Journal of
Dairy & Veterinary Sciences
ISSN: 2573-2196

Research Article
Volume 2 Issue 5 - June 2017
DOI: 10.19080/JDVS.2017.02.555599

Dairy and Vet Sci J

Effect of Selenium and Vitamin E on Development and Viability of Preimplanted Mouse Embryo

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Submission: February 06, 2017; **Published:** June 02, 2017

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Abstract

In vitro culture results higher level of reactive oxygen species (ROS) oxygen than in vivo environments that cause lipid peroxidation of cellular membranes. Selenium (Se) and Vitamin E (Vit-E) are the important antioxidants that protect mammalian cells against lipid peroxidation. Therefore, the present study was conducted to investigate whether Se or Vit-E and Se+Vit-E overcome the the undesirable oxidative stress produced by hydrogen peroxide (H_2O_2) and enhance the development of pre implanted mouse embryo. Co-incubating the embryos with 60nM Se and/or 100nM Vit-E were increased ($P<0.05$) the blastocyst development rate.

The addition of H_2O_2 reduced the development of mouse embryo, but the addition of Vit-E, Se and Se+Vit-E reduced the detrimental effect of H_2O_2 and influenced the higher rate of development to blastocysts, compared to CZB alone ($P<0.05$). The incorporation and oxidation of 14C-glucose in the blastocysts developed by the medium supplemented with Se and/or Vit-E in the presence or absence of H_2O_2 were significantly higher ($P<0.05$) than that of the control. Moreover, Vit-E is more effective than Se and Se+Vit-E in reversing ROS-induced mouse embryo toxicity. Therefore, Vit-E may be supplemented in the CZB medium for better development and viability of pre implanted mouse embryo.

Keywords: Mouse embryo; Selenium; Vitamin E

Abbrevations: Ros: Reactive Oxygen Species; -Oh: Hydroxyl Radical; O2-: Superoxide Anion Radical; Se: Selenium; Gpx: Glutathione Peroxidase; Cpm :Counts Per Minutes

Introduction

In vitro embryo culture suffers from excessive developmental failure. Its inefficiency is linked with the generation of reactive oxygen species (ROS), such as H_2O_2, hydroxyl radical (-OH) and superoxide anion radical (O_2^-), appears as the by-products of cell metabolism [1]. Superoxide may also spontaneously break down into oxygen and H2O2. As ROS are highly reactive molecules, their accumulation can lead to damage and breakage of DNA strands. There are many evidences have been found that ROS compromises embryo development in many species [2-6].

Selenium (Se), an essential trace element for mammals, is an integral part of anti- oxidant system [7]. Se dependent glutathione peroxidase (GPx) has an important role in free radical protective mechanisms. Vitamin E (Vit-E), the predominant lipid-soluble antioxidant in animal cells, protects cells from oxygen radical damage in vivo [8,9] and in vitro [10,11].Our previous study investigated that Se and Vit-E, as the integral parts of antioxidant systems which play important roles for the in vitro maturation, fertilization and culture of porcine oocyte [12]. However, there is a very limited studies were conducted with the effect of Se and Vit-E on the development of preimplanted mouse embryo. Therefore, the present study was conducted to investigate whether Se, Vit-E or Se+Vit-Eovercome the undesirable oxidative stress produced by hydrogen peroxide (H_2O_2) and enhance the development of preimplanted mouse embryo.

Material and Methods

Chemicals and reagents

The basic embryo culture medium used in this study was CZB [13], which contains 1mM glutamine, 0.1 mM EDTA and 5mg/ml

BSA, and considered as control. A part of CZB was added with 30% H_2O_2 to get a final concentration of 0.0003% that considered as negative control. According to the experimental layout, CZB (with or without H_2O_2) was supplemented with30, 60, 90Nm Se (sodium selenite; Sigma-Aldrich, St. Louis, MO, USA), 50, 100 and150nM of Vit-E (α-tocopherolacetate) and their combination (Se+Vit-E). Selenium, Vit-E and H_2O_2 were equilibrated in culture medium under CO_2 incubator for 8 hours before the start of culture. Vit-E was dissolved in ethanol, and an emulsion was formed by vortex mixing before adding it to the embryo. PMSG and HCG used in this study were obtained from Sankyo Chemical Industries Ltd., Tokyo, Japan. The copulation plug was checked 24 hours later. Radioactive 14C (U)-glucose was purchased from American Radio labeled Chemicals (St. Louis, Mo, USA). All other chemicals were of analytical grade and purchased from Nacalai Tesque (Kyoto, Japan) unless otherwise indicated.

Collection of embryos

Embryos were obtained from 7-8 weeks old female ICR mice. They were offered feed with a balanced standard diet and ad libitum clean drinking water. Animals were kept in polycarbonate cage with wood shavings under a 12h light: 12h dark regimen (light on at 6:00), at a temperature of 20±1 °C in accordance with the "Guideline for Regulation of Animal Experimentation, Faculty of Agriculture, Shinshu University." Female mice were induced to superovulation with PMSG (5 IU, i.p.) followed 48h later by hCG (5IU, i.p.) and met with male mice. Zygotes of 1-cell stages were collected at 25 hours HCG post injection by flushing out from the fallopian tubes. The embryos were subsequently incubated in CZB medium.

Experimental layout

In fact, there are four experiments were conducted under this study. In experiment I, effect of CZB supplanted with different levels of Se (0, 30, 60 and 90nM) and Vit-E (0, 50, 100 and 150nM) on the development of mouse embryo from 1-cell stage to the blastocyst were evaluated. The experiment II were conducted to investigate the effects of Se (60nM), Vit-E (100nM) and their combination (60nM Se+100nM Vit-E) on the development from 2-cell to blastocyst stages of embryo in the presence (1mM) or absence of H_2O_2 in CZB medium. Where, the experiment III was conducted to the effect evaluate Se (60nM), Vit-E (100nM) and their combination (60nM Se+100nM Vit-E) on the accumulation of ammonia (NH_4) in presence or absence of H_2O_2 due to metabolism of embryo during the development up to blastocyst stages. Incorporation and oxidation of 14C-glucose at blastocysts developed by the supplementation of Se (60nM), Vit-E (100nM) and their combination (60nM Se+100nM Vit-E) in the presence or absence of H2O2 were evaluated by conducting the experiment IV. These experiments were conducted five times repeated.

Culture of embryos

The collected zygotes of 1-cell stages were transferred to the culture dishes for washing, and grown in-vitro to the developmental stages up to blastocyst. Embryos were cultured according to the standard techniques, in groups of 10 zygotes were placed into 35mm-diameter culture dishes (Nunc Co., Denmark) containing 30µl of each CZB medium under a layer of paraffin oil and equilibrated overnight in an atmosphere of 5% CO_2 in air at 37 °C. The pH of all media was 7.4 after equilibration. The developing embryos seemed to be normal in their morphology, with almost no fragmentation.

Ammonia determination

During incubation period embryos which developed in the presence of Se and/or Vit-E with or without H_2O_2, the ammonia concentrations in the medium were assessed by using the Bertholot-indophenol method as described in our previous study [14]. To determine the ammonia concentration in the medium, 100µl of the culture medium was removed every 2-4h and frozen at -40 °C until measurement. The procedure was carried out five times for the analysis. A calibration curve in the range 0.0003 of ammonia was run with each experiment. The mean coefficient for determination of the calibration curve of five experiments was 0.994.

Incorporation and oxidation of 14C-glucose

The experiment was initiated with 14C-glucose 18.5kBq/0.1mol (specific activity 9.69MkBq/mol, Moravek Biochemicals, Inc., USA). Each of the ten blastocysts which developed in the presence of Se and/ or Vit-E with or without H_2O_2 was transferred in a microtube of 50µl CZB medium drop containing 14C-glucose then overlaid with mineral oil. On the other hand, 1ml of 2.5Mm NaOH solution was transferred into a 1.5ml micro tube as a trap for the evolved 14 CO_2. Both microtubes of NaOH and 14C-glucosewith embryos were confined into a scintillation vial using a rubber stopper. The scintillation vials were incubated for 5h in an incubator at 37 °C. After incubation period, the metabolic reactions of embryos were stopped with an injection of 100µl of 10% perchloric acid (PCA) kept at room temperature for 24h. The acid insoluble materials were carefully washed by millipore filtration (8.0µM white SCWP, 47mm; Millipore Corporation, Bedford, MA, USA) with 5% PCA 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 with 5ml of cocktail were set in a Liquid Scintillation Counter (LS-6500, Beckman Instruments, Inc. USA) to determine the levels of radio activity [15]. This experiment was conducted ten times to improve its accuracy. The values of incorporation and oxidation were expressed directly as counts per minutes (CPM).

Statistical analysis

Data obtained from this stud were analyzed by one-way ANOVA using the GLM procedure of SAS (SAS Institute, Cary, NC). The data expressed as percentage were tested by Chi-square test.

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Data were presented as mean ±SEM of at least 5 replicates and differences were considered significant at the level of P<0.05.

Results

The effects of supplementation of different levels of Se (0, 30, 60 and 90nM) in CZB medium on the development of mouse embryo are presented in (Table 1). The results revealed that 30 and 60nM of Se was more effective (P<0.05) for mouse embryo development than that of the control and 90nM of Se, where the level showed detrimental effect on the development of embryo as blastocyst. On the other hand, (Table 2) demonstrated that the supplementation of 100nM of Vit-E was the most effective (P<0.05) for embryonic development than that of the 0, (control), 50 and 150nM. The lowest (P<0.05) percentage of blastocyst was observed when zygotes were cultured in CZB supplemented with 150nM of Vit-E.

Table1: Effects of supplementation of different levels of selenium on development of mouse embryo cultured in CZB medium [abc]Mean values with different superscripts within the same column differ significantly (P<0.05).

Level of se in CZB	No. of Embryo Cultured	No. of Embryo Developed (%)	
		Morula	Blastocyst
CZB+0 Se (Control)	84	84(100)a	32(43.5)b
CZB+30nM Se	68	68(100)a	40(58.8)a
CZB+60nM Se	100	100(100)a	68(63.0)a
CZB+90nM Se	56	12(21)b	0(0)c

Table 2: Effects of supplementation of different levels of Vitamin E on development of mouse embryo cultured in CZB medium [abc]Mean values with different superscripts within the same column differ significantly (P<0.05).

Level of Vit-E In CZB	No. of Embryo Cultured	No. of Embryo Developed (%)	
		Morula	Blastocyst
CZB+0 Vit-E (control)	78	72(92)ab	36(46.2)b
CZB+50 nMVit-E	84	84(100)a	44(52.3)b
CZB+100 nMVit-E	80	80(100)a	54(67.5)a
CZB+150 nMVit-E	132	118(89)b	34(25.0)c

Detrimental effects of H_2O_2 and reducing or protecting ability of Se, Vit-E or their combination from the effects of H_2O_2 on the embryonic development were shown in (Table 3). The results demonstrated that the development of mouse embryos was reduced by the detrimental effect of H_2O_2 especially in CZB, the occurrence after 8 cell markedly decreased under H_2O_2 (P<0.05). However, the supplementation of Se, Vit-E and Se+Vit-E were able to reduce the detrimental effect of H_2O_2 and enhanced (P<0.05) the development of mouse embryo to be blastocyst. Highest percentage of blastocysts was obtained when the zygotes were cultured in the CZB medium supplemented with Vit-E in the presence or absence of H_2O_2.

Table 3: Effect of supplementation of Se, Vit-E or their combination (Se+Vit-E) on the development of mouse embryo in absence or presence of H2O2 abMean values with different superscripts within the same column differed significantly (P<0.05).

Medium +Reagents	No. of Embryo Cultured	No. of Embryo Developed (%)				
		2-cell	4-cell	8-cell	Morula	Blastocyst
a) in absence of H_2O_2						
CZB (control)	128	128(100)	128(100)	128(100)	120(94)[a]	60(48.4)[b]
CZB + Se	110	110(100)	110(100)	110(100)	110(100)[a]	70(63.6)[a]
CZB +Vit-E	146	146(100)	146(100)	146(100)	146(100)[a]	100(68.5)[a]
CZB + (Se+Vit-E)	126	126(10)	126(100)	126(10)	126(100)[a]	72 (57.1)[ab]
b) in presence of H_2O_2						
CZB (control)	156	156(100)	156(100)	78(71)b	46(42)[b]	4(3.6)[d]
CZB+Se	150	150(100)	150(100)	148(99)a	144(96)[a]	34(22.7)[b]
CZB+Vit-E	100	100(100)	100(100)	100(100)a	100(100)[a]	28(28.0)[a]
CZB+(Se+Vit-E)	116	116(100)	116(100)	116(100)a	104(90)[a]	20(17.2)[c]

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During the development of mouse embryos up to the blastocyst stage, accumulation of metabolic NH_4+ in the CZB medium supplemented with Se, Vit-E and Se+Vit-E in the presence or absence of H_2O_2 is showed in (Table 4). The results revealed that the lowest (P<0.05) accumulation of metabolic NH_4+ was observed in the CZB medium supplemented with Vit-E in the presence or absence of H_2O_2. The effects of supplementation of Se, VIt-E and Se+Vit-E in the CZB medium with or without H_2O_2 on incorporation and oxidation of 14C-glucose at the blastocyst stage are shown in (Table 5). The incorporation of 14C-glucose at the blastocyst stage cultured with Se, Vit-E and Se+Vit-E in the CZB medium were not significantly differed (P>0.05), but higher rate of incorporation (P<0.05) occurred by the supplementation of Se or Vit-E in the presence of H_2O_2. On the other hand, in the presence of H_2O_2, the oxidation of 14C-glucose by the blastocysts were higher (P<0.05) when zygotes were cultured in the CZB medium supplanted with Vit-E or Se alone or Se+Vit-E than the basic CZB medium. There was a slightly higher rate of oxidation of 14C-glucoseoccured in the blastocyst stage cultured with Se, Vit-E and Se+Vit-E.

Table 4: Effect of supplementation of Se, Vit-E or their combination (Se +Vit-E) on the accumulation of NH4 in the media developed to blastocyst stage of mouse embryo in the absence or presence of H2O2 Mean values with same superscripts within the same column did not differed significantly (P>0.05).

Medium +Reagents	Accumulation of NH_4 (Unit ?????)	
	In Absence of H_2O_2	In Presence of H_2O_2
CZB (control)	17.45±0.6	19.15±0.7
CZB + Se	15.39±0.8	18.94±1.0
CZB +Vit-E	14.51±1.0	18.71±0.5
CZB+(Se+Vit-E)	15.74±0.9	19.57±1.6

Table 5: Effect of supplementation of Se and or Vit-E with or without H2O2 on the incorporation and oxidation of 14C-glucose at the blastocyst stage abcMean valueswith different superscripts within the same column differ significantly (P<0.05).

Medium +Reagents	Incorporation of 14C-Glucose		Oxidation of 14C-Glucose	
	(-)H_2O_2	(+)H_2O_2	(-)H_2O_2	(+)H_2O_2
CZB	4000±20	385±8.4^{c}	109.1±2.3^{b}	23.1±0.6^{b}
Se	3692±17	1615±9.0^{a}	122.7±1.7^{ab}	100.0±0.7^{a}
Vit-E	3539±12	1692±6.7^{a}	144.5±1.	94.9±0.2^{a}
Se+Vit-E	4692±19	1385±8.7^{b}	138.2±1.8^{ab}	89.7±1.2^{a}

Discussion

A number of intrinsic and extrinsic factors have been shown to influence in vitro survival of the embryos to the blastocyst stage in extended culture. Previous studies suggest that *in vitro* extrinsic factors such as prolonged culture conditions and the autocrine and paracrine activities of the embryos may also contribute to the failure of optimal embryo development. Among the factors that might affect *in vitro* development of embryos is the balance between oxidative stress, and the ability of the embryos to neutralize their effects [16] reported a sustained increase in oxygen, glucose and pyruvate uptake during in vitro embryo development. The embryos were dependent on oxidative phosphorylation for energy (ATP) production at all stages of pre-elongation development, with perhaps a shift in dependence towards glycolysis in conjunction with compaction. This enhancement in oxidative metabolism of the embryo could be linked to the detected increase in ROS, which are characterized by the presence of an unpaired electron [17] and free-radical intermediaries [18].

Free radicals are generated from leakage of high-energy electrons as they proceed down the electron transport chain. The free radicals have many harmful effects including DNA damage [19]. Many embryos under oxidative stress step into a transient cell cycle arrest which is activated by DNA damage response before apoptosis [20]. Legge & Sellens [21] were suggested that the 2-cell block in mouse embryo is at least in part, of free radical damage incurred by embryos during collection and culture, and that medium supplementation with the radical scavenger, reduced glutathione, can improve embryo development in vitro reported that enhanced oxidative metabolism of embryos may be associated with increased ROS levels detected. The gradual increase in ROS levels from the 2-cell embryo up to the late morula stage could depend on the metabolic change undergone by the embryo during its development. It is necessary to prevent ROS as much as possible during culture embryos. However, it is unclear as to which embryos may be adversely affected and to what extent.

The present study showed that Se, Vit-E and SE+Vit-E increased blastocyst formation compared to control. Especially, it suggested that the formation of mouse blastocysts cultured in the presence of 60nM/ml Se and 100nM/ml Vit-E were significantly higher than control. The trace element Se is a component of antioxidative seleno enzymes, Glutathione Peroxidase (GPx) and ThioredoxinReductase (ThxRed) that decrease oxidative stress. Se, as sodium selenite, has been reported as a co-factor for glutathione peroxidase and other proteins and used as an anti-oxidant in medium [22]. In cell culture system, sodium selenite protected cell from oxidative damage, free radicals and obstructed lipid peroxide products [23,24]. Se played a role in the antioxidant defense system in the formation of mouse blastocyst, which was essential for the catalytic activity of glutathione peroxidase. Glutathione, a thiol tripeptide component in all cell types has an important role in the transportation of amino acid, synthesis of the protein and DNA, and reduction of disulfide bonds [25].

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In the present study, Se and Vit-E were used as combined supplements in the CZB medium for culture of mouse embryos, but this combination showed lower influence in the development of mouse blastocyst than Vit-E alone. Alpha-tocopherol (Vitamin E) is well known as an ROS scavenger in in vivo and in vitro conditions [26-28] and is the most important antioxidant present in ovarian tissue and follicular fluid. The antioxidant activity of α-tocopherol in preventing free-radical-induced tissue damage is accepted by most investigators and is believed to be the primary free radical scavenger and to inhibit lipid peroxidation in the mammalian cell membrane [26-29].The present study also demonstrated that the supplementation of Vit-E (specially, 100nM) played an important role in the development of mouse embryo. The results are in agreement with the results of our previous study and the study of [30] they also reported that the optimal concentration of Vit-E in embryo culture is 100nM.

There is a method to observe the effect of mild oxidative stress with retardation of embryo development in the medium supplemented with 1-5mM H_2O_2. The H_2O_2 used in this experiment was 1mM, and performed detrimental effects on the development embryo after 8 cells [31]. Reported that effects of H_2O_2 on blastocyst formation became more severe during the treatment of later stages of development. Embryos may also have different sensitivities to ROS at different developmental stages [32]. The exogenous oxidant H_2O_2 leads to over production of ROS, which may induce multiple cellular damages, including lipid peroxidation, nuclear DNA strand breaks, and mitochondrial alteration, consequently disturbing the development of pr implanted embryos *in vitro* [33-35]. Most of the embryos under oxidative stress step into a transient cell cycle arrest in vitro, which is activated by DNA damage response before apoptosis.

Vit-E (α-tocopherol) is a predominant lipid-soluble antioxidant that has been considered as a primary free radical scavenger in biological membranes [36-38]. α-tocopherol scavenges peroxyl radicals from polyunsaturated fatty acid in membrane phospholipids or lipoproteins that do not spread the radical chain, thereby protecting against lipid peroxidation [39]. Our previous study reported that supplementation of α-tocopherol maintains the development of mouse embryo and pig oocyte quality, fertilization rates and embryo development. Mouse preimplantation embryos can be cultured in a simple defined medium. Under such conditions energy substrates in the medium represent a major source of carbon for anabolism. Glucose is incorporated into macromolecules during in-vitro culture of cleaving mouse embryos and blastocysts [40]. In particular, both acid-soluble glycogen and desmoglycogen are rapidly synthesized from glucose presumably to act as a source of energy at implantation [41]. Overall metabolism, as assessed by oxygen consumption is low during the cleavage stages of development before rising sharply at the blastocyst stage. Moreover, metabolic activity has been shown to relate to developmental potential.

In this study, the accumulation of ammonia in the culture was measured regardless of the presence or absence of H_2O_2, the lower accumulation of ammonia in the blastocyst stage is better than the higher accumulation that occur incidence to the blastocyst [42] showed that high levels of ROS in culture media are associated with low rates of embryo development and blastocyst formation [43]. Suggested two possible mechanisms for the inhibitory effects of NH_4+. Perturbation of intracellular pH requires the involvement of Na/K ATPase to transport NH_4+ across membranes. Alternatively, may interact directly with enzymes, participating in a series of futile cycles which detoxify NH_4+ and result in consumption of ATP. Thus, by whichever mechanism, inclusion of NH_4+in culture media will divert ATP from growth to maintenance.

Azizimoghadam reported that pentose phosphate pathway activity of total glucose metabolism was increased at the compacted morula stage and was highest at the blastocyst stage [44]. In this study, incorporation and oxidation of 14C-glucose in the blastocyst was significantly influenced when cultured with Vit-E and Se in the presence of H_2O_2. The incorporation and oxidation of 14C-glucose at the blastocyst tended to resemble the development of embryo. The present study appeared that incorporation and oxidation of 14C- glucose was good for showing the activity of embryos.

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, generates NADP that serves as a coenzyme of the oxidative arm of the pentose phosphate pathway (PPP) [45]. The gradual increase in ROS levels from the 2-cell embryo up to the late morula stage could depend on the metabolic change undergone by the embryo during its development. It is necessary to prevent ROS as much as possible during culture embryos.

Conclusion

In conclusion, our results showed that culture of mouse zygotes in the CZB medium supplemented with 60nM Se and/or 100nM Vit-E improves the developmental rate of mouse blastocyst formation in the absence or presence of free radicals or their sources. Therefore, the present study reveals that selenium and vitamin E improves mice blastocyst viability by minimizing the level of free radicals that might occur during development of blastocyst in vitro and which may be useful for assisted reproductive techniques.

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How to cite this article: Hirotada T, Miah A, Salma U. Effect of Selenium and Vitamin E on Development and Viability of Preimplanted Mouse Embryo. Dairy and Vet Sci J. 2017; 2(5): 555599.DOI: 10.19080/JDVS.2017.02.555599

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