pISSN 1225-4991, eISSN 2288-0178
J. Emb. Trans. (2016) Vol. 31, No. 3, pp. 207~213
http://dx.doi.org/10.12750/JET.2016.31.3.207

Effect of glutathione on tetraploid embryo development in the pigs

Hwa-Young Kim[1], Sang-Hee Lee[1], Yong Hwangbo[1], Seung Tae Lee[1], Eunsong Lee[2], Choon-Keun Park[1,†]

[1]College of Animal Life Sciences, Kangwon National University and [2]College of Veterinary Medicine, Kangwon National University, Chunchoen 24341, Republic of Korea

ABSTRACT

The objective of this study was to investigate to influence of glutathione (GSH) on development and antioxidant enzyme activity in tetraploid porcine embryos. Tetraploid embryos were produced using parthenogenetic 2-cell embryo by electrofusion method. Tetraploid embryo development was observed every 24 hours and intracellular antioxidant enzyme activity was measured at 120 hours after electrofusion. The 4-cell to 16-cell stage tetraploid embryos was increased in 100 and 500 μM GSH-treated groups compared control group at 48 hours ($P < 0.05$) but cleavage rates were not significantly different among the GSH treatment groups at 48, 72, 96, and 120 hours. Blastocyst formation was significantly increased by 300 and 500 μM GSH at 120 hours in tetraploid embryos ($P < 0.05$). But blastocyst cell number were not significantly different among the GSH treatment groups (16.4 ± 0.8, 16.8 ± 2.6, 18.5 ± 2.8 and 17.5 ± 1.8). The intracellular antioxidant enzyme level was increased in 500 μM GSH compared to 0 and 100 μM GSH ($P < 0.05$). We suggest that GSH may be improve development of tetraploid embryo in pigs.

(Key words: Tetraploid, embryo development, electrofusion, glutathione, pig)

Introduction

General somatic cells have diploid (2n) chromosome, and reproductive cells have haploid (n) number chromosome through meiosis (He *et al*., 2013). Tetraploid (4n) define double times chromosomes compared to general diploid chromosome, which are used for studies of generating valuable information and preimplantation embryo development (Agrawal *et al*., 2016). Tetraploid embryos can be obtained by fusion of 2-cell stage embryos or inhibition of cell division using cytochalasin B or colchicine (Snow 1973; Kubiak and Tarkowski, 1985; McLaughlin 1993; Sembon *et al*., 2011). The tetraploid embryos are used to upgrade the cloned animal production (Sembon *et al*., 2011; Wang *et al*., 2016), widely used for study on embryogenesis (Sembon *et al*., 2011) and establishment of embryonic stem cell in mice (Nagy *et al*., 1990). Tetraploid embryos are occured rarely and not conciliable with normal development and viability and it has low efficiency of development (Eakin and Behringer, 2003).

In vitro production embryos such as *in vitro* fertilization (IVF), parthenogenetic activation and electrofusion were easily exposed to free radicals and oxidative stress for *in vitro* culture, which induce reactive oxygen species (ROS) in intracellular environment (Choi *et al*., 2013; Jin *et al*., 2016). The free radical, oxidative stress and ROS from physical and chemical damage are removed by antioxidants in mammal cells (Jin *et al*., 2016). Practically, vitamin C (Hu *et al*., 2012), vitamin E (Tareq *et al*., 2012) and resveratrol (Lee *et al*., 2015) have been used to remove ROS and increase antioxidant enzymes activity in oocytes culture system.

Glutathione (GSH) is one of the antioxidant enzymes, prevent cellular damage from free radical, oxidative stress and ROS in mammalian cells. It plays a critical role in antioxidation and detoxification of exo- and endogenous-oxidative stress compounds (Luberda, 2005). In addition, GSH prevented spindle of zygotes by oxidative stress and ROS, improve zygote formation (Zuelke *et al*., 1997).

Tetraploid embryos have value of study of embryogenesis and improvement of cloned animal production, but embryonic development is very low compared to diploid embryos (Kaufman and Webb, 1990; Eakin and Behringer, 2003). Practically, GSH improved development and blastocyst formation of diploid

*This research was supported by Agricultural Biotechnology Development Program (IPET31060-05), Food and Rural Affairs, Ministry of Agriculture, Republic of Korea.

†Correspondence: Choon-Keun Park
Tel: +82-33-250-8627
E-mail address: parkck@kangwon.ac.kr

embryos such as somatic cell nuclear transfer (SCNT), parthenogenesis (PA) and IVF (Bae *et al*., 2013; Bae *et al*., 2014) in pigs, but study of GSH on porcine tetraploid embryo development have not been reported. Therefore, the objective of this study was to detect developmental characteristic of tetraploid embryos and influence of GSH on development and intracellular antioxidant enzyme activity in porcine tetraploid embryos.

Materials and Methods

1. *In vitro* maturation

All procedures that involved the use of animals were approved by the Kangwon National University Institutional Animal Care and Use Committee (KIACUC-09-0139) and all reagents were from Sigma unless otherwise stated. Porcine ovaries were collected at a slaughter house from prepubertal gilts and transported to the laboratory at 35 to 36℃ within 2 hours. Ovaries were washed with 0.85% saline over 3 time and oocytes were aspirated with an 18-gauge needle from follicles of 3-6 mm in diameter. Cumulus–oocyte complexes (COCs) were washed in phosphate buffer saline-polyvinyl alcohol (PBS-PVA) and selected using mouth pipettes under microscope. Collected COCs were washed three times using PBS-PVA, then transferred to *in vitro* maturation medium (IVM) consisting of modified TCM-199 with 10% (v/v) porcine follicular fluid (pFF), 10 ng/ml of epidermal growth factor (EGF), luteinizing hormone (LH), follicle stimulating hormone (FSH) and 10 IU/ml human chorionic gonadotropin (hCG), for 22 hours. After COCs were cultured TCM-199 without hormones for 22 hours. All cultures were performed at 38.5°C in 5% CO_2.

2. Parthenogenetic activation

Cumulus cells were eliminated by mouth pipetting in TCM-199 containing 0.1% (w/v) hyaluronidase from cultured COCs. Then, metaphase II oocytes were selected, were washed three times in electric activation medium (0.01 mM $CaCl_2$; 0.05 mM $MgCl_2$; 0.28 M mannitol). For parthenogenetic activation, an electric pulse (1.2 kV/cm, 6 μs) generated by Electro-Cell Manipulator 2001 BTX (BTX, San Diego, CA, USA) was applied to matured oocytes. After 4 hours, activated oocytes were transport to embryo culture medium (porcine zygote medium 3; PZM-3) containing 0.3% bovine serum albumin.

3. Production of tetraploid embryo

After 24 hours of parthenogenetic activation, 2-cell stage embryos were selected to product tetraploid embryos. 2-cell stage embryos were washed three times in electrofusion medium before electrofusion. Then, for production of tetraploid embryos, an electric pulse (0.9 kV/cm, 30 μs) were applied to 2-cell embryos by Electro-Cell Manipulator (BTX).

4. *In vitro* culture

After 30 min of electrofusion, tetraploid embryo was washed three times in PZM-3 with 0, 100, 300 and 500 μM GSH and incubated at 38.5°C for 120 hours under 5% CO_2 in humidified air. The cleavage and blastocyst rate of tetraploid embryos were assessed at 48, 72, 96 and 120 hours after 2-cell embryos electrofusion. Tetraploid blastocysts were stained for detection of total cell number using hoechst 33342. In brief, tetraploid blastocysts were fixed 4% paraformaldehyde based on PBS-PVA for 5 min and incubated with 1 μg/mL of Hoechst 33342 in the dark room for 30 min. Then, stained blastocysts were washed three times in PBS-PVA and placed on the slide glass. Total cell number of tetraploid blastocyst was analyzed by epifluorescence microscope (DM 580, Nikon, Tokyo, Japan).

5. Intracellular antioxidant enzyme activity

Antioxidant enzyme activity of tetraploid embryos was measured using Cell Tracker Red (Invitrogen, Carlsbad, CA, USA) staining method. The tetraploid embryos were used at 120 hours after electrofusion, washed in PBS-PVA and fixed 4% paraformaldehyde based on PBS-PVA for 5 min. After, tetraploid embryos were incubated in the dark room for 30 min in 20 μM Cell Tracker Red based on PBS-PVA. Then, samples were washed in PBS-PVA and observed using epifluorescence microscope (Nikon). Intracellular antioxidant enzyme activity of tetraploid embryos was measured using ImageJ.

6. Statistical analysis

Data were performed using SAS ver. 9.4 software (SAS Institute, Cary, NC, USA). Data are expressed as means ± standard error. Treatment groups were compared for differences using the least significant difference test. All data were analyzed using ANOVA. $P < 0.05$ was considered significant.

Results

1. Effect of glutathione (GSH) on tetraploid embryo development in pigs

Fig. 1 was shown morphology of 2-cell stage parthenogenetic embryo (Fig. 1A) and tetraploid embryo (Fig. 1B and 1C). Tetraploid embryos were made from 2-cell stage parthenogenetic embryos (Fig. 1A), which were fused by electrofusion and blastomeres of parthenogenetic embryos were fused after 4 hours after electrofusion (Fig. 1C). Cleavage rate of tetraploid embryos with GSH treatment groups were not significantly differences at 48, 72, 96 and 120 hours after 2-cell embryo electrofusion (Fig. 2). However, 2-cell stage rate of tetraploid embryos with 300 μM GSH group at 48 hours was significantly higher than 100 μM GSH group (56.4 ± 8.7 % *vs* 41.9 ± 9.6 %; $P < 0.05$; Fig. 2A). However, 4-cell to 16-cell stage rate of tetraploid embryos with 100 and 500 μM GSH at 48 hours were significantly ($P < 0.05$) higher than 0 and 300 μM GSH (13.4 ± 3.1 %, 17.2 ± 5.7 % *vs* 24.6 ± 6.8 %, 29.7 ± 4.9 %, Fig. 2A). All treatment groups had not significantly 2-cell and 4-cell to 16-cell stage at 72 hours after electrofusion (Fig. 2B). In addition, all treatment groups had not significantly 2-cell, 4-cell to 16-cell, and morula stage at 96 hours but blastocyst rate of tetraploid embryos with 300 μM GSH group (58.6 ± 2.8 %) had significantly ($P < 0.05$) higher than other treatments at 96 hours after electrofusion (Fig. 2C). At 120 hours after electrofusion, blastocyst rate of tetraploid embryos with 300 and 500 μM GSH groups had significantly ($P < 0.05$) higher than 0 and 100 μM GSH groups (64.4 ± 4.8 %, 69.0 ± 2.4 % *vs* 40.5 ± 7.6 %, 40.3 ± 5.6 %, Fig. 2D). The blastocyst rate of tetraploid embryos at 48 and 72 hours after electrofusion were not significantly difference among the treatment groups (Fig. 2A and 2B), but 300 μM GSH significantly ($P < 0.05$) improved blastocyst rate at 96 hours after electrofusion, in addition blastocyst rate was significantly ($P < 0.05$) increased in 300 and 500 μM GSH groups compared to other treatment groups (Fig. 2C and 2D). The cell number of tetraploid at 120 hours after electrofusion was not significantly difference among the treatment groups (16.4 ± 0.8, 16.8 ± 2.6, 18.5 ± 2.8 and 17.5 ± 1.8; Fig. 4).

2. Influence of glutathione (GSH) on antioxidant enzyme activity in porcine tetraploid embryo

The intracellular antioxidant enzyme (GSH) activity of tetraploid embryos was measured at 120 hours after electrofusion. The results showed that intracellular antioxidant enzyme activity level of tetraploid embryos was significantly ($P < 0.05$) higher at 500 μM GSH group than other treatment groups (Fig. 4).

Discussion

Electrofusion condition was closely related to electrofusion machine and fusion buffer which influence electrofusion rate, which optimal condition is different among the species (Agrawal *et al*., 2016). Generally, 2DC 100 kV/cm pulses for 50 μs condition was used for production of tetraploid embryo in mice (Xiangyun *et al*., 2005), in bovine, Annelies *et al*. (1993) used 1.75 kV/cm 2DC pulse for 40 μs and Curnow *et al*. (2000) used 1.4 kV/cm for 100 μs. In addition, Prochazka *et al*. (2004) used 100 kV/cm 2DC pulses for 10 to 25 μs and He *et al*. (2013) used various 2DC pulses (0, 0.3, 0.6, 0.9, 1.2, 1.5, 1.8 and 2.1 kV/cm, 30 μs) in pigs. It is established to produce tetraploid embryo by electrofusion in mice, but optimal condition of production of porcine tetraploid embryo have not been clearly elucidated (Kubiak and Tarkowski, 1985). In this study, we used 2DC electro pulse of electric field 0.9 kV/cm for 30 μs to produce tetraploid embryo and electric field of 1.2 kV/cm for 6 μs is used for parthenogenesis activation. In addition, 2-cell stage parthenogenetic embryos were successfully fused when we use 0.9 kV/cm for 30 μs, the tetraploid embryos ordinarily developed to blastocyst.

In this study, high blastocyst formation rate and low cell number of blastocyst in tetraploid embryo were similar to previous other studies. However, mechanism of developmental speed and high blastocyst formation of tetraploid embryos have not been clearly elucidated. Therefore, we suggested that many groups need to study about the mechanism of tetraploid embryo development in pigs.

During *in vitro* culture, embryos were produced the excessive intracellular ROS compared to *in vivo* environment, eventually is negative affect to development in mammals (Harvey *et al*., 2002). The study was confirmed that 300 μM GSH improved *in vitro* development of tetraploid embryos and 300 and 500 μM GSH improved blastocyst rate of tetraploid embryos in the pig. Also, we investigated that influence of GSH on development in porcine tetraploid embryo. Low intracellular GSH level was caused to lower development capability during IVM in porcine

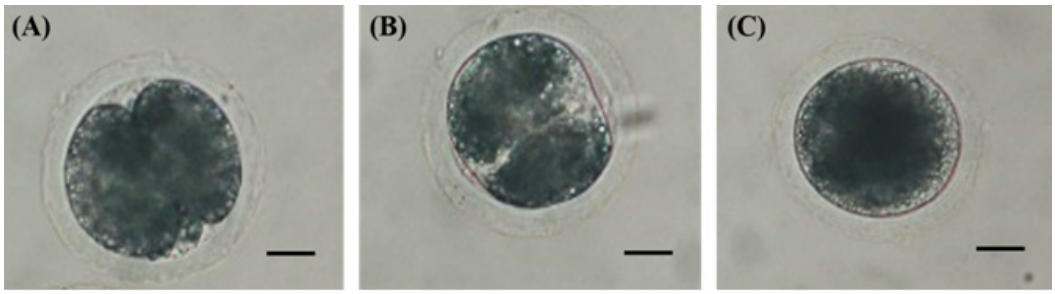

Fig. 1. Morphology of 2-cell stage parthenogenetic and tetraploid embryos. 2 cell stage parthenogenetic embryos (A) were used for production of tetraploid embryo (B and C) by electrofusion in pigs, B, fused tetraploid embryo at 30 min (B) and 4 hours (C) after the electrofusion, Scale bar: 50 μm.

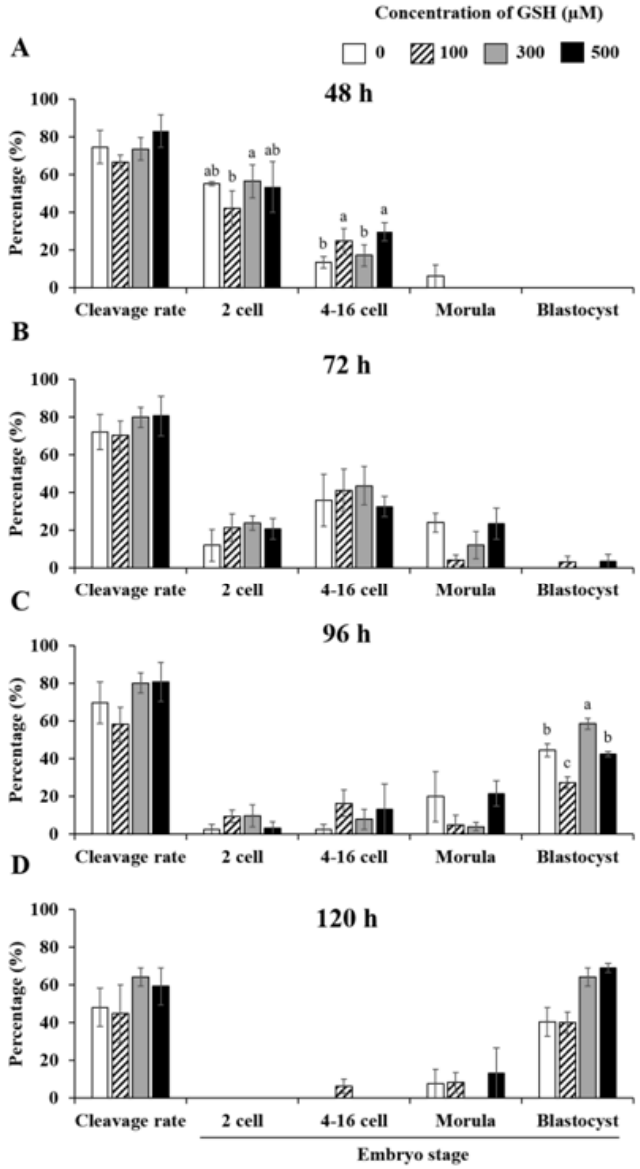

Fig. 2. Effect of GSH on tetraploid embryos developmentin pigs, tetraploid embryo stage and blastocyst was measured at 48 (A), 72 (B), 96 (C) and 120 (D) hours after 2-cell stage parthenogenetic embryo electrofusion.

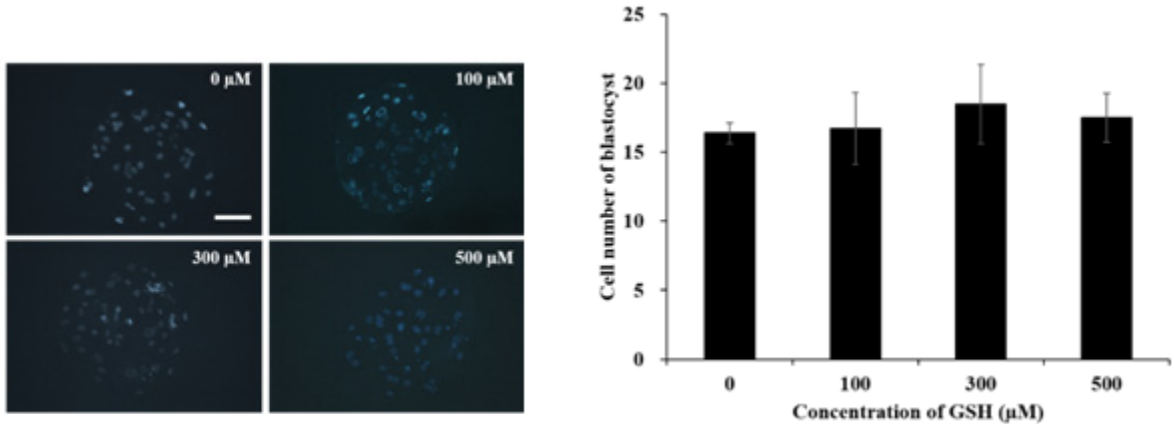

Fig. 3. Effect of GSH on tetraploid blastocyst cell number in pigs, Scale bar: 50 μm, blue is indicated nucleus of tetraploidblastocyst, nucleus is stained by 1 μg/mL Hoechst 33342.

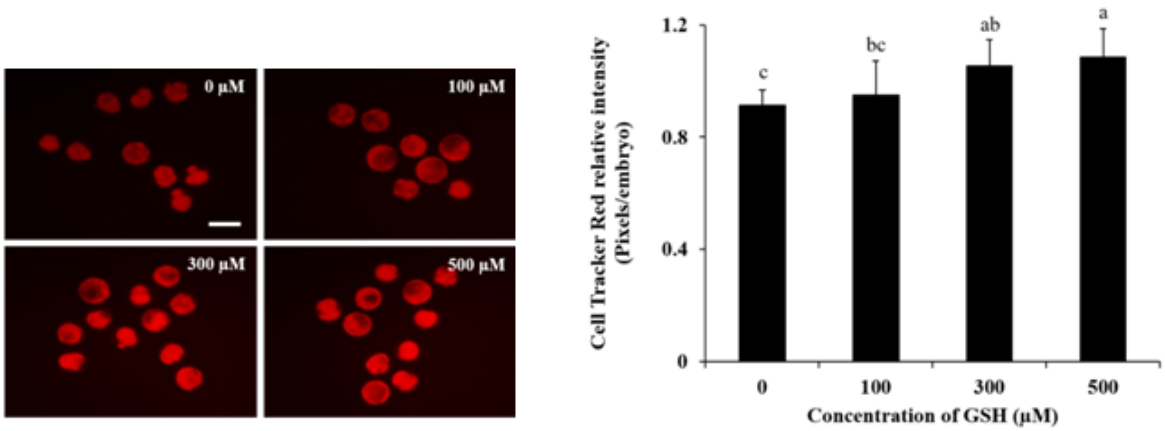

Fig. 4. Measurement of relative intracellular antioxidant enzyme levels (glutathione; GSH) using CellTracker Red in tetraploid embryos, tetraploid embryo was used at 120 hours after 2-cell stage parthenogenetic embryo electrofusion, Scale bar:50 μm.

oocytes (Brad *et al*., 2003), and the effect of GSH supplementation on the IVF and development of the mammalian embryos (Boquest *et al*., 1999) Consequently, the scavenging function of GSH improved fertilization and development of the mammalian embryo. GSH is a potentially high proper biochemical marker of the developmental ability of mammalian oocytes (Zuelke *et al*., 2003). However, there is no study that effect of GSH on *in vitro* development in tetraploid embryos, therefore we performed treatment of GSH during *in vitro* culture of tetraploid embryos. Consequently, 500 μM GSH improve intracellular antioxidant enzyme activity in tetraploid embryos. Other studies reported that GSH is active in maintaining meiotic spindle morphology on oocyte and protects the oxidative damage of the spindle (Zuelke *et al*., 1997). Intracellular GSH improves the capacity of the embryo to relieve the cytotoxic effects of H_2O_2 (Nasr-Esfahani and Johnson, 1992). So we presumed that GSH protects the tetraploid embryos during *in vitro* development from oxidative stress.

In summary, GSH had positive effect on development and protection of oxidative damage on porcine tetraploid embryos. Therefore, these results provide understanding of the tetraploid embryonic development and interaction between antioxidant enzymes and embryos.

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Received August 19 2016, Revised September 06, 2016, Accepted September 12, 2016