—Original Article—

Selenium and Vitamin E Improve the *In Vitro* Maturation, Fertilization and Culture to Blastocyst of Porcine Oocytes

K. M. A. TAREQ^{1,2)}, Quzi Sharmin AKTER²⁾, M. A. M. Yahia KHANDOKER³⁾ and Hirotada TSUJII¹⁾

¹⁾Laboratory of Animal Biotechnology, Department of Bioscience and Biotechnology, Faculty of Agriculture, Shinshu University, Nagano 399-4598, Japan

²⁾Faculty of Animal Science and Veterinary Medicine, Patuakhali Science and Technology University, Barisal-8210, Bangladesh

³⁾Department of Animal Breeding and Genetics, Faculty of Animal Husbandry, Bangladesh Agricultural University, Mymensingh-2202, Bangladesh

Abstract. Selenium (Se) and vitamin E (Vit-E), as integral parts of antioxidant systems, play important roles for sperm and embryos *in vitro*. In this study, the effects of Se and Vit-E on the maturation, *in vitro* fertilization and culture to blastocysts of porcine oocytes and accumulation of ammonia in the culture medium during different development stages were investigated. The maturation was performed in modified tissue culture medium (mTCM)-199 supplemented with 10% (v/v) porcine follicular fluid, the fertilization medium was modified Tyrode's albumin lactate pyruvate (mTALP), and the embryo culture medium was modified North Carolina State University (mNCSU)-23. Se in the form of sodium selenite (SS) and seleno-L-methionine (SeMet) and Vit-E at different concentrations were also used. The incorporation and oxidation of ¹⁴C(U)-glucose were assessed with a liquid scintillation counter. In this study, SeMet and SeMet+Vit-E increased oocyte maturation, fertilization and incorporation and oxidation of ¹⁴C(U)-glucose significantly ($P<0.05$) compared with the control and other treatments. In addition, embryo development, specifically in terms of the numbers of morulae and blastocysts, significantly increased ($P<0.05$) with SeMet and SeMet+Vit-E. In contrast, the accumulation of ammonia was reduced with SeMet and SeMet+Vit-E compared with other treatments. These findings indicate that SeMet and SeMet+Vit-E may play important roles in reducing the accumulation of ammonia and subsequently in increasing the rate of maturation of porcine oocytes and fertilization, as well as development of the blastocyst and utilization of glucose in *in vitro* maturation, fertilization and culture to blastocysts of porcine oocytes.

Key words: Ammonia, Incorporation and oxidation, Porcine embryo, Selenium, Vitamin E

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Ammونيا produced from amino acids present in culture medium inhibits cell growth *in vitro*. The levels of ammonia increase significantly during the culture period because of the deamination and spontaneous breakdown of amino acids, especially glutamine, in *in vitro* fertilization of porcine oocytes [1]. The toxic effect of glutamine can be avoided by making use of the fact that dipeptides can be directly used by mammalian cells *in vitro* [2]. We reported previously that the accumulation of ammonia in the medium can be reduced by supplementation with the dipeptides L-alanyl-L-glutamine and L-glycyl-L-glutamine, which can play important roles in motility and the acrosome reaction (AR) in porcine spermatozoa [3].

In recent years, the involvement of free radicals and oxidative damage has been proposed as an alternate mechanism for ammonia toxicity [4]. Selenium (Se), an essential trace element for mammals, is an integral part of antioxidant systems [5]. Se-dependent glutathione peroxidase (GPx) has an important role in free radical

protective mechanisms. Se may have a role in the motility of bovine spermatozoa by protecting them from oxidative damage [6]. Se, which commonly replaces sulfur in proteins, is normally incorporated through selenoamino acids (L-selenomethionine, L-selenocysteine) and selenoenzymes, such as GPxs, which oxidize glutathione in the antioxidant system and protect cells from oxidative damage [7].

Vitamin E (Vit-E) is one of the most important antioxidative molecules, residing mainly in the cell membranes [8]. We reported previously that antioxidant Vit-E protects mouse embryos from oxidative damage [9]. Olson and Seidel [10] reported that the development of bovine embryos *in vitro* improved with Vit-E addition. We previously reported that seleno-L-methionine (SeMet) and SeMet+Vit-E may play important roles in reducing the accumulation of ammonia and subsequently in increasing the rate of AR and the utilization of glucose in porcine spermatozoa [11].

Although several studies have shown the importance of Se levels in embryo development, the direct influences of Se and Vit-E on reducing the accumulation of ammonia and on embryo development in porcine *in vitro* have not been definitively demonstrated yet.

In this study, we have investigated the roles of sodium selenite (SS), SeMet and Vit-E in embryo development and accumulation of ammonia in the medium during *in vitro* maturation, fertilization

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Correspondence: H Tsujii (e-mail: htsujii@shinshu-u.ac.jp)

and embryo development in porcine and assessed the utilization of $^{14}\text{C}(\text{U})$ -glucose.

Materials and Methods

Chemicals

Radioactive $^{14}\text{C}(\text{U})$ -glucose was purchased from American Radiolabeled Chemicals (St. Louis, MO, USA); SS, SeMet, Vit-E (α -tocopheryl acetate, E) and all other chemicals were of analytical grade and purchased from Nacalai Tesque (Kyoto, Japan), unless otherwise indicated. Vit-E was dissolved in ethanol, and an emulsion was formed by vortex mixing before being added to the embryo. The final concentration of ethanol was <2% (the volume of ethanol was 0.15 ml in the total volume) [12]. The control and other treatments involved an equivalent amount of ethanol.

Oocyte recovery and *in vitro* maturation

Ovaries were collected from gilts (Landrace, Large White and Duroc) at a local slaughterhouse and transported to the laboratory in physiological saline supplemented with 100 U/ml penicillin G and 100 mg/ml streptomycin sulfate at around 30–35 °C within 1–3 h of collection. Cumulus-oocyte complexes (COCs) from follicles 3 to 6 mm in diameter were aspirated using an 18-gauge needle attached to a 10 ml disposable syringe. Intact COCs were selected using mouth pipettes and washed three times in HEPES-buffered North Carolina State University medium (NCSU)-23 supplemented with 0.3% bovine serum albumin (BSA). Washed COCs were transferred to IVM medium consisting of modified tissue culture medium (mTCM)-199, which was supplemented with 10 ng/ml epidermal growth factor, 4 IU/ml pregnant mare serum gonadotropin (PMSG; Sankyo Zoki, Tokyo, Japan) and human chorionic gonadotropin (hCG; Sankyo Zoki), and 10% (v/v) porcine follicular fluid (pFF). The pFF was collected from antral follicles of prepubescent gilts, centrifuged at $1600\times g$ for 30 min and filtered through 1.2 and 0.45- μm syringe filters (Toyo Roshi Kaisha, Tokyo, Japan). The filtered pFF was stored in aliquots at –20 °C. Fifty COCs in 500 μl of IVM medium were cultured at 39 °C in 5% CO_2 in air. After culturing for 22 h, COCs were washed three times and cultured in PMSG and hCG-free mTCM-199 medium for an additional 22 h at 39 °C in 5% CO_2 in air.

Sperm preparation and *in vitro* fertilization

Ejaculated sperm were obtained from Duroc boars and diluted according to the method of Johnson *et al.* [13]. After being washed three times by centrifugation at $900\times g$ for 5 min, the sperm pellet was resuspended in *in vitro* fertilization medium, which was modified Tyrode's albumin lactate pyruvate (mTALP) medium containing 3 mg/mL BSA and 2 mM caffeine, mTALP medium, to give a final concentration of 2×10^6 spermatozoa/ml [1]. The oocytes and spermatozoa were co-cultured for 6 h at 39 °C in an atmosphere of 5% CO_2 in air. At 44 h of maturation culture, oocytes were freed from cumulus cells by repeated pipetting in 0.1% hyaluronidase in mTCM-199 medium and washed three times with pre-equilibrated mTALP. After washing, 20–25 oocytes were placed in 45 μl drops of the mTALP (fertilizing drop) covered with pre-warmed paraffin oil and 5 μl of sperm suspension was added to each fertilization drop to give a final sperm concentration of 2×10^6 sperm/ml. After

co-incubation of gametes for 6 h, the presumptive zygotes were washed and transferred into *in vitro* culture (IVC) medium.

Embryo culture

After IVF, COCs were washed several times in a fertilization drop to remove spermatozoa loosely attached to inseminated oocytes and then washed a final time in glucose-free mNCSU-23 [14] medium containing 0.5 mM sodium pyruvate, 5 mM sodium lactate, and 0.4% BSA (A-6003, fraction V). Ten to fifteen putative zygotes were then freed from cumulus cells and transferred to a 30- μl microdrop of culture medium covered with warm mineral oil. Embryos were cultured up to 168 h after IVF in a humidified atmosphere of 39 °C and 5% CO_2 in air without any replacement with fresh medium [15].

Assessment of meiotic maturation, sperm penetration and embryo cell number

Oocyte maturation, fertilization, cleavage and blastocyst formation and blastomere number in blastocysts were examined at 44 h after IVM and at 12, 48 and 168 h after IVF, respectively. Oocytes or blastocysts at the time of examination were mounted on a glass slide and fixed for 10 min in 25% (v/v) acetic acid in ethanol. Fixation was carried out on a 33.8 °C warm plate for the removal of lipids within 10 min. They were then stained with 1% (w/v) orcein in 45% (v/v) acetic acid solution and examined under a phase-contrast microscope (IX-50, Olympus, Tokyo, Japan) at $400\times$. Meiotic stage of the oocytes was assessed according to Hunter and Polge [16]. Oocytes at the metaphase-II (MII) stage were regarded as mature. Oocytes were considered to be penetrated when they had one or more swollen sperm heads or male pronuclei with corresponding sperm tails.

Differential staining

The quality of blastocysts was assessed by differential staining of the inner cell mass (ICM) and trophectoderm (TE) cells according to the modified staining procedure of Thouas *et al.* [17]. Briefly, hatched blastocysts were used without treatment, and unhatched blastocysts were treated with 0.25% pronase (w/v, Sigma-Aldrich, St. Louis, MO, USA) for 5 min to dissolve the zonae pellucidae. After rinsing in mNCSU-23 medium, zona-free blastocysts were stained with 0.01% (w/v) bisbenzimidazole for 1 h. The blastocysts were rinsed in mNCSU-23 medium again and treated with 0.04% (v/v) Triton X-100 (Sigma-Aldrich) for 3 min followed by treatment with 0.005% (w/v) propidium iodide (Sigma-Aldrich) for 10 min. After another rinse in mNCSU-23 medium, stained blastocysts were mounted on glass slides under a cover slip and examined under an inverted microscope (Nikon, Tokyo, Japan) equipped with epifluorescence. The ICM nuclei labeled with bisbenzimidazole appeared blue, and the TE cell nuclei labeled with both bisbenzimidazole and propidium iodide appeared pink. Any blastocysts without dual staining were excluded from the study [17].

Incorporation and oxidation of radiolabeled glucose by porcine oocytes

The radioactive substrates, $^{14}\text{C}(\text{U})$ -glucose (specific activity: 185 MBq/mmol), was as described in our previous study [18]. Briefly, each of the five oocytes and embryos at a particular stage of development was transferred into a 100- μl drop of the culture

medium containing 18.5 kBq $^{14}\text{C}(\text{U})$ -glucose and then overlaid with mineral oil. $^{14}\text{C}(\text{U})$ -glucose was incorporated into a sodium pyruvate, sodium lactate and glucose-free medium containing oocytes at the MII stage at 44 h after culture of oocytes, cleaved oocytes at the 2-cell stage at 48 h and blastocysts at 168 h after IVF. On the other hand, 1 ml of 2.5 mM NaOH solution was transferred into a 1.5 ml microtube as a trap for the evolved $^{14}\text{C}(\text{U})$ - CO_2 . Microtubes of both NaOH and $^{14}\text{C}(\text{U})$ -glucose containing oocytes at the maturation, fertilization, cleavage and blastocyst stages were confined in a scintillation vial using a rubber stopper. The scintillation vials were incubated for 0 to 3 h in a cell culture incubator with a humidified atmosphere of 5% CO_2 in air at 39 °C. Just after completion of the specific incubation period, the metabolic reactions of oocytes and embryos were stopped with an injection of 100 μl of 10% perchloric acid (PCA) for glucose kept at room temperature for 24 h. The acid-insoluble materials were carefully washed by filtration (8.0 μM white SCWP, 47 mm; Millipore, Bedford, MA, USA) with 5% PCA or TCA, and the filter papers were kept overnight under a lamp. After drying, the filter papers were transferred into scintillation vials. The NaOH solution was transferred into a new scintillation vial by washing 3–4 times with cocktail (0.5% PPO+0.03% POPOP solution in toluene). All the scintillation vials plus three blanks for each group with 5 ml of cocktail were set in a liquid scintillation counter (LS-6500, Beckman-Coulter, Brea, CA, USA) to determine the levels of radioactivity. This experiment was conducted five times to improve its accuracy. The values of incorporation and oxidation are expressed directly as counts per minute (cpm) [9].

Ammonia assay

The ammonia concentrations in the medium were assessed using the Berthelot-indophenol method as described in our previous study [19]. This method was further used to analyze the maturation at the MII stage within 44 h, fertilization for 6 h and culture of oocytes, cleavage to the 2-cell stage at 48 h and then the blastocyst stage at 168 h. After completion of the stages mentioned above, 50 μl of the culture medium was taken out immediately to measure the concentration of ammonia. This procedure was performed five times for each analysis. A calibration curve in the range of 0–0.40 mM ammonia was run with each experiment. The mean correlation coefficient for the calibration curves of five experiments was 0.994.

Experimental design

In the first experiment, maturation was performed for 44 h to find out the optimal additive concentration of SS, SeMet, and Vit-E in medium mTCM-199 (0, 0.5, 5 and 10 $\mu\text{g/l}$ and 0, 0.5, 1.0 and 2.0 mM). In the second experiment, the various combinations of the optimal concentration of SS, SeMet, and Vit-E were used to show the effect of mTCM-199 on IVM for 44 h, the effect of mTALP on IVF for 6 h and the effect of mNCSU-23 on IVC for 168 h respectively. Finally, the incorporation and oxidation of the $^{14}\text{C}(\text{U})$ -glucose, accumulation of ammonia after IVM, IVF and IVC and the number of cells of blastocysts were investigated. After supplementation of all the chemicals, the pHs (HM-30s, Tokyo TOA Electronics, Tokyo, Japan) and osmolarities (Model 3300, Advanced Instruments, Norwood, MA, USA) of these media were adjusted to 7.4 with 1 N HCl or 1 N NaOH and 270 mOsm with NaCl, respectively.

Statistical analysis

In each experimental group, oocytes were randomly distributed. Each experiment was repeated five times. All data were subjected to one-way ANOVA followed by Tukey's test to determine differences among experimental groups using GraphPad Version 4.0 [20]. Statistical significance was determined when a P value was less than 0.05.

Results

Figure 1 shows effects of sodium selenite (SS), seleno-L-methionine (SeMet) and vitamin E (Vit-E) on metaphase II development in porcine oocytes. It is clear from the figure that 5 $\mu\text{g/l}$ of the supplements of SS and SeMet was significantly more effective ($P<0.05$) for MII development than the control and other two concentrations (0.5 and 10 $\mu\text{g/l}$). In the case of Vit-E, 1.0 mM was significantly more effective ($P<0.05$) than the control and other two concentrations (0.5 and 2.0 mM).

Table 1 presents the effect of SS, SeMet, Vit-E and their combinations on the percentages of maturation and fertilization of porcine oocytes. This table accumulated the concentrations (5 $\mu\text{g/l}$, 1.0 mM and their combination) found to be more effective in Fig. 1. The percentages of development to the MII stage, monospermic fertilization and male pronuclear formation significantly increased ($P<0.05$) when oocytes were treated with SeMet and SeMet+Vit-E compared with the control and other treatments. However, there was no significant difference among SS, Vit-E, SS+Vit-E and SS+SeMet+Vit-E in terms of maturation and fertilization.

The rates of incorporation and oxidation of $^{14}\text{C}(\text{U})$ -glucose and the accumulation of ammonia upon treatment with SS, SeMet, Vit-E and their combinations for maturation and fertilization of porcine oocytes are shown in Table 2. The incorporation and oxidation rates of $^{14}\text{C}(\text{U})$ -glucose were significantly higher ($P<0.05$) in oocytes treated with SeMet, SeMet+Vit-E and SS+SeMet+Vit-E compared with those of the control during maturation and fertilization ($P<0.05$).

The accumulation of ammonia in the medium was significantly reduced ($P<0.05$) after the addition of SeMet and SeMet+Vit-E, with the exceptions of SS, Vit-E and SS+SeMet+Vit-E, compared with the other treatments during maturation and fertilization.

The effects of SS, SeMet, Vit-E and their combinations on the development of embryos in terms of percentages of porcine IVC are shown in Table 3. The development ratios for the 2-cell, morula and blastocyst stages were higher with SeMet and SeMet+Vit-E. In the presence of SS, Vit-E and SS+SeMet+Vit-E, no differences ($P<0.05$) were observed at the 2-cell, morula and blastocyst stages compared with the control after IVF.

The rates of incorporation and oxidation of $^{14}\text{C}(\text{U})$ -glucose and the accumulation of ammonia upon treatment with SS, SeMet, Vit-E and their combinations in medium for the 2-cell and blastocyst stages are shown in Table 4. The incorporation and oxidation rates of $^{14}\text{C}(\text{U})$ -glucose were higher ($P<0.05$) in embryos treated with SeMet and SeMet+Vit-E at the 2-cell and blastocyst stages compared with those of the control. The accumulation of ammonia in the culture medium was significantly reduced ($P<0.05$) in the presence of SeMet and SeMet+Vit-E compared with those of the other treatments.

The effects of SS, SeMet, Vit-E and their combinations on blastocyst

Fig. 1. Effects of sodium selenite (SS), selen-L-methionine (SeMet) and vitamin E (Vit-E) on metaphase II in porcine oocytes. Different letters indicate significant differences ($P<0.05$) among the treatments at the different concentrations.

cell number are shown in Table 5. The ICM and TE cell numbers were taken into consideration. The numbers of ICM cells for SeMet and SeMet+Vit-E were significantly higher ($P<0.05$) than for the

control. In addition, more TE cells were observed for SeMet and SeMet+Vit-E in comparison with the control and all other treatments.

Discussion

Oocyte growth and maturation appear to be affected by nutritional imbalances, hormonal disturbances, and physical conditions of the microenvironment, such as oxidative stress [21–23]. Oxygen concentration is higher in *in vitro* cultures than in *in vivo* conditions, and free radicals are produced during aerobic metabolism of cells [24–26]. The oxidative modification of cell components due to the action of reactive oxygen species (ROS) is one of the most potentially damaging processes for proper cell function and may lead to cell death by necrosis or apoptosis [27, 28]. During IVM of oocytes, ROS are produced [29–31], and it has been shown that cumulus cells play a major role in the antioxidant defense of oocytes using GPx and catalase activities. Since these enzymes are active in mature oocytes, it seems that oocytes also have their own capacity to control ROS production during maturation by their antioxidant system [29–31]. Basini and Tamanini [32] showed that Se stimulated proliferation of bovine granulosa cells and had some stimulatory effects on estradiol secretion. In this study, the effect of the antioxidation was seen during *in vitro* maturation of oocytes,

Table 1. Effect of sodium selenite (SS), selen-L-methionine (SeMet), vitamin E (Vit-E) and their combinations on the percentages of maturation and fertilization of porcine oocytes

Treatment	Maturation (%)			Fertilization (%)			
	n	Germinal vesicle ^a	Metaphase II ^b	n	Penetration ^a	Monospermic fertilization ^b	MPN ^{c,b}
Control (0)	325	13.0 ± 4.0	67.6 ± 3.7 ^a	285	67.0 ± 5.0 ^a	53.4 ± 4.0 ^a	47.1 ± 1.7 ^a
SS (5 µg/l)	316	12.7 ± 2.1	74.4 ± 3.5 ^a	272	56.9 ± 2.0 ^a	58.7 ± 6.1 ^a	41.9 ± 5.3 ^a
SeMet (5 µg/l)	304	5.6 ± 2.1	80.2 ± 2.1 ^b	298	59.0 ± 4.0 ^a	65.3 ± 1.5 ^b	73.3 ± 3.1 ^b
Vit-E (1.0 mM)	333	12.1 ± 2.0	72.2 ± 2.0 ^a	222	62.1 ± 2.0 ^a	52.2 ± 2.1 ^a	53.6 ± 3.5 ^a
SeMet+Vit-E (5 µg/l+1.0 mM)	302	7.1 ± 1.5	85.1 ± 4.2 ^c	235	74.8 ± 5.0 ^b	70.4 ± 3.2 ^c	75.0 ± 3.1 ^b
SS+Vit-E (5 µg/l+1.0 mM)	295	10.2 ± 1.5	76.1 ± 4.5 ^a	255	65.0 ± 5.0 ^a	59.6 ± 3.5 ^a	60.2 ± 6.1 ^c
SS+SeMet+Vit-E (5 µg/l+5 µg/l+1.0 mM)	310	9.0 ± 2.0	73.5 ± 2.5 ^a	245	68.1 ± 3.0 ^a	44.3 ± 4.0 ^a	56.2 ± 4.0 ^d

Within a column, values with different letters (a–d) are significantly different ($P<0.05$). n: number of oocytes, five replicates. ^a Percentage of the number of oocytes. ^b Percentage of the number of penetrated oocytes. ^c Male pronuclear formation.

Table 2. The rates of incorporation and oxidation of ¹⁴C(U)-glucose and the accumulation of ammonia upon treatment with sodium selenite (SS), selen-L-methionine (SeMet), vitamin-E (Vit-E) and their combinations for maturation and fertilization of porcine oocytes

Treatment n (15)	Incorporation ¹⁴ C(U)-glucose (cpm/oocyte)		Oxidation ¹⁴ C(U)-glucose (cpm/oocyte)		Ammonia concentration (×10 ⁻¹ mM)	
	Maturation ^a	Fertilization ^b	Maturation ^a	Fertilization ^b	Maturation ^a	Fertilization ^b
Control (0)	1890.0 ± 254.6 ^a	1723.3 ± 194.7 ^a	143.7 ± 11.7 ^a	140.3 ± 4.2 ^a	1.3 ± 0.1 ^a	1.8 ± 0.1 ^a
SS (5 µg/l)	2912.0 ± 32.9 ^a	3384.3 ± 159.2 ^b	174.7 ± 9.0 ^b	193.7 ± 5.5 ^a	1.3 ± 0.1 ^a	1.7 ± 0.1 ^a
SeMet (5 µg/l)	6157.3 ± 334.1 ^b	7531.0 ± 102.8 ^c	226.7 ± 3.1 ^c	284.3 ± 62.9 ^b	1.2 ± 0.1 ^b	1.1 ± 0.1 ^b
Vit-E (1.0 mM)	1627.3 ± 44.3 ^a	1856.7 ± 44.9 ^a	153.7 ± 3.5 ^a	150.3 ± 4.2 ^a	1.4 ± 0.2 ^a	1.7 ± 0.4 ^a
SeMet+Vit-E (5 µg/l+1.0 mM)	8232.3 ± 980.6 ^c	9298.0 ± 427.9 ^d	334.0 ± 3.1 ^d	346.3 ± 31.8 ^c	1.1 ± 0.1 ^c	1.0 ± 0.2 ^b
SS+Vit-E (5 µg/l+1.0 mM)	3006.0 ± 66.0 ^a	4206.3 ± 679.8 ^c	149.3 ± 7.0 ^a	258.7 ± 61.2 ^d	1.3 ± 0.1 ^a	1.6 ± 0.1 ^c
SS+SeMet+Vit-E (5 µg/l+5 µg/l+1.0 mM)	7991.7 ± 243.7 ^d	8875.0 ± 594.0 ^d	220.3 ± 53.5 ^c	262.3 ± 8.1 ^d	1.2 ± 0.1 ^b	1.8 ± 0.2 ^a

Within a column, values with different letters (a–e) are significantly different ($P<0.05$). n: number of oocytes, five replicates. ^a Maturation (44 h incubation period in IVM). ^b Fertilization (the incubation period was 6 h after IVM). Mean ± SEM obtained from the average of each replicate.

Table 3. Effect of sodium selenite (SS), seleon-L-methionine (SeMet), vitamin E (Vit-E) and their combinations on the development of embryos

Treatment	n	Percentages of embryo development (mean±SEM) ^b			
		2–4 cells ^a	8–16 cells ^a	Morula ^a	Blastocyst ^a
Control (0)	275	(189) 68.7 ± 1.8 ^a	(109) 57.7 ± 2.5 ^a	(58) 30.7 ± 1.4 ^a	(36) 19.0 ± 2.5 ^a
SS (5 µg/l)	263	(186) 70.7 ± 1.8 ^a	(124) 66.7 ± 4.4 ^a	(60) 32.3 ± 0.9 ^a	(38) 20.4 ± 2.7 ^a
SeMet (5 µg/l)	271	(204) 75.3 ± 0.8 ^b	(142) 69.6 ± 3.0 ^b	(77) 37.8 ± 3.5 ^b	(51) 25.0 ± 3.1 ^b
Vit-E (1.0 mM)	259	(187) 72.2 ± 1.4 ^a	(114) 60.9 ± 4.3 ^a	(63) 33.7 ± 2.6 ^a	(26) 13.9 ± 3.3 ^a
SeMet+Vit-E (5 µg/l+1.0 mM)	255	(199) 78.0 ± 2.9 ^b	(141) 70.8 ± 1.5 ^b	(80) 40.2 ± 2.0 ^b	(54) 27.1 ± 1.1 ^b
SS+Vit-E (5 µg/l+1.0 mM)	295	(208) 70.5 ± 1.6 ^a	(134) 64.4 ± 4.3 ^a	(71) 34.1 ± 2.0 ^a	(43) 20.7 ± 2.0 ^a
SS+SeMet+Vit-E (5 µg/l+5 µg/l+1.0 mM)	277	(204) 73.6 ± 2.1 ^a	(136) 66.7 ± 2.3 ^a	(73) 35.8 ± 2.5 ^a	(36) 17.5 ± 1.1 ^a

Within a column, values with different letters (a–b) are significantly different ($P<0.05$). n: number of embryos, five replicates. ^a Embryo development calculated from cleaved embryos. ^b Mean ± SEM obtained from the average percentages of each replicate.

Table 4. The rates of incorporation and oxidation of ¹⁴C(U)-glucose and the accumulation of ammonia upon treatment with sodium selenite (SS), seleon-L-methionine (SeMet), vitamin E (Vit-E) and their combinations in medium for the 2-cell and blastocyst stages

Treatment n (15)	(mean±SEM) ^a					
	Incorporation ¹⁴ C(U)-glucose (cpm/oocyte)		Oxidation ¹⁴ C(U)-glucose (cpm/oocyte)		Ammonia concentration (×10 ⁻¹ mM)	
	2- cell	Blastocyst	2- cell	Blastocyst	2-cell	Blastocyst
Control (0)	982.7 ± 161.5 ^a	1410.0 ± 160.0 ^a	230.7 ± 29.5 ^a	390.0 ± 10.0 ^a	1.3 ± 0.1 ^a	1.5 ± 0.2 ^a
SS (5 µg/l)	1082.0 ± 95.0 ^a	2354.0 ± 200.0 ^a	323.0 ± 38.0 ^a	451.3 ± 21.0 ^a	1.4 ± 0.1 ^a	1.2 ± 0.1 ^a
SeMet (5 µg/l)	2137.0 ± 135.0 ^b	3509.7 ± 197.5 ^b	475.0 ± 20.0 ^b	518.3 ± 12.6 ^b	0.9 ± 0.1 ^b	1.0 ± 0.1 ^b
Vit-E (1.0 mM)	1164.0 ± 119.0 ^a	2345.0 ± 213.0 ^a	242.0 ± 40.0 ^a	450.7 ± 19.5 ^a	1.2 ± 0.1 ^a	1.6 ± 0.1 ^a
SeMet+Vit-E (5 µg/l+1.0 mM)	2927.0 ± 169.0 ^c	4890.7 ± 655.5 ^c	373.0 ± 70.0 ^c	540.0 ± 50.0 ^c	0.9 ± 0.1 ^c	1.1 ± 0.2 ^c
SS+Vit-E (5 µg/l+1.0 mM)	1660.0 ± 140.0 ^b	2873.7 ± 332.5 ^c	289.0 ± 30.0 ^a	441.0 ± 20.0 ^a	1.2 ± 0.1 ^a	1.4 ± 0.1 ^a
SS+SeMet+Vit-E (5 µg/l+5 µg/l+1.0 mM)	1229.3 ± 104.5 ^a	2398.3 ± 233.5 ^a	295.0 ± 40.0 ^a	487.0 ± 30.0 ^a	1.2 ± 0.1 ^a	1.5 ± 0.2 ^a

Within a column, values with different letters (a–c) are significantly different ($P<0.05$). n: number of embryos, five replicates. ^a Mean ± SEM obtained from the average of each replicate.

Table 5. Effect of sodium selenite (SS), seleon-L-methionine (SeMet), vitamin E (Vit-E) and their combinations on blastocyst cell number

Treatment	Cell numbers (mean±SEM)			
	ICM	TE	Total	TE/ICM
Control (0)	9.0 ± 1.0 ^a	27.0 ± 1.6 ^a	36.1 ± 2.0 ^a	2.8 ± 1.0
SS (5 µg/l)	12.6 ± 1.5 ^a	29.0 ± 1.6 ^a	41.0 ± 3.0 ^a	2.4 ± 0.4
SeMet (5 µg/l)	16.0 ± 2.0 ^b	35.0 ± 3.2 ^b	51.0 ± 5.0 ^b	2.3 ± 0.1
Vit-E (1.0 mM)	11.6 ± 1.5 ^a	28.0 ± 2.4 ^a	39.0 ± 4.0 ^a	2.5 ± 0.1
SeMet+Vit-E (5 µg/l+1.0 mM)	18.0 ± 3.0 ^b	38.0 ± 1.6 ^b	54.1 ± 2.0 ^b	2.3 ± 0.1
SS+Vit-E (5 µg/l+1.0 mM)	10.6 ± 1.5 ^a	26.0 ± 0.8 ^a	36.0 ± 2.0 ^a	2.6 ± 0.2
SS+SeMet+Vit-E (5 µg/l+5 µg/l+1.0 mM)	10.0 ± 2.0 ^a	25.0 ± 2.4 ^a	35.0 ± 2.0 ^a	2.7 ± 0.1

Values shown were obtained with 15 embryos from three replicates. Within the same column, values with different superscripts letters (a–b) are significantly different ($P<0.05$).

and this was agreement with these previous results. In particular, SeMet and SeMet+Vit-E significantly enhanced the development to the MII stage, monospermic fertilization and male pronuclear formation. Generally, SeMet and other Se compounds are dynamically metabolized into a wide array of products, and these derivatives may directly influence the chemopreventive effects of Se [33, 34]. These compounds might be better precursors for generating methylated Se

intermediates. In general, Se undergoes reductive metabolism and forms methylated excretory products, whereas sulfur tends to form more oxidized excretory products [35]. The optimum concentration of Se and Vit-E used in the present study is similar to the concentration used by porcine embryos and sperm [12, 36].

At the time of fertilization, one capacitated spermatozoon binds to the zona pellucida (ZP) and undergoes the acrosome reaction before

crossing the perivitelline space to reach the plasma membrane of the oocyte. Low levels of ROS play a critical role in induction of sperm capacitation and the AR during fertilization in humans [37, 38] and bulls [39]. Moreover, tyrosine phosphorylation of sperm proteins in humans [40] and bulls [39] is in part regulated by ROS and is an essential trigger for the AR after sperm-ZP binding. Although the proper balance between ROS and antioxidants adjusts oxidative stress to the optimal levels in the reproductive tract *in vivo* [41], addition of tannic acid to IVF medium for the purpose of preventing polyspermy in porcine oocytes has strong ROS scavenging activity [42]. We have found that monospermic fertilization and male pronuclear formation were significantly increased by SeMet and SeMet+Vit-E compared with other treatments (Table 1). Brzezinska-Slebodzinska *et al.* [43] indicated that Vit-E's role in boar semen was as an antioxidant. Boar spermatozoa contain glutathione peroxidase (GPx) and α -tocopherol [44], suggesting that both Se and Vit-E have roles in preventing peroxide damage to sperm cells. Se seems to be involved in the structure of the spermatozoon tail mid piece, the shape, and/or the array of the mitochondria within the coil, as well as contact of the coil with the outer plasma membrane of the tail mid piece [45]. When Se and α -tocopherol containing the antioxidant SS were added to bovine semen, spermatozoon motility increased, suggesting that adding Se to semen extender may enhance spermatozoon function [46].

Uhm *et al.* [47] showed that the addition of SS to culture media increased the blastocyst rate and cell number, decreased the apoptotic index and increased the expression of GPx in porcine embryos. In our study, the numbers of cells and blastocysts were increased with SeMet and SeMet+Vit-E in comparison with SS. Ebert *et al.* [48] demonstrated that Se supplementation of bone marrow stromal cell culture media was effective in restoring antioxidative capacity and in reducing cell damage. Se acts via intracellular signaling including protein kinase C, nuclear factor-kappa B and inhibitors of apoptosis proteins [49]. Many of the biological actions of selenium have been attributed to powerful antioxidant properties, including direct quenching of ROS, chelation of metal ions and regeneration of Se-GPx antioxidants. Yeo and Kang [50] investigated the prevention of cell death by selenium using cultured brain-derived neural progenitor cells (NPCs) and showed that selenite inhibits H₂O₂-induced apoptosis of NPCs.

A vast number of studies have been conducted on glucose metabolism in mammalian preimplantation embryos; nutrient uptake studies carried out on porcine embryos have shown that the embryos consume glucose and produce lactate at all stages of development. Therefore, glucose-containing media are commonly used for producing porcine embryos *in vitro* [51, 52]. The incorporation and oxidation of glucose have become indicators of the vitality of oocytes and eggs [9, 18]. In this study, the incorporation and oxidation rates of ¹⁴C(U)-glucose were significantly higher in oocytes treated with SeMet and SeMet+Vit-E than those of the control during maturation and fertilization. Generation of ROS induced by glucose utilization was assumed to be caused by the activation of NADPH oxidase, an enzyme that catalyzes the oxidation of NADPH, which generates NADP that serves as a coenzyme of the oxidative arm of the pentose phosphate pathway (PPP) [53-57]. On the other hand, NADPH generated as a result of the PPP activity is also reported to act for the reduction of intracellular glutathione (GSH), a tripeptide thiol

compound that plays a major role in regulating ROS concentrations within cytoplasm [58]. During early development, the embryos seem to be dependent on the stored GSH that may be packaged during oocyte development in the ovary [59]. Gardiner and Reed [60] observed that the GSH content of mouse embryos decreases continuously from the unfertilized oocytes to blastocyst stage. Thus, it is likely that the observed decrease in GSH content could be due to a depletion of GSH stored in the oocytes combined with an inability of the embryo to synthesize GSH [60, 61]. Therefore, we think that the addition of Se-GPx antioxidants is necessary for culturing embryos *in vitro*.

Amino acids support normal preimplantation development *in vivo* as evidenced by studies *in vitro*. Undergoing, spontaneous degradation, the breakdown of the amino acids results in the formation of pyrrolidone carbonic acid and potentially harmful ammonia. Ammonia toxicity for living cells is well recognized *in vitro* and *in vivo*. The increased levels of ammonia decrease the pH and increase the osmolarity, leading to a progressive loss in sperm motility [62] and decreased rates of porcine oocyte development to the MII stage and monospermic fertilization *in vitro* [1]. In our previous study, ammonia was added to medium to investigate the effects of Se and Vit-E, and the sperm motility, viability, AR and ammonia accumulation were observed [11]. The results showed that the accumulation of ammonia was reduced by SeMet and SeMet+Vit-E compared with those of the other treatments. We suggest that SeMet and SeMet+Vit-E play important roles in reducing the accumulation of ammonia in culture medium and increase the rates of oocyte maturation, fertilization and development to blastocysts. These results were in agreement with the study of Keskes-Ammar *et al.* [63], who reported that the combination of Se and Vit-E, in comparison with single supplementation, improves human sperm *in vitro*. Kosenko *et al.* [4] suggested ammonia toxicity as an alternate mechanism for the involvement of free radicals and oxidative damage. GPx, GSH and Vit-E (enzymatic and nonenzymatic antioxidant) are involved in reducing ammonia toxicity and the production of free radicals [64, 65]. We suggested that SeMet and SeMet+Vit-E might protect against the oxidative damage of ammonia.

In conclusion, our present results provide evidence that Se and Vit-E are involved in modulating porcine oocyte maturation, fertilization and embryo development. Furthermore, SeMet and the combination of SeMet+Vit-E may be involved in reducing the accumulation of ammonia and subsequently in increasing the rates of maturation, fertilization, embryo development and the utilization of glucose in porcine embryos.

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