	0.4286	0.537	ns
	Selenium content	0.29	1	0.009025	51.57	0.0004	***
	Fat content	99.63	1	3.098	13,280	< 0.0001	***
	Error	0.0338	6	0.000175			

factors, and their expression decreases in inflammation. In our experiments, the mRNA levels of the cytokines IL-1α, IL-1β, IL-6, IL-8, IL-9, iNOS, COX-2, NF-κB, and TNF-α in group L-Se, group H-fat, and group L-Se-H-fat all increased, and the other cytokines are lower than group C. Also, group L-Se-H-fat underwent a remarkable change. This is consistent with previous studies showing that high fat and low Se induced inflammatory responses and changes in immune function in lymphocytes [10, 32]. Therefore, we conclude that low Se or high fat could induce inflammation and decrease immune function. We also conclude that low Se might cause more serious injury that is induced by high-fat diet in the lymphocyte.

The major biological form of Se is the amino acid selenocysteine, which is present in the active sites of selenoproteins. Se plays a role through selenoproteins. A low-Se diet can cause a variety of selenoprotein expression changes in animals such as the pig, rat, and chicken [17, 33, 34]. At the same time, animals that lack Se show immune function decline and immune disorders of the organism [1]. Selenoproteins in the organism play a number of roles, such as regulating antioxidant capacity, which is closely related to the organism's immune function [35]. Dietary Se deficiency and SelW knockdown increased mitochondrial Ca^{2+} levels and oxidative stress [36, 37]. Immune cell function is obviously changed in Selk knockout mice [21]. Long-term high-fat diets lead to chronic inflammation, which can increase the expression of pro-inflammatory cytokines, leading to immune function disorders [38]. Existing research shows that Selw regulates the expression of other selenoproteins (Gpx3, Gpx4, Txnrd1, Selt, Selh, Sepp1, Sels, and Sep15) and also regulates inflammation-related cytokines (IL-1, IL-6, IL-8, IL-17, IL-4, IL-10, and IFN-γ) [39, 40]. Yu Dong et al. also proved that silencing of Selw significantly up-regulates inflammation-related genes (iNOS, COX-2, NF-κB, PTGEs, and TNF-α) in lymphocytes [32]. High-fat diet-induced inflammation in pigs is associated with thioredoxin and oxidoreductase systems and related selenoproteins [41]. Lymphocytes are important immune cells to test the immune injury induced by low Se and high fat. Experiments showed that low Se and high fat can induce an inflammatory response and change lymphocyte function [4, 42]. Thus, expression of 16 selenoprotein genes was determined in pig peripheral blood lymphocytes in 4 types of responses to dietary Se deficiency: normal, high fat, and Se deficiency/high fat. Our results showed that the mRNA level of selenoproteins was lower in group L-Se and group L-Se-H-fat than in group C in pig peripheral blood lymphocytes. This is consistent with previous research. Also, a high-fat diet changes the selenoprotein mRNA levels in pig peripheral blood lymphocytes. Our result showed that four selenoproteins (Gpx1, Gpx2, Selt, and Selm) examined in obese pigs were elevated. This is different from a previous report where 12 selenoproteins (Gpx3, Gpx4, Gpx6, Dio1, Dio2, Txnrd1, Selv, Seli, Sels, Selm, Selo, and Sep15) examined in high-fat pigs were elevated in 6 tissues. This may be related to the differences among the tissues. Existing studies have shown that Se deficiency and high-fat diet may change the mRNA level of selenoprotein genes in pig immune cells, and our results also verify this model [43]. Our two-way ANOVA showed that the primary source of variation in selenoprotein parameters in pig peripheral blood lymphocytes was the concentration of dietary Se, and the concentration of dietary fat content was a minor factor in most cases. These

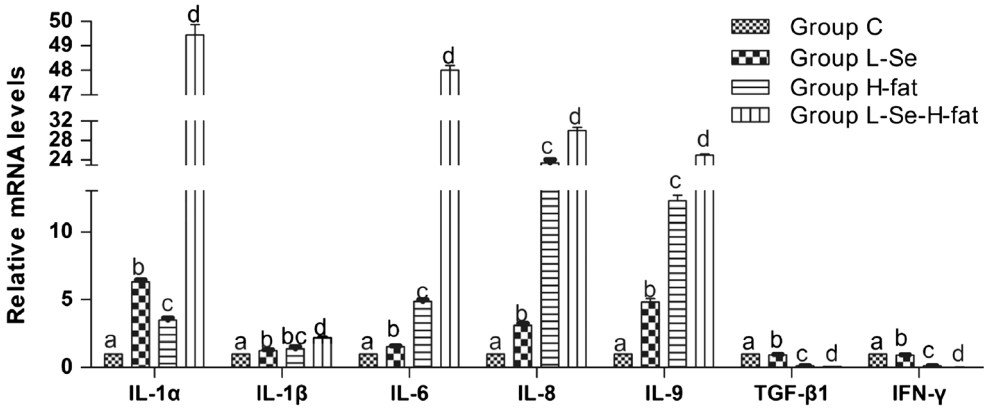

Fig. 4 The cytokines mRNA levels. Analysis of the mRNA levels of cytokines in pig peripheral blood lymphocytes. The results are taken from at least four independent experiments. Data are presented as the mean ± SD ($n = 4$). Bars with different superscript letters represent statistically significant differences ($P < 0.05$) among group C, group L-Se, group H-fat, and group L-Se-H-fat

results suggested that dietary Se content and fat content can regulate the levels of selenoprotein genes in peripheral blood lymphocytes. The precise mechanism needs further research.

HSPs are proteins that are abundantly expressed during stress. They protect cells against stimulation, improve tolerance, and deal with the adverse effects of stimulation. HSPs can directly stimulate cells and enhance the organism's immune function. However, when cells undergo stress, such as oxidative stress, which is induced by Se deficiency and high fat, the expression quantity will increase. For example, a certain concentration of manganese can induce high expression of HSP40, HSP60, HSP70, and HSP90 in spleen lymphocytes in vitro [44]. That may be explained by the negative feedback regulation. Previous studies demonstrated that Se deficiency or high fat could induce the expression of HSPs. Khoso's results showed that immunosuppression was accompanied by a downregulation of mRNA expression levels of selenoproteins and an upregulation of the HSP mRNA expression levels, and the expression levels of IL-2, IL-6, IL-8, IL-10, IL-17, IFN-α, IFN-β, and IFN-γ were lower [45]. Selenium can reduce oxidative stress and inflammatory response induced by lead [46, 47]. A Se-deficient diet induced oxidative stress and impaired immune function [48, 49]. HSP70 is one of the most strongly induced after stresses such as oxidative stress [50]. HSP can induce production of cytokines such as TNF-α and other cell types, and they can deliver maturation signals and peptides to antigen-presenting cells through receptor-mediated interactions [51]. HSP60 treatment of mouse and human macrophages increased the production of IL-12, IL-15, INF-γ, and TNF-α. Khoso et al. and Chen et al. have reported that Se deficiency activates HSP expression in splenic lymphocytes, the thymus and neutrophils [52, 53]. HSP60 and HSP90 may have protective effects during Se deficiency-induced erythrocyte injury [54]. Upon hyperlipidemia, HSP expression increased significantly in the intracranial cerebral arteries of rabbits [55]. In response to a high-fat diet, interactions of HSPs, which act as danger signals, influenced the signaling pathways and the inflammatory responses of cells [56]. A high-fat diet inhibits lymphocyte immune function in rats, and lipoprotein is one of the inhibiting factors [10]. Our result is consistent with the findings that HSPs are extensively elevated compared with the control group. Not only group L-Se but also group H-fat and group L-Se-H-fat have an observable change. Se deficiency and high-fat diet can cause injury to lymphocytes in the organism, and our results show that their interaction is not a simple superposition. Low Se will promote high-fat damage to lymphocytes, causing the organism to be in a more serious state of stress. This serves as a basis for the potential correlation of Se and lipids in the organism.

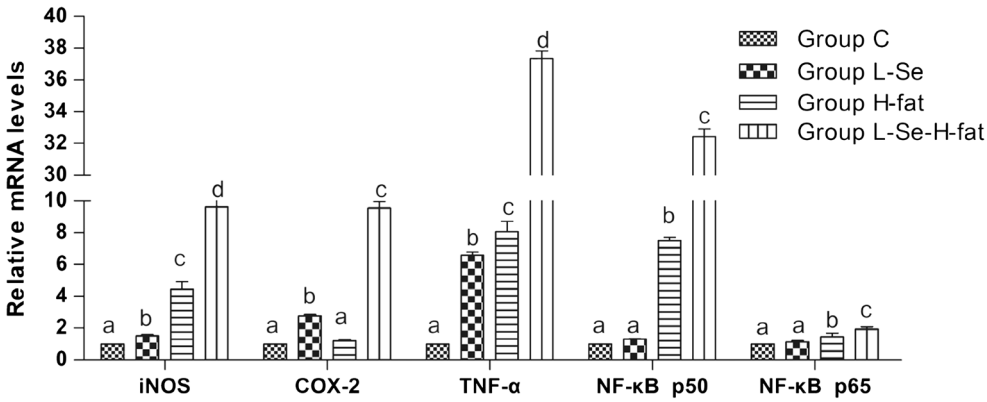

Fig. 5 The mRNA levels of inflammatory cytokines. Analysis of the mRNA levels of inflammatory cytokines in the pig peripheral blood lymphocytes. The results are from at least four independent experiments. Data are presented as the mean ± SD ($n = 4$). Bars with different superscript letters represent statistically significant differences ($P < 0.05$) among group C, group L-Se, group H-fat, and group L-Se-H-fat

Conclusion

In summary, our results indicated that low-Se/high-fat diet could cause relevant selenoprotein expression changes and promote the expression of pro-inflammatory factors and HSPs. Also, low Se will promote changes associated with high-fat diet. This information is helpful in understanding pig peripheral blood lymphocytes with low-Se and high-fat diet.

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Compliance with Ethical Standards

All procedures used in the present study were approved by the Institutional Animal Care and Use Committee of Northeast Agricultural University.

Conflict of Interest The authors declare that they have no conflicts of interest.

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