2-Hydroxy-4-Methylselenobutanoic Acid (HMSeBA) Promote Follicle Development By Antioxidant Pathway in Gilts

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Research

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2-Hydroxy-4-methylselenobutanoic acid (HMSeBA) promote follicle development by antioxidant pathway in gilts

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ABSTRACT

Background: Dietary 2-hydroxy-4-methylselenobutanoic acid (HMSeBA) supplementation can exert antioxidant effects in poultry, pigs and weaned pigs. However, it is unknown whether HMSeBA could improve the development of follicle by anti-oxidize effects in gilt. This study was conducted to evaluate the effects of dietary HMSeBA supplementation on the follicle development in gilt. A total of 36 gilts were randomly fed the control diet (CON, negative control), Na_2SeO_3 diet containing 0.3 mg Se/kg (positive control) or the HMSeBA diet containing 0.3 mg Se/kg from weaning to the 19th day after the second estrus. In another study, the effect of HMSeBA on the cells viability, proliferation, release of 17β-estradiol (E_2) and antioxidant capacity were investigated in the mouse ovarian granulosa cells *in vitro*.

Results: Results showed that HMSeBA group increased the average daily body weight gain (ADG) and decreased the ratio of feed: gain during day 120 to 176 in gilts ($P < 0.05$). The selenium (HMSeBA and Na_2SeO_3) increased the weight of uterine at the third estrus. There was no effect of HMSeBA on the number of large follicles (diameter >5mm), but HMSeBA decreased the gene expression of *growth differentiation factor-9* (*GDF-9*) and *bone morphogenetic protein-15* (*BMP-15*) in cumulus-oocyte complexes (COCs). HMSeBA group increased the total selenium content in serum ($P < 0.05$) and liver ($P < 0.01$) and tended to increase the total selenium content in ovary ($P = 0.08$). HMSeBA group decreased the malondialdehyde (MDA) concentration in the serum, liver and ovary ($P < 0.05$), increased the total antioxidant capacity (T-AOC) in the liver, thioredoxin reductase (TrxR) in the ovary ($P < 0.05$) and increased the activity of GPx in the serum, liver and ovary ($P < 0.05$). Na_2SeO_3 supplementation decreased MDA and increased the T-AOC in liver, increased the T-SOD and TrxR in the ovary compared with control. At the transcription level, HMSeBA group increased the *glutathione peroxidase 2* (*GPx2*) and *TrxR1* ($P < 0.05$) expression in the liver, and increased the *GPx1* expression ($P < 0.05$) in the ovary

of gilts compared with Na_2SeO_3 treatment. Besides, HMSeBA group increased the expressions of *superoxide dismutase 1* (*SOD1*) and *Thioredoxin l* (*Trx1*) in the liver. *In vitro* experiment, HMSeBA improved granulosa cells' proliferation and E_2 secretion ($P < 0.05$). HMSeBA and Na_2SeO_3 both increased the T-AOC and decreased MDA in granulosa cells *in vitro*. Meanwhile, HMSeBA increased T-SOD, GPx, glutathione reductase (GR) and TrxR activity in granulosa cells *in vitro*. In addition, HMSeBA up-regulated *SOD2* and *GPx1* gene expression in the granulosa cells *in vitro*.

Conclusion: These results demonstrate directly, HMSeBA was more conducive to absorption and storage of selenium in the liver and ovary in gilt, and beneficial to exert the effect of HMSeBA on the antioxidant function in the liver and ovary of gilt. Moreover, HMSeBA has stronger antioxidant capacity in granular cells *in vitro*, which is more conducive to promoting follicle development. Therefore, the new type of organic selenium, HMSeBA, could be potentially useful for the control of reproductive processes in gilt.

Key words: Hydroxy-analogue of selenomethionine (HMSeBA), puberty, follicle development, antioxidant capacity, gilt, granulose cell

Introduction

Ovarian follicular development is the most important event in the gilt. Ovarian follicular development is dependent on the proliferation and differentiation of the granulosa cells (GCs) [1]. 17β-estradiol (E_2) is synthesized from androgen in granulose cell via the aromatization by cytochrome P450 aromatase. E_2 represents one of the key ovarian hormones produced by the developing ovulatory follicle, and is reflecting the differentiation of ovarian granulose cell. E_2 is crucial for female reproduction, as proved by the severe fertility defects when its synthesis or action are suppressed [2,3]. With the metabolism of ovarian, the reactive oxygen species (ROS) and free radicals would be generated [4]. Those metabolites must be neutralized locally to maintain tissue integrity and function. The antioxidants such as vitamins C and the Se-dependent glutathione peroxidase (GPx) system would protect against ROS and free radicals.

Selenium (Se) is one of the essential trace elements for animals, which can effectively improve its reproductive performances [5-7]. Types of selenium in nature include inorganic and organic selenium compounds [8]. Se may has contributes to the basal high ovulation rate, such as the high basal Se level (0.3 mg/kg) in Dalto et al. (2016) [9] control diet increased the average number of corpora lutea (19.4 vs. 17.4; number of corpus luteum represents the number of ovulations of last estrus) compared with Dalto et al. (2015) [10] (basal Se level 0.2 mg/kg). Fortier et al. (2012) [11] studied the effects of organic selenium and inorganic selenium on the antioxidant capacity and reproductive performance of sow. The results showed that the GPx activity in the blood of control group sows decreased by 3.2%, while the inorganic (sodium selenite) and organic selenium (Se-enriched yeast) groups increased by 13.7% and 19.6%, respectively. The above studies showed that sow diet supplementation with selenium can improve the antioxidant capacity and then protect itself from oxidative stress.

78 Recently, one new organic selenium product called Selisseo® (SO) has been developed, which
79 containing selenium in the form of 2-hydroxy-4-methylselenobutanoic acid (HMSeBA). Studies have
80 shown that HMSeBA to be more bioavailable than sodium selenite or selenium yeast in poultry, growing
81 pigs and weaned pigs [12-15]. Our lab also found HMSeBA supplementation during sow pregnancy
82 increased the number of total born piglets, decreased the piglet birth interval, improved concentrations
83 of total selenium, and also improved activity of antioxidant enzymes compare with the control and
84 sodium selenite treatment [16]. However, the available data concerning HMSeBA action on the gilts'
85 reproduction and function on the granulose cells remain insufficient. In particular, since the outbreak of
86 African swine fever in China in 2018, in order to restore production capacity as soon as possible, many
87 pig farms in China have begun to use Duroc × Landrace × Yorkshire (DLY) commercial gilts for breeding.
88 However, before the outbreak of African swine fever, there were fewer reports on the use of DLY gilt as
89 breeding gilt. Therefore, in the current study, the effect of HMSeBA supplementation on the onset of
90 puberty and follicle development in DLY gilts, as well as its potential mechanisms on the granulose cell
91 *in vitro* were examined.
92
93 **Materials and methods**
94 The present experiment was conducted at the Research Farm of Animal Nutrition Institute, Sichuan
95 Agricultural University, Ya'an, China. All experimental procedures followed the current law regarding
96 animal protection and were approved by the Guide for the Care and Use of Laboratory Animals prepared
97 by the Animal Care and Use Committee of Sichuan Agricultural University.
98 **Animals and diets**
99 A total of 36 gilts (Duroc × Landrace × Yorkshire, 5.50 ± 0.09 kg) were randomly allocated to 3

100 treatment groups as follows: 1) control diet (CON, negative control, basal diet, n = 6, 6 repeatments in
101 each treatment, 2 gilts/repeatment), 2) sodium selenite (Na_2SeO_3) supplemented diet (Na_2SeO_3, positive
102 control, basal diet + Na_2SeO_3 at 0.3 mg Se/kg, n = 6), 3) hydroxy-analogue of selenomethionine
103 supplemented diet (HMSeBA, Selisseo® 2% Se provided by Adisseo France, basal diet + HMSeBA at
104 0.3 mg Se/kg, n = 6). Control (CON) diet was corn-soybean meal-based, which was formulated according
105 to the nutrient requirements recommended by the National Research Council (NRC, 2012) except
106 selenium (Table 1). The experiment was terminated at the 19th day after the second estrus. Gilts were
107 fed four times a day from weaning to 90 days of age (08:00; 12:00; 16:00; 20:00), and from 90 days to
108 slaughter, they were fed twice a day (08: 00; 16:00). Gilts were feed freely before 176 days of age, the
109 feed limited 2.5 kg/d/gilt from 176 days of age to slaughter. After 176 days of age, gilts were exposed to
110 a rotation of mature boars twice a day (08:00; 16:00). Estrous detection was carefully conducted by an
111 experienced stockperson based on behavioral and vulvar characteristics. Recorded the estrus interval and
112 backfat thickness of gilts.

113 **Sample collection**

114 Gilts (n=5/treatment) were slaughtered on the morning of the 19th day after the second estrus after
115 fasting for 12 hours. Before slaughter, 10 mL blood sample was collected by acute jugular puncture to
116 obtain the serum. The serum was stored at -20 °C for further analysis.

117 After the gilt was slaughtered, the liver and the reproductive tract were dissected. The liver sample
118 from the left side were collected from each gilt and rapidly frozen in liquid nitrogen then stored at -80°C.
119 The right and left ovaries were separated from the uterine horns and weighed. The uterus was
120 subsequently trimmed of mesentery and weighed. All the surface antral follicles greater than 1 mm in
121 diameter were individually measured and recorded within different size categories (small: 1-5 mm in

diameter and large: >5 mm in diameter). Meanwhile, the number of corpora lutea was recorded. Then, the follicular content of left ovary was aspirated from all the follicles with >5 mm in diameter using a 10-mL syringe equipped with an 18-gauge needle. Following aspiration, cumulus-oocyte complexes (COCs) were recovered from the aspirate using a dissecting microscope (40 × magnification). Then the remaining follicular contents were centrifuged for 5 minutes at 2000 rpm. The supernatant (follicular fluid) was collected and stored at -20°C until analysis was conducted.

Granulosa cell culture and cell proliferation assay

Mouse ovarian granulosa cells (GC, Shanghai Sixin Biological Technology Co., Ltd.) at passage 3 or 4 at 2 x 10^5 cells (viable cell, determined by trypan blue) per well were seeded in 6-well tissue culture-treated plate in 2mL DMEM supplemented with extra 1X antibiotic/antimicotic and 10% FBS. And added sodium selenite or HMSeBA working solution at 1 ng Se /uL according to the final concentration of 0, 2.5, 5, 10 ng Se /mL, respectively. Then the plate incubated in a humidified atmosphere of 95% air and 5% CO_2 at 37ºC. After 24 hrs of culture, the cells should be around 40-50% confluent. Culture medium was vacuum-aspirated and attached cells were washed once with sterile 1* PBS. Add the new culture medium with the final treatment concentration of selenium and incubated for 24 hrs (about 90% confluent). Then the supernatant and the cells were collected and stored at -80 °C for further analysis.

The MTT (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide) assay was applied to analysis the GCs' proliferation. The method of using the MTT reagent and the detection of the OD value are carried out according to the manufacturer's protocol (Nanjing Jiancheng Institute, Jiangsu, China). Briefly, GCs were cultured with different concentration of sodium selenite or HMSeBA at 96-well tissue culture plates. After cultured 44hrs, the supernatant was removed and replaced by a solution composed of complete medium and MTT salt solution at the ratio of 4:1. After 4 h of incubation at 37°C in 5 %

144 CO_2/air, the supernatant was removed, then added DMSO to each well and shaked for 10 minutes to fully
145 melt the crystals. Finally, the measurements were performed using a microplate reader (Model 680
146 Microplate Reader, Bio-Rad Laboratories) with 570 nm interference filters. Each experiment was carried
147 out in triplicate.

148 **Selenium content analyses**

149 The concentrations of selenium in feed, tissue and serum samples were detected refer to "National
150 Food Safety Standard Determination of Selenium in Food" (GB5009.93-2017). Weighed about 1 g/mL
151 of sample and mixed acid (nitric acid: perchloric acid = 4:1) for overnight digestion. Then heat the
152 digestion on an electric hot plate at 36.5℃ for one day until the solution became clear and colorless.
153 Used hydrochloric acid and potassium borohydride to reduce the hexavalent selenium in the sample to
154 hydrogen selenide in a hydrochloric acid medium. Subsequently, the total selenium content in the sample
155 was determined by hydride atomic fluorescence spectrometry (AFS-9230, Beijing Auspicious Day
156 Instrument Co., LTD, Beijing, China).

157 **Hormone levels analyses**

158 Serum and follicular fluid estradiol, follicle stimulating hormone (FSH) and luteinizing hormone
159 (LH) were measured using sow enzyme-linked immunosorbent assay (ELISA) Kits (R&D Systems Inc.,
160 Minneapolis, MN, USA). Optical density (OD) values were determined at 450 nm by a MuLtisKan MK3-
161 Thermo Labsystems microplate reader (Thermo Labsystems, CA, USA). Serum three iodine thyroid (T_3)
162 and thyroxine (T_4) were measured using sow enzyme-linked immunosorbent assay (ELISA) Kits
163 (Jiancheng Institute of Biological Technology, Nanjing, China) according to the manufacturer's protocol.

164 GCs were cultured with different concentration of Na_2SeO_3 or HMSeBA. After cultured 48hrs with
165 once media change, the media was collected. The spent media were assayed for the presence of estradiol

by ELISA kit (R&D Systems Inc., Minneapolis, MN, USA) according to the manufacturer's protocol. Each experiment was carried out in triplicate *in vitro* experiment.

Analysis of oxidant and antioxidant contents

The supernatant and the GCs were collected after treated with different concentration of Na_2SeO_3 or HMSeBA. Commercial assay kits (Nanjing Jiancheng Institute, Jiangsu, China) were used to determine total antioxidative capability (T-AOC), total superoxide dismutase (T-SOD), glutathione peroxidase (GPx), glutathione reductase (GR), thioredoxin reductase (TrxR) and malondialdehyde (MDA) of gilts' serum, liver, ovary, and supernatant and the GCs of *in vitro* experiment. Briefly, MDA content was measured based on the thiobarbituric acid (TBA) method. The activities of SOD, GR and GPx were assayed as described by Jia et al (2009) [17]. All samples were measured in triplicate *in vitro* experiment.

Quantitative PCR analysis

Total RNA was isolated from gilt samples and GCs of *in vitro* experiment using the Trizol reagent (TaKaRa Biotechnology, Dalian, China), according to the manufacturer's instructions. Agarose gel electrophoresis was conducted to detect the integrity of the RNA. RNA purity was determined using a nucleic acid/protein analyzer (Beckman DU-800, Beckman Coulter, Inc., CA, USA) by evaluating the OD260/OD280 ratio. Both genomic DNA removal and reverse transcription (RT) were performed using a PrimeScript RT reagent kit with gDNA eraser (TaKaRa Biotechnology, Dalian, China) according to the manufacturer's instructions. Real-time PCR was performed to analyze mRNA expression using SYBR® Premix Ex TaqTM Kits (TaKaRa Biotechnology, Dalian, China). A total volume of 10μL reaction system contained 5.0 μL SYBR Premix Ex Taq (2×), 0.4 μL forward primer (10 μM, Table 2), 0.4 μL reverse primer (10 μM), 0.2 μL ROX Reference Dye (50×), 1.0 μL cDNA and 3.0 μL double-distilled water.

188 Cycling conditions were 95°C for 30s, then 40 cycles at 95°C for 5s and 60°C for 34s. At the end of
189 amplification, melting curve analysis was performed to verify specific amplifications by an ABI-7900HT
190 Fast Real-Time PCR System (Applied Biosystems, CA, USA). The products were electrophoresed on
191 agarose gel to confirm the product size. Negative controls were performed in which water was substituted
192 for cDNA. The experiment was repeated three times. The *β-actin* was used as housekeeping gene to
193 normalize the expression of target genes according the $2^{-\triangle\triangle Ct}$ method [18].

194 **Statistical analysis**

195 Before using parametric analyses, all data were checked for normality and homogeneity of variance
196 with Shapio-Wilk W test and Levene's test, respectively. All data were analyzed by one-way ANOVA
197 using SPSS Statistics 22 (IBM® SPSS® Statistics, New York, NY, USA) and GraphPad Prism 6.0
198 (GraphPad Inc., La Jolla, CA, USA; figures). Then multiple comparison by DUNCAN analysis (Tukey's
199 multiple range test were used in all figures) were used to determine statistical differences between groups.
200 The results are presented as mean ± SE. $P < 0.05$ was considered as a statistically significant difference.
201 A trend was assumed at $0.05 \leq P < 0.1$.

202

203 **Results**

204 **Effects of selenium on growth performance and pubertal onset in gilts**

205 The average daily body weight gain (ADG) ($P < 0.05$) was greater in gilts receiving HMSeBA
206 during day 148 to 176 and day 120 to 176. The average daily feed intake (ADFI) of gilts in HMSeBA
207 group had lower tendency than that in Na_2SeO_3 and control groups ($P = 0.05$). The ratio of feed: gain was
208 lower in HMSeBA group than that in control group during day 148 to 176 and day 120 to 176. Body
209 weight at puberty was lower in the Na_2SeO_3 group than in the HMSeBA and control group ($P < 0.05$).

The age at puberty, backfat at puberty and duration of estrus cycle were not affected by selenium (Table 3).

Effects of selenium on the development of reproductive tracts, internal organs and follicles in gilts

The selenium (HMSeBA and Na_2SeO_3) increased the weight of uterine and relative weight of uterine ($P < 0.05$, table 4). There's no significant difference in the bodyweight at slaughter, ovaries weight, length of uterine horn and internal organs.

The number of small follicles (diameter, 1-5mm), large follicles (>5mm; these follicles will ovulate in the estrus) and corpora lutea (No. of corpus luteum represents the number of ovulations at last estrus.) were not affected by selenium (Table 5). However, the HMSeBA decreased the gene expression of *growth differentiation factor-9* (*GDF-9*) in COCs than in the control ($P < 0.05$). HMSeBA and Na_2SeO_3 decreased *bone morphogenetic protein-15* (*BMP-15*) expression in COCs than in the control ($P < 0.05$).

Effects of selenium on the selenium content and hormone concentration in gilts

Dietary supplementation with HMSeBA significantly increased the total selenium content in serum and liver ($P < 0.05$, Table 6) compared with control groups in gilts, while Na_2SeO_3 group increased the total selenium content in serum and liver compared with control group in gilts too ($P < 0.05$). Dietary supplementation with HMSeBA tended to increase the total selenium content in ovary compared with control group in gilts ($P = 0.08$).

There was no difference among the treatments in the concentration of T_3 and T_4 in the serum. Dietary supplementation with selenium (HMSeBA and Na_2SeO_3) didn't change the FSH, LH and estradiol concentration in the serum and follicular fluid of gilts compare with control group.

Effects of selenium on the antioxidant and oxidative indicators in gilts

Dietary supplementation with HMSeBA significantly decreased the concentration of MDA ($P <$

232 0.05, Table 7) as compared with control group in the serum, liver and ovary of gilts, and increased the
233 GPx activity in the serum, liver and ovary compared with control group ($P < 0.05$). HMSeBA and
234 Na_2SeO_3 supplementation increased the concentration of T-AOC in the liver compared with control group
235 ($P < 0.05$). HMSeBA and Na_2SeO_3 supplementation increased the concentration of TrxR compared with
236 control group in the ovary of gilts ($P = 0.02$). Na_2SeO_3 supplementation increased the activity of T-SOD
237 compared with HMSeBA and control groups in the ovary of gilts ($P < 0.05$).

238 **Effects of selenium on the gene expression in gilts**

239 HMSeBA supplementation significantly increased the expression of *GPx2* and *TrxR1* ($P < 0.05$)
240 compared with Na_2SeO_3 supplemented group in the liver of gilts, while increased the expression of *GPx1*
241 compared with Na_2SeO_3 group in the ovary of gilts (Table 8). Na_2SeO_3 supplementation decreased the
242 expression of *SelW* and *SelO* compared with control group in the ovary of gilts. Besides, HMSeBA
243 supplementation significantly increased the expressions of *SOD1* ($P = 0.03$) in the liver of gilts compared
244 with both control and Na_2SeO_3 groups. HMSeBA supplementation increased the expression of *Trx1* (P
245 $= 0.02$) compared with Na_2SeO_3 group in the liver of gilts. In addition to the above genes, although the
246 difference was not significant, it was observed a numerical improvement in gene expression with
247 HMSeBA compared to Na_2SeO_3 for all of the selenoproteines in liver.

248 **Effect of selenium on the granulosa cell proliferation and estradiol secretion in vitro**

249 HMSeBA significantly increased the cell proliferation at 5 ng/mL ($P < 0.05$, Fig. 1a), Na_2SeO_3 had
250 tendency to increased cell proliferation at 2.5 ng/mL ($P = 0.097$). However, there was no significant
251 difference ($P > 0.05$) among the treatments.

252 Estradiol secretion is an important manifestation of the physiological function of granular cells.
253 HMSeBA significantly increased the E_2 concentration compared with the control group at 5 and 10 ng/mL

($P < 0.05$, Fig. 1b). However, adding different concentrations of Na_2SeO_3, there was no significant difference in E_2 concentration in granulosa cells.

Effect of selenium on the oxidative stress status in granulosa cells in vitro

Compared with the control group, the total antioxidant capacity of cells *in vitro* was improved by adding of inorganic selenium and organic selenium (HMSeBA, $P < 0.05$, Fig. 2a). The addition of HMSeBA at a concentration of 2.5 ng/mL increased the intracellular T-SOD content ($P < 0.05$, Fig. 2b). The addition of Na_2SeO_3 and HMSeBA at a concentration of 5 ng/mL increased the intracellular GPx level ($P < 0.05$, Fig. 2c), compared with the control group. HMSeBA increased the GR concentration compared with the control group at 2.5ng/mL ($P < 0.05$, Fig. 2d). HMSeBA at 2.5 ng/mL increased the content of TrxR in the cells ($P < 0.05$) (Fig. 2e). Compared with the control group, Na_2SeO_3 and HMSeBA both decreased the concentration of MDA ($P < 0.05$, Fig. 2f) at the concentration of 5 ng/mL of Na_2SeO_3 and 5, 10 ng/mL of HMSeBA.

Effect of selenium on the oxidative stress relative gene expression in granulosa cells in vitro

HMSeBA increased the expression of *SOD2* and *GR* gene in granulosa cells at 10 ng/mL and 2.5 ng/mL compared with control group ($P < 0.05$, Fig. 3b and 3c). HMSeBA and inorganic selenium Na_2SeO_3 both up-regulated antioxidant-related *GPx1* gene expression in the granulosa cells at the concentration of 5, 10 ng/mL of Na_2SeO_3 and 5 ng/mL of HMSeBA ($P < 0.05$, Fig. 3e). However, there was no significant difference at transcription level of *SOD1* and *TrxR1* when granulosa cells treated with different concentrations of Na_2SeO_3 or HMSeBA (Fig. 3a and 3d).

Discussion

Studies of selenium (Se) status indicate that Se is necessary for fertility [5-7,9]. HMSeBA as an

organic Se source has been studied in poultry, weaned pigs, growing pigs and sows [12,15,16,19]. This is the first study focused on the effect of dietary supplementation with HMSeBA on the follicle development in gilts. The elemental distribution in the bovine ovary showed that Se is consistently localized to the granulosa cell layer of large (410 mm) healthy follicles which was identified by X-ray fluorescence (XRF) imaging [20]. As far as we know, folliculogenesis is characterized by a rapid growth of small primary follicles with few granulosa cells to mature preovulatory follicles with several layers of cells [21]. Granulosa cell growth is therefore an important feature in this developmental process. We also investigated the effect of HMSeBA on the *in vitro* proliferation and function in granulosa cells. The results of this study will provide a reference for the application of HMSeBA in gilt diet.

In the context of African swine fever, DLY gilts was used in this study. We found that the age and weight of puberty in DLY gilts were higher than those in LY gilts (220-250d vs. 190-210d, 120-140kg vs. 105-120kg, Zhou et al., 2010) [22]. In the current study, the gilts were applied the same treatments with their maternal generation during gestation [16]. Dietary supplementation with HMSeBA increased the average daily body weight gain (ADG) and decreased the ratio of feed: gain during day 120 to 176 in gilts. However, Jlali et al. (2014) [14] and Chao et al (2019) [15] didn't find the dietary HMSeBA supplementation affect growth performance in growing pigs (a 32-d experiment) and weaned piglets (a 28-d experiment). This could be attributed to the long-term effect of HMSeBA on the gilt. We also found the long-term HMSeBA treatment increased the weight of uterine at the third estrus in gilts. In addition, although the statistical difference is not significant, dietary supplementation with HMSeBA increased the average number of large follicles (>5mm, 17.6 vs. 13.2) and corpora lutea (23.2 vs. 33.0).Growth differentiation factor-9 (GDF-9, Shimizu et al., 2004) [23] and bone morphogenetic protein-15 (BMP-15, Otsuka et al., 2000) [24] are important oocyte-secreted factors which stimulate granulosa cell

298 proliferation and suppress progesterone production in the early stages of follicular development. Porcine
299 *GDF-9* and *BMP-15* genes were reported to be highly expressed in immature oocytes and declined during
300 the oocyte maturation process [25,26]. In present study, dietary supplementation with HMSeBA
301 decreased *GDF-9* and *BMP-15* gene expression compared with control group which reflected the
302 maturity of oocytes were much better than control group. Similar results were obtained by Zhou et al.
303 (2010) [22] the down-regulated of GDF-9 and BMP-15 expression conducive to the improvement of
304 oocyte quality by increased energy feeding in gilt.

305 Selenium concentrations in serum and liver was increased by both HMSeBA and Na_2SeO_3 diet in
306 comparison with gilts receiving the unsupplemented control diet. This result agreed with previous studies
307 on pigs [14,15,27] and even on other species such as poultry [12,19], sheep [28] and cattle [29]. HMSeBA
308 increased the total selenium content in liver compared with Na_2SeO_3 group, similar results were obtained
309 by Hu et al. (2011) [30] and Zhan et al. (2011) [31], which found that Se concentration elevation was
310 greater when the organic Se source was provided. We also found that dietary supplementation with
311 HMSeBA tended to increase the total selenium content in ovary compared with control group in gilts. In
312 the present study, consistent with previous study [11], we found that selenium didn't increase T_3, T_4, FSH
313 and LH concentration in serum at the third estrus of gilts. Fortier et al. (2012) [11] found that there's no
314 difference of FSH concentration among the selenium treatments on the third estrus but the FSH
315 concentration decreased with the estrus progress when dietary supplementation with 0.3 mg/kg sodium
316 selenite and Se-enriched yeast. There is little known about the interaction between FSH and Se status.
317 The relationship between FSH and GPx has been observed in rats [32] which have found that apoptosis
318 in preovulatory follicles induced by oxidative stress is prevented by FSH through stimulation of follicular
319 glutathione synthesis and suppression of ROS production. In present study, we found that HMSeBA

increased the GPx activity in serum, liver and ovary, increased the T-AOC in the liver and the TrxR in the ovary, decreased the MDA in the serum, liver and ovary. For E_2, there was no treatment effect in the serum and follicular fluid among treatments. However, there was a numerical improvement in follicular fluid in HMSeBA treatment (45.8 pg/mL in HMSeBA vs. 39.8 pg/mL in Na_2SeO_3 and 43.7 pg/mL in control). Fortier et al (2012) [11] found that plasma E_2 concentration peaked on d −1 of peri-ovulatory in gilts and rapid decline with the ovulation progress. They also found that the E_2 concentration was less in sodium selenite treatment than in Se-enriched yeast and control (without supplemental Se) treatment on d −1 of peri-ovulatory and no difference among the treatments with other time of the peri-ovulatory. This response might be related to the different sampling time *in vivo* or the complexity of the *in vivo* test did not reflect the treatment effect. In our *in vitro* experiment, HMSeBA significantly increased the E_2 concentration compared with control.

It is well known that maintaining homeostatis of redox system plays an important role female reproductive function. However, if oxygen free radicals are not eliminated in time, the redox state in the body will be out of balance, leading the body in a state of oxidative stress [33]. There are two types of antioxidant systems in the body, one is the enzyme antioxidant system, including SOD, catalase (CAT), GPx and so on. The other is the non-enzymatic antioxidant system, including thioneine, vitamin C, vitamin E, glutathione, selenium (Se), etc [34]. Oxidative stress impaired many physiological processes of reproductive system, from oocyte maturation and fertilization to pregnancy and embryo development [11,35,36]. Once oxidative stress occurs in granulose cell, it impairs its function. In turn, it affects ovulation of the ovary and the reproductive performance of gilt [9]. Our *in vitro* experiment suggested that HMSeBA, a new organic selenium, played a regulatory role in GC proliferation and E_2 synthesis. These findings agreed with those from previous studies reporting that sodium selenite supplementation

342 plays a regulatory role on E_2 synthesis and the proliferation of bovine GCs [37] and goat luteinized
343 granulosa cells (LGCs) [38] *in vitro*. However, the mechanism by which selenium regulates the
344 proliferation of granulosa cells is not yet clear.

345 Cell proliferation is closely related to oxidative stress [39]. Free radicals are produced continuously
346 in aerobic cells. Therefore, we investigated the antioxidant status of GCs. Administration of 5 ng/mL
347 Na_2SeO_3 and HMSeBA to the culture medium significantly both increased total antioxidant capacity and
348 GPx activity in the cells, which was also accompanied by higher proliferation rates especially in
349 HMSeBA treatment. The enzyme GR recycles oxidized glutathione (GSSG) by converting it to the
350 reduced form (GSH) in an NADPH-dependent manner. During the estrous cycle in the rat, the strongest
351 expression of GR was observed in oocytes, followed by granulosa cells [40]. GSH is known to increase
352 gamete viability and the efficiency of fertility, thus GR is predicted to play a pivotal role in the
353 reproduction process as a source of GSH [40]. In our *in vitro* study, HMSeBA (2.5 ng/mL) treatment
354 increased the GR concentration compared with the control, which was consistent with the results of gene
355 expression. However, no difference in GR in the ovary at the transcription level and translation level in
356 *in vivo* experiment. This may be due to HMSeBA regulates the expression of GR at the post-
357 transcriptional level or too many cell types in the ovary mask the effect of HMSeBA on granulosa cells
358 *in vivo*. However, this hypothesis requires more research to verify. The increasing of GR with the
359 supplementation of HMSeBA indicated that the HMSeBA might be beneficial with GCs to increase
360 oocyte viability and the fertility efficiency. Additionally, we found that HMSeBA treatment (10 ng/mL)
361 increased mRNA expression of *SOD2 in vitro*, HMSeBA (5 ng/mL) and Na_2SeO_3 (5, 10 ng/mL) increased
362 mRNA expression of *GPx1 in vitro* and HMSeBA group increased mRNA expression of *GPx1* in the
363 ovary *in vivo*, which agreed with results from previous study, which suggested that Se might affect cell

proliferation by regulating the activity of antioxidant enzymes in testis [41,42].

SOD exists in intracellular cytoplasmic spaces in major form as CuZn-SOD or SOD1, with Cu or Zn deficiency directly resulting in decreased Cu-/Zn-SOD activity [43]. SOD2 (Mn-SOD) is exclusively localized in the mitochondrial spaces, which been demonstrated that high expression in rodent steroidogenic [44,45]. A recent study found that, SOD2-deficiency in mice induced oxidative stress inhibits ovarian granulosa cell steroidogenesis, which mainly by interfering with cholesterol transport to mitochondria and attenuating the expression of Star protein gene and key steroidogenic enzyme genes [46]. Similarity to the results of this study, in our *in vitro* study, the SOD2 mRNA expression and E_2 level both increased in the HMSeBA treatment at 10 ng/mL , Ceko et al. (2015) [20] found that only *GPx1* was significantly up-regulated in large healthy follicles compared to the small healthy or atretic follicles rather than *GPx3*, *VIMP* and *SELM* in bovine and human granulosa cells. GPx1 is abundant in the liver and erythrocytes, with its concentration being dependent on the nutritional Se status. It is also one of the most highly sensitive selenoproteins to change with Se status. The levels of mRNA and protein are dramatically reduced under low Se conditions [47]. The relationship of Se presence with both size of follicle and state of health suggests an important biological role with the most likely function being that the selenoproteins are providing a defense against oxidative stress and inevitable atresia or damage to DNA in the oocyte. In particular the highest levels of Se and GPx1 were observed in granulosa cells of large follicles [20] which are known to express higher levels of cytochrome P450s (cholesterol side-chain cleavage and aromatase) [48] to enable large dominant follicles to synthesize progesterone and estradiol. It has reported that *GPx1* has the highest expression level in the granulosa cells from the most estrogenic follicle prior to emergence of the dominant follicle and then after emergence the dominant follicle has higher levels than the subordinate follicles [49]. From these studies the expression of *GPx1* was so

strongly associated with large follicles suggests that antioxidant could be involved in the signaling process leading to dominance, or that it is simply scavenging the ROS which would otherwise accelerate atresia in competing follicles, or that it protects oocytes from the increasing levels of ROS associated with increasing steroidogenesis in maturing follicles. In present study, the expression of *GPx1* both increased in inorganic selenium Na_2SeO_3 and organic selenium HMSeBA *in vitro*, and in organic selenium HMSeBA in the ovary *in vivo*, which further proof of the important role of selenium in reproduction.

Thioredoxin reductase (TrxR) in mammalian is a family member of selenium-containing pyridine nucleotide-disulphide oxidoreductases. TrxR and Thioredoxin (Trx) plus NADPH are the components of the Trx/TrxR system, which have been implicated a key antioxidant system in defense against oxidative stress [50,51]. It's reported that, using ^{75}Se, the Se incorporated into TrxR increased with increasing concentration of selenium in the cell medium of cancer cell [52]. The mRNA of TrxR increased 2-5-fold at 1 microM selenium compared with that in the absence of selenium [52]. The studies in rats also found that fed selenium deficiency diet decreased TrxR activity in the liver, kidney and lung for several weeks [53,54]. In broiler chicks, it had been found that the TrxR activity in the pectoral and thigh muscles (20-37%) was increased with the diet supplementation of HMSeBA compared with selenium-deficient treatment [55]. Similar to previous study, in this study compared with control, although the difference was not significant at the transcription level, HMSeBA rather than Na_2SeO_3 significantly increases the activity of TxrR at the protein level in the GCs *in vitro* and in the ovary *in vivo*. These results indicated that HMSeBA might have much more effects on the Trx/TrxR system to help cells to defense against oxidative stress.

MDA is an oxidized lipid metabolite and can be used to measure the level of lipid peroxidation [56].

The results of this study indicate that Na_2SeO_3 and HMSeBA both decreased the concentration of MDA thus beneficial to the redox balance *in vitro* in granulosa cells and in the liver *in vivo* in gilts. At same time, HMSeBA decreased the concentration of MAD in the serum and ovary *in vivo* in gilts. The maintenance of redox balance is crucial for effective immunity and health and biomarkers of oxidative stress have been linked to cellular functionality [57]. Therefore, Na_2SeO_3 and HMSeBA both have the positive impact on the redox balance.

Conclusion

Collectively, dietary HMSeBA supplementation in gilts increased the selenium deposition and antioxidant capacities in gilts. HMSeBA promoted follicle development by antioxidant pathway from the *in vivo* and *in vitro* data. Therefore, the new type of organic selenium, HMSeBA, could be potentially useful for the gilt reproductive processes.

Availability of data and materials

The data generated or analyzed during this study are available from the corresponding author on reasonable request.

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Author Contributions

Shengyu Xu and De Wu designed and supervised the experiments. Yanpeng Dong, Zimei Li, Sirun Chen, Yalei Liu and Xinlin Jia conducted the experiments. Shengyu Xu, Yanpeng Dong, Zimei Li, Jian Li, Yan Lin, Lianqiang Che, Zhengfeng Fang, Bin Feng, Yong Zhuo, Jianping Wang and Zhihua Ren performed the data measurements, statistical data analysis and supervision. Shengyu Xu, Sirun Chen and M. Briens wrote and revised the manuscript. All authors have read and agreed to the published version of the manuscript.

Ethics declarations

Ethics approval and consent to participate

The experimental protocol, including animal care and use, was reviewed and approved by the Animal Care and Use Committee of Sichuan Agricultural University.

Conflicts of Interest

The authors declare no conflict of interest.

Abbreviations

ADFI: Average daily feed intake

ADG: Average daily body weight gain

BMP-15: Bone morphogenetic protein-15

COCs: Cumulus-oocyte complexes

E2: 17β-estradiol

FSH: Follicle stimulating hormone

GCs: Granulosa cells

GDF-9: Growth differentiation factor-9

GPx: Glutathione peroxidase

GR: Glutathione reductase

HMSeBA: 2-hydroxy-4-methylselenobutanoic acid

LH: Luteinizing hormone

MDA: Malondialdehyde

ROS: Reactive oxygen species

Se: Selenium

SOD: Superoxide dismutase

T3: Three iodine thyroid

T4: Thyroxine

T-AOC: Total antioxidant capacity

Trx: Thioredoxin

TrxR: Thioredoxin reductase

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624

Table 1 Composition and nutrient level of the basal diet (as-fed basis)

	7-25kg	25-75kg	75kg-end
Ingredient, %			
De-hulled soybean meal, 46% CP	15.00	-	-
Extruded maize meal, 8.24% CP	12.40	-	-
Expanded soybean, 35.5% CP	10.00	-	-
Whey powder, 2% CP	5.00	-	-
Sucrose	3.90	-	-
Corn, 8.24% CP	45.00	69.48	72.00
Soybean, 44% CP	-	19.00	14.00
Wheat bran, 15% CP	-	5.00	7.57
Fish meal, 62.5% CP	3.00	0.50	1.50
Soybean oil	2.00	2.00	2.00
$_{L}$-Lys HCl, 98%	0.60	0.49	0.28
$_{DL}$-Met, 98.5%	0.22	0.10	0.04
$_{L}$-Thr, 98%	0.19	0.17	0.08
$_{L}$-Trp, 98%	0.05	0.05	0.03
Choline chloride, 50%	0.16	0.16	0.12
Limestone	0.85	0.94	0.95
$CaHPO_4$	0.86	1.60	0.95
Sodium chloride	0.40	0.28	0.25
Mineral premix	0.32[1]	0.20[2]	0.20[2]
Vitamin premix	0.05[3]	0.03[4]	0.03[4]
Total	100.00	100.00	100.00
Nutrient level[5]			
Digestible energy, Mcal/kg	3.538	3.449	3.430
Crude protein, %	17.06	14.21	14.52
Ca, %	0.85	0.85	0.79
Se, (mg/kg)	0.09	0.06	0.04
Total P, %	0.52	0.51	0.52

[1] Per kilogram of diet provided: 125 mg Fe; 14 mg Cu; 30 mg Mn; 110 mg Zn; 0.3 mg I.

[2] Per kilogram of diet provided: 120 mg Fe; 12 mg Cu; 30 mg Mn; 100 mg Zn; 0.28 mg I.

[3] Per kilogram of diet provided: 12000 IU VA; 2400 IU VD_3; 100 IU VE; 4.8 mg VK_3; 2 mg VB_1; 7.2 mg VB_2; 3.6 mg VB_6; 0.025 mg VB_{12}; 0.48 mg biotin; 25 mg pantothenic acid; 4 mg folic acid; 40 mg niacin.

[4] Per kilogram of diet provided: 7200 IU VA; 1440 IU VD_3; 60 IU VE; 2.88 mg VK_3; 1.2 mg VB_1; 4.32 mg VB_2; 2.16 mg VB_6; 0.015 mg VB_{12}; 0.288 mg biotin; 15 mg pantothenic acid; 2.4 mg folic acid; 30 mg niacin.

[5] Excepted the calculated values of digestible energy, the rest were all measured values. For the control, the three phases of the measured selenium concentration were 0.09, 0.06 and 0.04 mg/kg, the Na_2SeO_3 treatment the three phases of the measured selenium concentration were 0.35, 0.33 and 0.31 mg/kg, the HMSeBA treatment the three phases of the measured selenium concentration were 0.37, 0.41 and 0.41 mg/kg, respectively.

637 **Table 2** Primer sequences of the target genes

Genes	Primer	Sequence (5'→3')	Accession no.
for gilt tissue			
SelP	Forward	AACCAGAAGCGCCAGACACT	EF113596
	Reverse	TGCTGGCATATCTCAGTTCTCAGA	
SelW	Forward	CACCCCTGTCTCCCTGCAT	NM_213977
	Reverse	GAGCAGGATCACCCCAAACA	
SelO	Forward	CTTCCGACCCCAGATGGAT	AK236851
	Reverse	GGTTCGACTGTGCCAGCAT	
SelH	Forward	TGGTGGAGGAGCTGAAGAAGTAC	HM018602
	Reverse	CGTCATAAATGCTCCAACATCAC	
GPx1	Forward	GATGCCACTGCCCTCATGA	AF532927
	Reverse	TCGAAGTTCCATGCGATGTC	
GPx2	Forward	AGAATGTGGCCTCGCTCTGA	DQ898282
	Reverse	GGCATTGCAGCTCGTTGAG	
GPx3	Forward	TGCACTGCAGGAAGAGTTTGAA	AY368622
	Reverse	CCGGTTCCTGTTTTCCAAATT	
GPx4	Forward	TGAGGCAAGACGGAGGTAAACT	NM_214407
	Reverse	TCCGTAAACCACACTCAGCATATC	
TrxR1	Forward	GATTTAACAAGCGGGTCATGGT	AF537300
	Reverse	CAACCTACATTCACACACGTTCCT	
TrxR2	Forward	TCTTGAAAGGCGGAAAAGAGAT	GU181287
	Reverse	TCGGTCGCCCTCCAGTAG	
SPS2	Forward	TGGCTTGATGCACACGTTTAA	EF033624
	Reverse	TGCGAGTGTCCCAGAATGC	
SOD1	Forward	GAGCTGAAGGGAGAGAAGACAGT	NM_001190422.1
	Reverse	GCACTGGTACAGCCTTGTGTAT	
SOD2	Forward	CTGGACAAATCTGAGCCCTAAC	NM_214127.2
	Reverse	GACGGATACAGCGGTCAACT	
CAT	Forward	CGAAGGCGAAGGTGTTTG	NM_214301.2
	Reverse	AGTGTGCGATCCATATCC	
GR	Forward	AGCTCCTCACATCCTGATTGC	AY368271.
	Reverse	CCAGCTATCTCCACAGCAATGT	
SCLY	Forward	ATCGTGGGCCACAAGTTCTATG	FJ860901
	Reverse	GCTCTTGTCCACCTCCAAACA	
Trx1	Forward	CAAGCCTTTCTTCCATTC	NM 214313.2
	Reverse	ACCCACCTTCTGTCCCT	
GDF-9	Forward	CCCCTAGTGGTCTCCAAACAA	AY649763
	Reverse	CAGACAGCCCTCTTTTCTGG	
BMP-15	Forward	AGCTTCCACCAACTGGGTTGG	AF458070
	Reverse	TCATCTGCATGTACAGGGCTG	
β-actin	Forward	TCTGGCACCACACCTTCT	DQ178122
	Reverse	TGATCTGGGTCATCTTCTCAC	
for granulosa cell			
SOD1	Forward	GGTTCCACGTCCATCAGT	NM_011434.2
	Reverse	ACATTGCCCAGGTCTCC	
SOD2	Forward	ATTGACGTGTGGGAGCA	NM_013671.3
	Reverse	AATGTGGCCGTGAGTGA	
TrxR1	Forward	CAGTGTTGCTGGCGGTA	NM_001042523.1
	Reverse	AGGCACATTGGTCTGCTC	
GPx1	Forward	ATCAGTTCGGACACCAGGA	NM_001329528.1
	Reverse	TCTCACCATTCACTTCGCA	
GR	Forward	ATCATTTCGGCCACTCC	NM_010344.4
	Reverse	GTGCAGGTTTTGTTTCCC	
β-actin	Forward	GGCTGTATTCCCCTCCATCG	NM_007393.5
	Reverse	CCAGTTGGTAACAATGCCATGT	

638 *SelP*: selenoprotein P; *SelW*: selenoprotein W; *SelO*: selenoprotein O; *SelH*: selenoprotein H; *GPx*: glutathione
639 peroxidase; *TrxR*: thioredoxin reductase; *Trx1*: Thioredoxin l; *GR*: Glutathione reductase; *SOD*: superoxide
640 dismutase; *CAT*: catalase; *SCLY*: Selenocysteine lyase; *SPS2*: Selenophosphate synthetase 2; *GDP-9*: growth
641 differentiation factor-9; *BMP-15*: bone morphogenetic protein-15; *β-actin*, beta-actin.
642

643 **Table 3** Effects of dietary selenium supplementation on growth performance and pubertal onset in gilts

	Control	Na_2SeO_3	HMSeBA	*P*-value
Initial age, d	21	21	21	
Bodyweight, kg				
Initial 21d	5.54±0.19	5.34±0.45	5.63±0.19	0.99
120d	55.13±3.23	50.27±5.23	53.85±2.74	0.66
148d	77.85±4.61	76.25±5.93	78.23±3.17	0.95
176d	99.00±5.20	100.90±5.42	104.14±4.03	0.75
ADG, g/d				
120-148d	811.43±53.35	908.89±37.40	870.46±33.76	0.30
148-176d	726.43±50.38^{b}	851.23±31.34^{ab}	896.75±44.99^{a}	0.03
120-176d	768.93±39.11^{b}	880.06±17.70^{a}	883.61±31.10^{a}	0.02
ADFI, kg/d				
120-148d	2.42±0.16	2.49±0.20	2.42±0.09	0.94
148-176d	3.34±0.16	3.33±0.18	2.91±0.05	0.05
120-176d	2.88±0.14	2.91±0.18	2.66±0.06	0.37
Feed: Gain				
120-148d	3.00±0.11	2.71±0.15	2.79±0.09	0.21
148-176d	4.79±0.37^{a}	4.01±0.33^{ab}	3.33±0.18^{b}	0.01
120-176d	3.89±0.21^{a}	3.36±0.23^{ab}	3.06±0.10^{b}	0.01
Age at puberty, d	247.29±9.16	227.33±9.51	249.67±8.66	0.18
BW at puberty, kg	141.93±5.98^{a}	126.61±2.39^{b}	139.88±2.68^{a}	0.01
Backfat at puberty, mm	17.48±1.46	16.42±0.65	16.98±0.93	0.77
Duration of estrus cycle, d	20.71±0.36	20.44±0.41	21.33±0.33	0.23

644 Data are expressed as means values and standard error, n = 5 (one repeatment was eliminated, because of sick at
645 younger). Mean values with different superscript letters were significantly different ($P < 0.05$). Control, basal diet;
646 Na_2SeO_3, 0.3 mg Se/kg Na_2SeO_3; HMSeBA, 0.3 mg Se/kg HMSeBA.
647
648 **Table 4** Effects of dietary selenium supplementation on the development of reproductive tracts and
649 internal organs of gilts

	Control	Na_2SeO_3	HMSeBA	*P*-value
BW at slaughter, kg	162.50±8.22	148.80±3.11	165.50±3.68	0.11
Weight of ovaries, g	9.91±1.32	11.98±1.16	12.22±0.36	0.26
Relative weight of ovaries, g/kg	0.062±0.009	0.080±0.007	0.074±0.002	0.17
Weight of uterine, kg	0.87±0.06^{b}	1.22±0.09^{a}	1.19±0.07^{a}	<0.01
Relative weight of uterine, g/kg	5.37±0.38^{b}	8.20±0.51^{a}	7.21±0.49^{a}	<0.01
Left uterine horn, cm	195.00±22.17	226.30±33.78	184.12±20.09	0.51
Right uterine horn, cm	188.08±12.81	188.52±28.11	186.72±18.31	0.99
Weight of liver, kg	1.94±0.14	1.86±0.11	2.03±0.05	0.52
Relative weight of liver, g/kg	11.92±0.43	12.46±0.5	12.261±0.26	0.68
Weight of spleen, g	203.00±10.79	213.00±14.71	221.00±22.27	0.75
Weight of thymus, g	72.19±12.41	47.24±3.57	66.8±18.7	0.39

650 Data are expressed as means values and standard error, n = 5. Mean values with different superscript letters were
651 significantly different ($P < 0.05$). Control, basal diet; Na_2SeO_3, 0.3 mg Se/kg Na_2SeO_3; HMSeBA, 0.3 mg Se/kg
652 HMSeBA.
653

654 **Table 5** Effects of dietary selenium supplementation on the development of follicles and gene
655 expression of COCs of gilts

	Control	Na_2SeO_3	HMSeBA	*P*-value
No. of follicle, n				
Small follicles, 1-5mm	8.00±2.32	20.75±6.86	9.60±3.22	0.12
Large follicles, >5mm	13.20±2.40	16.00±2.47	17.60±0.93	0.34
No. of corpora lutea, n	23.20±3.00	24.00±5.43	33.00±4.09	0.24
Gene expression of COCs				
GDF-9	1.00±0.00^{a}	0.70±0.27^{ab}	0.17±0.09^{b}	0.01
BMP-15	1.00±0.00^{a}	0.76±0.21^{a}	0.23±0.11^{b}	< 0.01

656 Data are expressed as means values and standard error, n = 5. Mean values with different superscript letters were
657 significantly different ($P < 0.05$). Control, basal diet; Na_2SeO_3, 0.3 mg Se/kg Na_2SeO_3; HMSeBA, 0.3 mg Se/kg
658 HMSeBA.

659

660 **Table 6** Effects of dietary selenium supplementation on the selenium content and hormone

661 concentration in gilts

	Control	Na_2SeO_3	HMSeBA	*P*-value
Selenium content				
Serum (mg/L)	0.18±0.01^{b}	0.21±0.00^{a}	0.22±0.01^{a}	0.02
Liver (mg/kg)	0.46±0.03^{c}	0.67±0.04^{b}	0.91±0.02^{a}	< 0. 01
Ovary (mg/kg)	0.31±0.01	0.34±0.02	0.35±0.01	0.08
Hormone concentration in serum				
T_3 (nmol/L)	1.25±0.04	1.35±0.17	1.09±0.07	0.28
T_4 (ng/mL)	1.21±0.13	1.11±0.06	1.12±0.05	0.81
LH (ng/mL)	23.62±0.85	24.49±3.36	21.82±1.09	0.47
FSH (ng/mL)	22.60±3.21	24.50±1.93	27.02±1.32	0.42
E_2 (pg/mL)	60.10±2.50	59.50±4.64	56.75±1.85	0.74
Hormone concentration in ovarian follicular fluid				
LH (ng/mL)	19.96±1.27	24.08±0.38	23.69±1.70	0.12
FSH (ng/mL)	6.38±0.95	7.32±1.77	4.80±1. 17	0.43
E_2 (pg/mL)	43.71±0.58	39.85±6.44	45.76±6.13	0.76

662 Data are expressed as means values and standard error, n = 5. Mean values with different superscript letters were
663 significantly different ($P < 0.05$). Control, basal diet; Na_2SeO_3, 0.3 mg Se/kg Na_2SeO_3; HMSeBA, 0.3 mg Se/kg
664 HMSeBA. T_3, three iodine thyroid; T_4, thyroxine; LH, luteinizing hormone; FSH, follicle stimulating hormones;
665 E_2, Estradiol.
666

667 **Table 7** Effects of dietary selenium supplementation on the antioxidant and oxidative indicators in gilts

	Control	Na_2SeO_3	HMSeBA	*P*-value
Serum				
MDA (nmol/mL)	1.61±0.11[a]	1.26±0.11[ab]	1.14±0.06[b]	0.01
T-AOC (U/mL)	0.96±0.16	1.04±0.04	1.09±0.06	0.68
T-SOD (U/mL)	90.70±8.39	86.61±2.62	89.25±5.16	0.85
GR (U/L)	5.98±1.64	4.58±2.72	6.19±1.89	0.85
GPx (U/mgprot)	961.61±45.81[b]	1031.06±44.19[ab]	1125.68±36.06[a]	0.05
Liver				
MDA (nmol/mgprot)	3.61±0.19[a]	2.17±0.29[b]	2.24±0.23[b]	<0.01
T-AOC (U/mgprot)	0.70±0.07[b]	1.14±0.10[a]	1.18±0.14[a]	0.02
T-SOD (U/mgprot)	284.77±14.79	338.27±28.91	328.69±18.27	0.21
GR (U/gprot)	0.85±0.05	0.79±0.05	0.84±0.05	0.62
TrxR (U/mgprot)	0.54±0.04	0.71±0.06	0.53±0.10	0.17
GPx (U/mgprot)	344.25±11.37[b]	415.79±18.92[a]	416.57±10.03[a]	<0.01
Ovary				
MDA (nmol/mgprot)	1.07±0.16[a]	0.77±0.21[ab]	0.46±0.01[b]	0.04
T-AOC (U/mgprot)	0.69±0.06	0.74±0.20	0.61±0.11	0.80
T-SOD (U/mgprot)	47.32±1.13[b]	57.88±4.37[a]	39.34±3.06[b]	<0.01
GR (U/gprot)	1.61±0.32	1.32±0.38	0.92±0.03	0.27
TrxR (U/mgprot)	0.15±0.02[b]	0.27±0.03[a]	0.28±0.04[a]	0.02
GPx (U/mgprot)	90.39±2.95[b]	103.14±3.93[ab]	110.19±5.40[a]	0.02

668 Data are expressed as means values and standard error, n = 5. Mean values with different superscript letters were
669 significantly different ($P < 0.05$). Control, basal diet; Na_2SeO_3, 0.3 mg Se/kg Na_2SeO_3; HMSeBA, 0.3 mg Se/kg
670 HMSeBA. MDA, malondialdehyde; T-AOC, total antioxidant capacity; T-SOD, total superoxide dismutase; GR,
671 gluathione reductase; TrxR, thioredoxin reductase; GPx, glutathione peroxidase.
672

673 **Table 8** Effects of dietary selenium supplementation on the expression of genes related to selenoprotein

674 and antioxidant capacity in the liver and ovary of gilts

	Control	Na_2SeO_3	HMSeBA	*P*-value
Liver				
SelP	1.00±0.00	0.85±0.26	1.33±0.39	0.45
SelW	1.00±0.00	1.03±0.20	1.30±0.18	0.37
SelO	1.00±0.00	0.78±0.14	1.20±0.14	0.07
SelH	1.00±0.00	1.04±0.27	1.54±0.28	0.20
GPx1	1.00±0.00	0.80±0.13	1.27±0.17	0.06
GPx2	1.00±0.00^{ab}	0.68±0.14^{b}	1.26±0.19^{a}	0.04
GPx3	1.00±0.00	0.41±0.06	1.30±0.46	0.09
GPx4	1.00±0.00	0.93±0.26	1.08±0.32	0.89
TrxR1	1.00±0.00^{ab}	0.81±0.03^{b}	1.09±0.08^{a}	<0.01
TrxR2	1.00±0.00	0.79±0.06	1.04±0.14	0.13
SPS2	1.00±0.00	0.88±0.18	0.98±0.22	0.85
SOD1	1.00±0.00^{b}	0.83±0.19^{b}	1.52±0.22^{a}	0.03
SOD2	1.00±0.00	1.13±0.27	1.35±0.32	0.61
CAT	1.00±0.00	0.87±0.30	1.55±0.47	0.33
GR	1.00±0.00	0.74±0.16	0.98±0.24	0.49
SCLY	1.00±0.00	1.10±0.21	1.39±0.31	0.43
Trx1	1.00±0.00^{ab}	0.83±0.08^{b}	1.41±0.19^{a}	0.02
Ovary				
SelP	1.00±0.00	0.77±0.48	0.80±0.16	0.84
SelW	1.00±0.00^{a}	0.48±0.08^{b}	0.84±0.16^{ab}	0.01
SelO	1.00±0.00^{a}	0.37±0.06^{b}	0.62±0.17^{ab}	<0.01
SelH	1.00±0.00	0.45±0.11	0.92±0.27	0.08
GPx1	1.00±0.00^{a}	0.32±0.03^{b}	0.86±0.16^{a}	<0.01
GPx2	1.00±0.00	0.82±0.26	1.31±0.32	0.38
GPx3	1.00±0.00	1.17±0.39	1.27±0.33	0.81
GPx4	1.00±0.00	0.79±0.20	1.02±0.34	0.74
TrxR1	1.00±0.00	0.86±0.12	1.63±0.41	0.10
TrxR2	1.00±0.00	0.69±0.15	1.07±0.17	0.15
SPS2	1.00±0.00	0.64±0.24	1.00±0.45	0.65
SOD1	1.00±0.00	0.63±0.19	0.90±0.23	0.34
SOD2	1.00±0.00	1.11±0.45	1.39±0.32	0.67
CAT	1.00±0.00	1.06±0.56	1.38±0.46	0.79
GR	1.00±0.00	1.02±0.58	1.52±0.28	0.56
SCLY	1.00±0.00	0.66±0.20	0.94±0.22	0.36
Trx1	1.00±0.00	0.93±0.46	0.81±0.14	0.88

675 Data are expressed as means values and standard error, n = 5. Mean values with different superscript letters were
676 significantly different ($P < 0.05$). Control, basal diet; Na_2SeO_3, 0.3 mg Se/kg Na_2SeO_3; HMSeBA, 0.3 mg Se/kg
677 HMSeBA. *SelP*: selenoprotein P; *SelW*: selenoprotein W; *SelO*: selenoprotein O; *SelH*: selenoprotein H; *GPx*:
678 glutathione peroxidase; *TrxR*: thioredoxin reductase; *SPS2*: Selenophosphate synthetase 2. *SOD*: superoxide
679 dismutase; *CAT*: catalase; *GR*: Glutathione reductase; *SCLY*: Selenocysteine lyase; *Trx1*: Thioredoxin l.
680

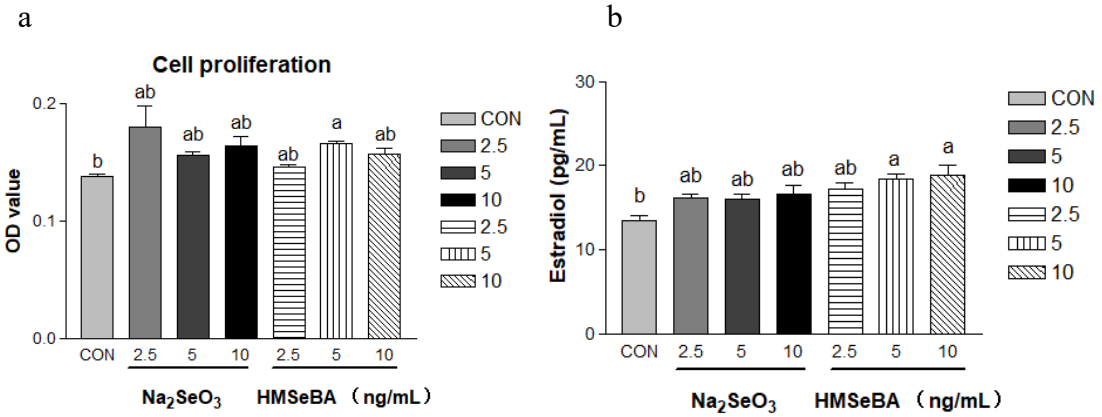

Fig. 1. Effects of selenium from different sources and concentrations on the granulosa cells' proliferation (a) and estradiol (b) secretion in granulosa cells *in vitro*

Control: basal media, Na_2SeO_3 2.5, 5 and 10 ng Se/mL, HMSeBA 2.5, 5, and 10 ng Se/mL. a,b Mean values with different superscript letters were significantly different ($P < 0.05$). Data are expressed as means values and standard error.

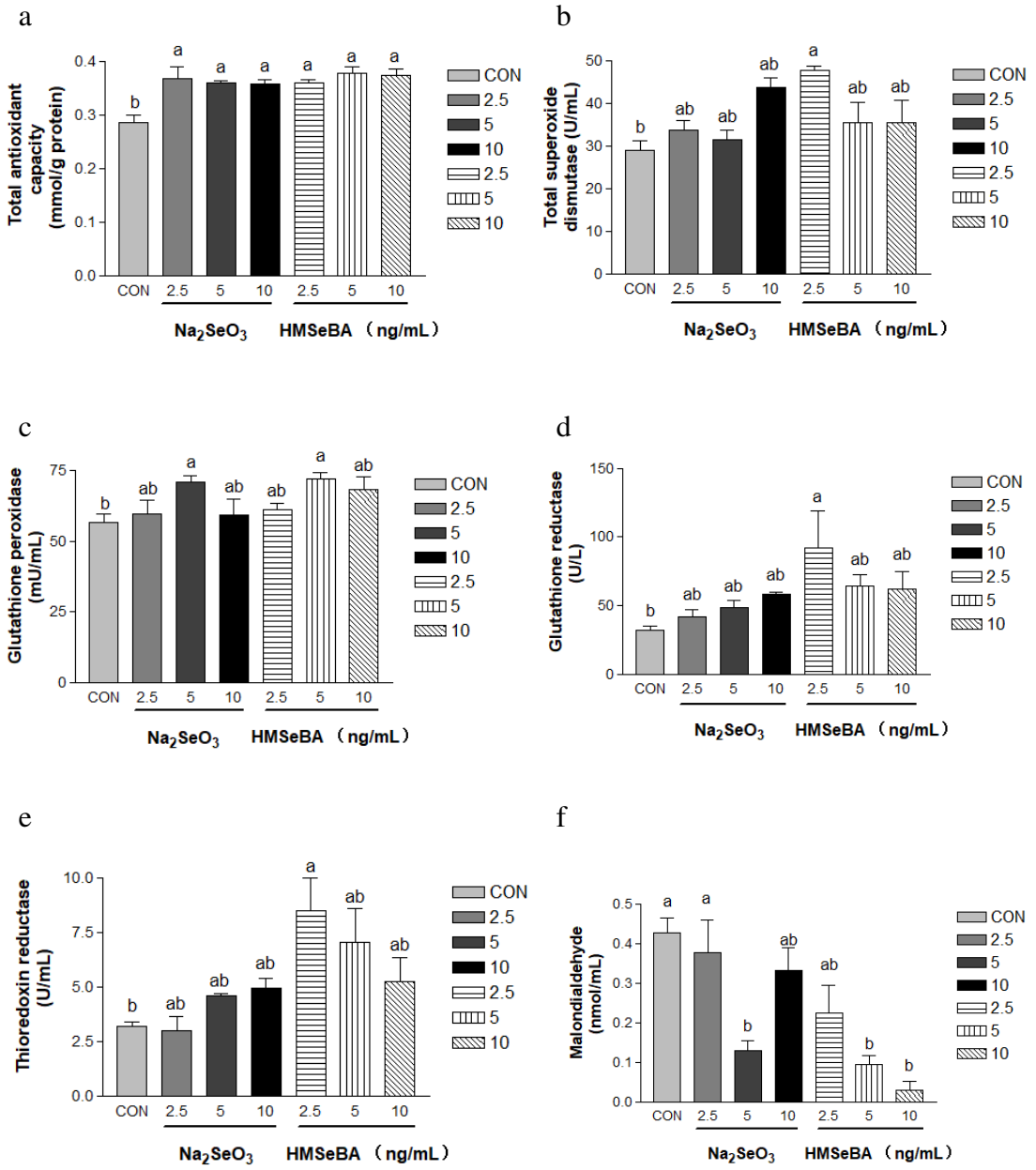

Fig. 2. Effects of selenium from different sources and concentrations on the oxidative stress status (a-f) in the granulosa cells *in vitro*

Control: basal media, Na_2SeO_3 2.5, 5 and 10 ng/mL, HMSeBA 2.5, 5, and 10 ng/mL. a,b Mean values with different superscript letters were significantly different ($P < 0.05$). Data are expressed as means values and standard error.

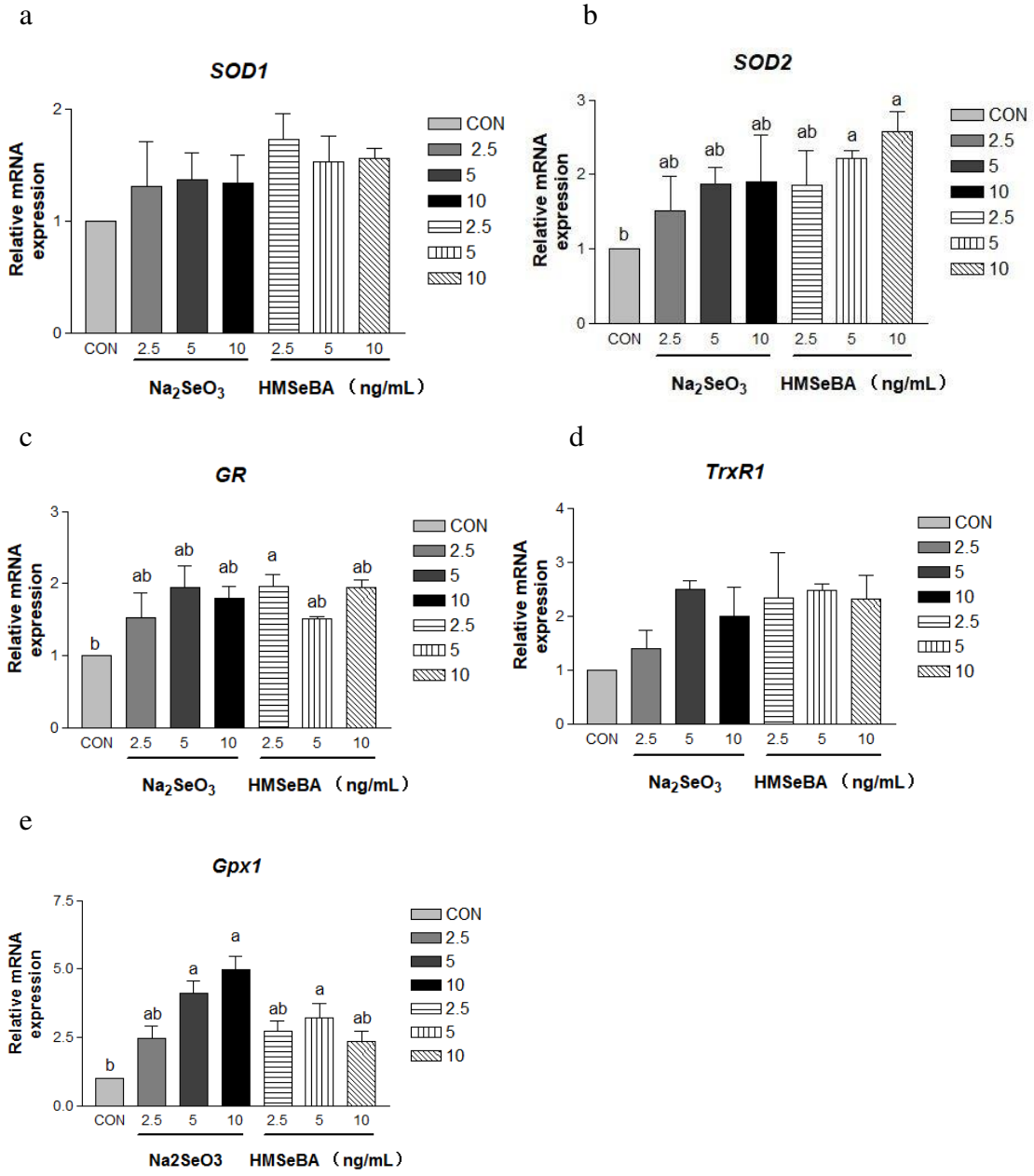

Fig. 3 Effects of selenium from different sources and concentrations on the oxidative stress relative gene expression (a-e) in granulosa cells *in vitro*

SOD1, superoxide dismutase 1; *SOD2*, superoxide dismutase 2; *GR*, glutathione reductase; *TrxR*, thioredoxin reductase; *GPx1*, glutathione peroxidase. Control: basal media, Na_2SeO_3 2.5, 5 and 10 ng/mL, HMSeBA 2.5, 5, and 10 ng/mL. a,b Mean values with different superscript letters were significantly different ($P < 0.05$). Data are expressed as means values and standard error.