Zygote **15** (November), pp. 317–324. © 2007 Cambridge University Press
doi:10.1017/S0967199407004273 Printed in the United Kingdom

Glutamine and hypotaurine improves intracellular oxidative status and *in vitro* development of porcine preimplantation embryos

C. Suzuki[1], *K. Yoshioka*[1], *M. Sakatani*[2] *and M. Takahashi*[2]
National Institute of Animal Health, Ibaraki and National Agricultural Research Center for Kyushu Okinawa Region, Kumamoto, Japan

Date submitted: 02.12.06. Date accepted: 15.02.07

Summary

We previously developed an *in vitro*-production system for porcine embryos and reported that the addition of glutamine (Gln) and hypotaurine (HT) during *in vitro* culture improved embryo development. This study examined the effects of Gln and HT on *in vitro* development, intracellular oxidative status and DNA damage of porcine preimplantation embryos. Porcine zygotes produced by *in vitro* maturation (IVM) and *in vitro* fertilization (IVF) were cultured until day 2 (day 0 = day of IVF) in porcine zygote medium (PZM) including 2 mM Gln and 5 mM HT, namely PZM-5. On day 2, the cleaved embryos were selected and cultured for 24 h in PZM-5 to which one of the following substances was added: (1) none (control); (2) Gln; (3) HT; or (4) Gln + HT. After 24 h of culture in each medium, the embryos were then returned to PZM-5 and cultured until day 5. Day-5 blastocyst yield was significantly higher in the Gln and Gln + HT groups ($p < 0.05$) than in the control and HT groups. In addition, Gln + HT significantly increased the total number of cells in blastocysts ($p < 0.05$) compared with the control. Although the number of cells and the intracellular GSH levels in day-3 cleaved embryos did not differ among treatments, addition of Gln, HT or Gln + HT significantly ($p < 0.05$) reduced the intracellular H_2O_2 content and the extent of DNA damage compared with the control. These results indicate that the presence of Gln and HT in PZM-5 from day 2 to day 3 promotes the development of porcine embryos by improvement of intracellular oxidative status.

Keywords: Comet assay, *In vitro* culture, Porcine embryos, Reactive oxygen species

Introduction

Successful large-scale *in vitro* production of porcine embryos enables us to reduce the cost and time required to obtain embryos and is valuable for research in reproductive physiology, agriculture and biotechnology, including cloning and transgenesis in pigs. Previously, we demonstrated that the addition of glutamine (Gln) and hypotaurine (HT) to porcine zygote medium (PZM) improved the *in vitro* development of porcine embryos after *in vitro* maturation (IVM) and fertilization (IVF) (Yoshioka *et al.*, 2002; Suzuki & Yoshioka, 2006). Gln is a pleiotropic amino acid used as an energy substrate (Fox *et al.*, 1996) and a precursor for nucleotides (Boza *et al.*, 2000) of most cells. In pigs, glucose or Gln have been shown to support embryo development as the sole energy source from the 1-cell stage to the blastocyst stage (Petters *et al.*, 1990). Abundant taurine and HT exist in the reproductive tract and embryos (Miller & Schultz, 1987) and supplementation of taurine and HT to culture medium improves the *in vitro* development of murine (Dumoulin *et al.*, 1992) and bovine embryos (Guyader-Joly *et al.*, 1998). In pig embryos, addition of HT to the medium was shown to increase the degree of embryo development *in vitro* (Reed *et al.*, 1992; Suzuki & Yoshioka, 2006).

Reactive oxygen species (ROS) such as superoxide anions (O_2^-), hydrogen peroxide (H_2O_2) and hydroxyl radicals (OH^-) cause damage to the cell

All correspondence to: C. Suzuki, Research Team for Production Diseases, National Institute of Animal Health, Kannondai 3–1–5, Tsukuba, Ibaraki 305–0856, Japan. Tel: +81 29 838 7784. Fax: +81 29 838 7880. e-mail: schie@affrc.go.jp
[1]Research Team for Production Diseases, National Institute of Animal Health, Kannondai 3–1–5, Tsukuba, Ibaraki 305–0856, Japan.
[2]Research Team for Effects of Climate Change on Agriculture, National Agricultural Research Center for Kyushu Okinawa Region, 2421 Suya, Koshi, Kumamoto 861–1192, Japan.

membrane (Aitken *et al.*, 1989) and DNA fragmentation (Halliwell & Aruoma, 1991). Early cleavage-stage embryos are sensitive to oxidative stress and ROS inhibit embryonic development (Nasr-Esfahani *et al.*, 1990; Yang *et al.*, 1998; Guérin *et al.*, 2001). Especially, these ROS have been directly implicated in the 2-cell block in mouse embryos (Nasr-Esfahani *et al.*, 1990). To protect against oxidative stress, there are several defense mechanisms in embryos, follicular and oviductal fluids. The glutathione (GSH) system is one major protective mechanism from oxidative stress (Kosower & Kosower, 1978) and an increase in GSH levels in oocytes matured *in vitro* improves the developmental competence of pig embryos after *in vitro* fertilization (Abeydeera *et al.*, 1999; Guérin *et al.*, 2001). HT is also an antioxidant that neutralizes hydroxyl radicals (Fellman *et al.*, 1987; Guérin *et al.*, 2001). However, how Gln and HT in PZM exert beneficial effects for embryo development remains unknown.

The aims of the present study were: (1) to evaluate the effects of Gln and HT in PZM from day 2 to day 3 after IVF, when developmental arrest at the 4-cell stage is observed in pig embryos, on *in vitro* embryo development to the blastocyst stage; and (2) to determine whether these substrates influence intracellular oxidative status and DNA damage of embryos.

Materials and methods

IVM, IVF and IVC

IVM and IVF of porcine oocytes were conducted using our previously described method (Yoshioka *et al.*, 2003). Briefly, intact cumulus–oocyte complexes (COCs) were aspirated from antral follicles (3–6 mm in diameter) of ovaries of slaughtered prepuberal gilts. COCs were matured in modified North Carolina State University-37 medium supplemented with 10% (v/v) porcine follicular fluid, 0.6 mM cysteine (Sigma Chemical Co.), 1 mM dibutyryl cyclic AMP (Sigma), 10 IU/ml equine chorionic gonadotropin (Peamex, Sankyo), 10 IU/ml human chorionic gonadotropin (Puberogen, Sankyo) and 50 μg/ml gentamicin sulphate (Sigma) for 20 to 22 h and subsequently in the same medium without dibutyryl cyclic AMP and hormones for 24 h. IVF was performed using the same batch of frozen-thawed ejaculated semen. The sperm-rich fraction of the ejaculation collected from Landrace boars were cryopreserved by previously described method (Yoshioka *et al.*, 2003). The sperm suspension was transferred to 0.5 ml straws, which were frozen in liquid nitrogen vapour and finally stored in liquid nitrogen until use. After IVM, COCs were incubated for fertilization in a porcine gamete medium supplemented with theophylline, adenosine and cysteine (PGMtac) (Yoshioka *et al.*, 2003) with modification of Ca-(lactate)$_2$.5H$_2$O concentration from 2.5 to 5 mM, with 2.5 or 5×10^6 Percoll-separated spermatozoa for 12 h. After coincubation with spermatozoa, the COCs were vortexed for 4 min in modified TALP–HEPES (Yoshioka *et al.*, 2002) to remove cumulus cells from the oocytes. Some of the denuded oocytes were randomly selected, mounted on slides, fixed with acetic alcohol (1:3), stained with 1% (w/v) aceto-orcein and examined under a phase-contrast microscope for sperm penetration and pronuclear formation (Yoshioka *et al.*, 2003). The frequency of normal fertilization was determined as the proportion of oocytes with a second polar body, a pair of pronuclei and corresponding sperm tail out of the total number of oocytes evaluated. Approximately 25 presumptive zygotes were cultured in 40 μl droplets of PZM (Suzuki & Yoshioka, 2006) including 2 mM Gln and 5 mM HT, namely PZM-5. At 48 h postinsemination, the cleaved embryos ($\geqslant$ 2-cell stage) were selected and cultured for 24 h in PZM-5 to which one of the following substances was added: (1) none (control); (2) 2 mM Gln; (3) 5 mM HT; or (4) 2 mM Gln and 5 mM HT (Gln + HT). All cultures were maintained at 39 °C in a humidified atmosphere of 5% CO_2, 5% O_2 and 90% N_2.

Evaluation of embryo development

After 24 h culture in each medium, some of the cleaved embryos were randomly selected, mounted on slides, fixed with acetic alcohol (1:3), stained with 1% (w/v) aceto-orcein and counted under a phase-contrast microscope for the total number of cells per embryo (Yoshioka *et al.*, 2003). The remaining cleaved embryos were returned to PZM-5 and further cultured for 2 days (until day 5), before evaluating the rate of blastocysts formation and counting the total number of cells per blastocyst using an air-dry method, as described previously (Yoshioka *et al.*, 2003).

Assay of GSH

The intracellular GSH levels of the embryos were determined by the 5,5′-dithio*bis*(2-nitrobenzoic acid)–glutathione disulphide (DTNB–GSSG) reductase recycling assay according to Funahashi *et al.* (1994). After 24 h culture in each medium, day-3 cleaved embryos were washed three times in TALP–HEPES. Groups of 36 embryos in 5 μl of distilled water were transferred to 1.5 ml microfuge tubes and 5 μl of 1.25 M H_3PO_4 was added. Samples were frozen at −80 °C until assay. The samples were mixed with 700 μl of 0.33 mg/ml NADPH in 0.2 M sodium phosphate buffer containing 10 mM EDTA (pH 7.2, stock buffer), 100 μl of 6 mM DTNB (Sigma) in stock buffer and 190 μl of distilled water. After rapid mixing with 10 μl

https://doi.org/10.1017/S0967199407004273 Published online by Cambridge University Press

of 250 IU/ml glutathione reductase (Oriental Yeast Co. Ltd), the increase in absorbance at 412 nm was measured with a spectrophotometer (Beckman Coulter Inc., CA, USA) from 30 s to 5 min. GSH standards (0.1–1.0 nmol) and a sample lacking GSH were also assayed. The amount of GSH was determined according to Calvin *et al.* (1986).

Measurement of H_2O_2 content

The intracellular level of H_2O_2 in each embryo was examined according to the 2′,7′- dichlorohydrofluorescein diacetate (DCHFDA) method described by Sakatani *et al.* (2004). After 24 h culture in each medium, day-3 cleaved embryos were immediately transferred to PZM-5 containing 10 μM DCHFDA (Molecular Probes Inc.) and incubated at 39°C for 15 min in a humidified atmosphere of 5% CO_2, 5% O_2 and 90% N_2. After incubation, embryos were washed three times with TALP–HEPES and placed on glass slides. The fluorescent emission from the embryos were recorded as TIFF files using a CCD camera attached to a fluorescence microscope (OLYMPUS Co.) with filters at 450 nm for excitation and 520 nm for emission. The recorded fluorescent images were analyzed using IPLab software (Scanalytics Inc.) by counting the number of pixels after colour inversion.

Comet assay for detecting DNA damage

Detection of DNA damage in individual embryos was carried out using the method described by Takahashi *et al.* (2000). After 24 h culture in each medium, 10 to 20 day-3 cleaved embryos were washed twice in PBS supplemented with 3 mg/ml polyvinyl alcohol and then transferred to a 200 μl drop of 1% (w/v) low-melting-point agarose (Bio-Rad Co., CA, USA) in PBS at 39°C. The embryos were placed as a drop of the 1% low-melting-point agarose onto a glass slide initially coated with 1% (w/v) high-melting point agarose (Bio-Rad). The embryos were then lysed by incubating the slides for 3 h at ambient temperature in lysing buffer (10 mM Tris, pH 10, containing 1% [w/v] sodium sarcosinate, 2.5 mM NaCl, 100 mM Na_2–EDTA, 1% [v/v] Triton X-100 and 10 μg/ml proteinase K [Sigma]). The slides were then placed on a horizontal gel electrophoresis unit and electrophoresis was conducted for 30 min at 25 V using an electrophoresis power supply (Bio-Rad). DNA was stained with SYBR Green solution (Cambrex Bio Science Rockland Inc.). Observation of DNA was carried out under a fluorescence microscope with filters at 490 nm for excitation and 520 nm for emission and recorded as TIFF files using a CCD camera attached to a fluorescence microscope. DNA damage was quantified according to Takahashi *et al.* (2000).

Statistical analysis

Data were analyzed using a general linear models procedure (SAS Institute, Inc.). Treatment differences were determined using the Tukey–Kramer multiple range test. Percentage data and the total number of cells per blastocyst were subjected to arcsine and logarithmic transformation, respectively, before statistical analysis. A p-value of less than 0.05 was considered statistically significant.

Results

In our IVF system, mean percentage of penetrated oocytes and the normal fertilization rate were 55.6% and 45.9% of examined oocytes, respectively. Moreover, 8% of examined oocytes were identified as polyspermy. A total of 2969 presumptive zygotes were cultured in PZM-5 following IVF and 1595 cleaved embryos (53.7%) were obtained on day 2 after IVF.

Effects of treatment with Gln and/or HT on embryo development

The rates of development to the blastocyst stage of embryos cultured in medium with Gln + HT and Gln only from day 2 to day 3 were significantly higher ($p < 0.05$) than those of embryos cultured in medium with no addition and HT only (Table 1). Moreover, the total number of cells in blastocysts cultured in medium with Gln + HT from day 2 to day 3 was significantly greater ($p < 0.05$) than that in culture with no addition (Table 1). However, the total number of cells in cleaved embryos cultured in all mediums from day 2 to day 3 did not differ among treatments (Fig. 1).

Table 1 Effects of Gln and HT in PZM-5 from day 2 to day 3 on development to blastocysts*

Treatment group	No. of cleaved embryos cultured	Blastocysts at day 5	
		No. (%) blastocysts at day 5	Total no. cells in blastocysts
None	90	11 (11.1 ± 5.2)[a]	25.8 ± 1.9[a]
Gln	88	36 (41.2 ± 6.3)[b]	30.5 ± 1.7[a,b]
HT	89	19 (21.1 ± 1.6)[a]	29.4 ± 1.8[a,b]
Gln + HT	90	47 (51.8 ± 3.5)[b]	34.5 ± 1.8[b]

*Percentage and cell numbers are expressed as the mean ± SEM from six replicates.

[a,b]Values with different superscripts within each column are significantly different ($p < 0.05$).

https://doi.org/10.1017/S0967199407004273 Published online by Cambridge University Press

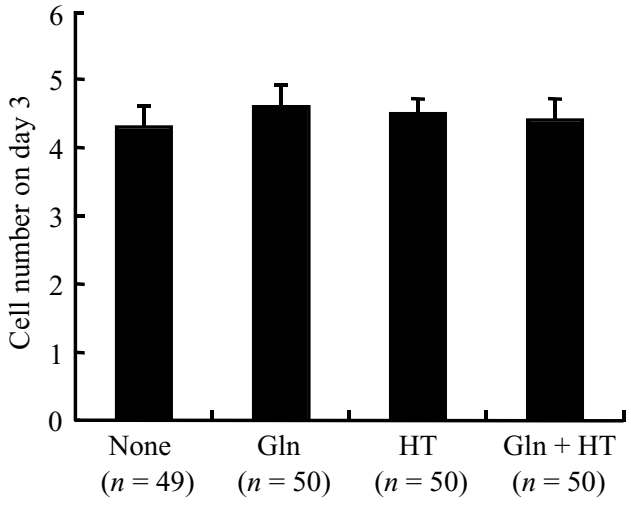

Figure 1 Effects of Gln and HT in PZM-5 from day 2 to day 3 on the cell numbers in day-3 cleaved embryos. Data represent the mean and SEM of four replicates. Numbers in parentheses indicate the number of embryos tested per group.

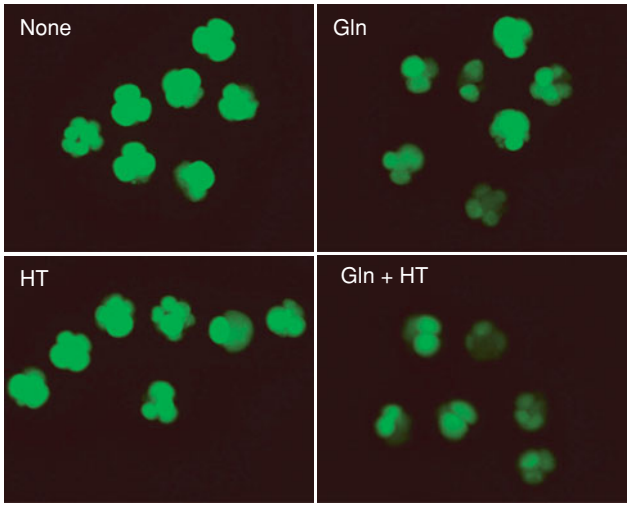

Figure 3 Fluorescent photomicrographs of day-3 cleaved embryos with DCHFDA showing the intracellular H_2O_2 contents.

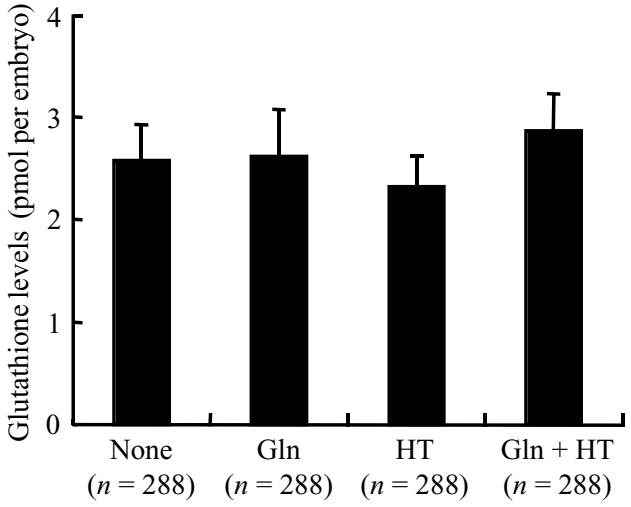

Figure 2 Effects of Gln and HT in PZM-5 from day 2 to day 3 on intracellular GSH levels in day-3 cleaved embryos. Data represent the mean and SEM. Numbers in parentheses indicate the total number of embryos in eight replicates.

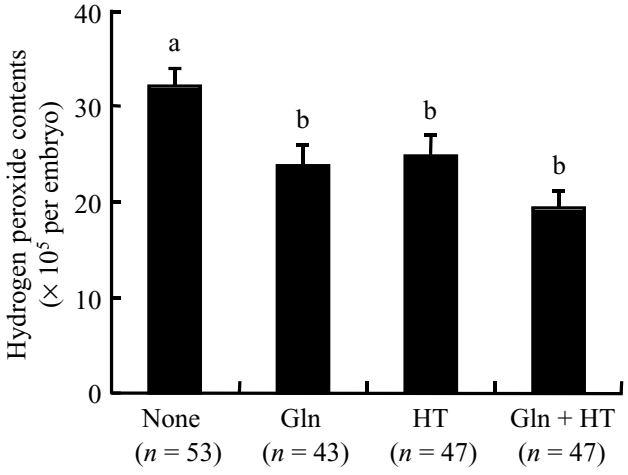

Figure 4 Effects of Gln and HT in PZM-5 from day 2 to day 3 on the intracellular H_2O_2 content of day-3 cleaved embryos. Data represent the mean and SEM. Different letters above the bars denote significant differences ($p < 0.05$). Numbers in parentheses indicate the total number of embryos in four replicates.

Effects of treatment with Gln and/or HT on intracellular GSH content, H_2O_2 content and DNA damage in day-3 embryos

There were no significant differences among treatments in the intracellular GSH levels of day-3 cleaved embryos (Fig. 2).

The intensities of fluorescence generated by oxidized DCHFDA in day-3 cleaved embryos are shown in Fig. 3. After calculation of the intensity, the intracellular H_2O_2 content of cleaved embryos cultured in medium with no addition was significantly higher ($p < 0.05$) than that in culture with Gln, HT and Gln + HT, respectively (Fig. 4).

The lengths of DNA tails in day-3 cleaved embryos are shown in Fig. 5. When the length of the DNA tails was measured, the comet tails of cleaved embryos cultured in medium with no addition were significantly longer ($p < 0.05$) than after culture in medium with Gln, HT and Gln + HT, respectively (Fig. 6).

https://doi.org/10.1017/S0967199407004273 Published online by Cambridge University Press

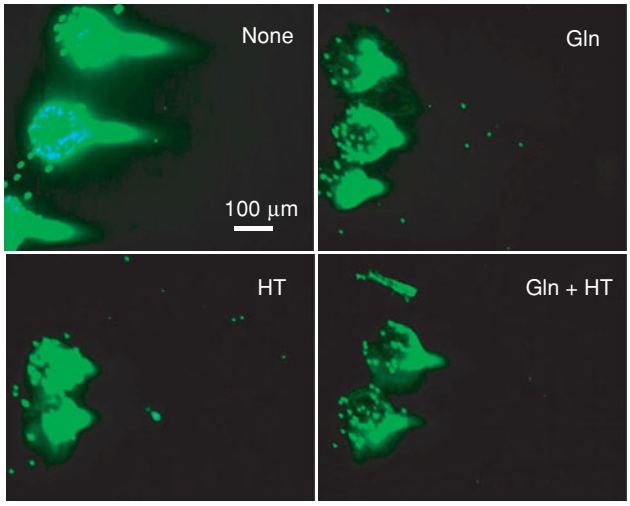

Figure 5 Fluorescent photomicrographs of typical DNA migration patterns in day-3 cleaved embryos.

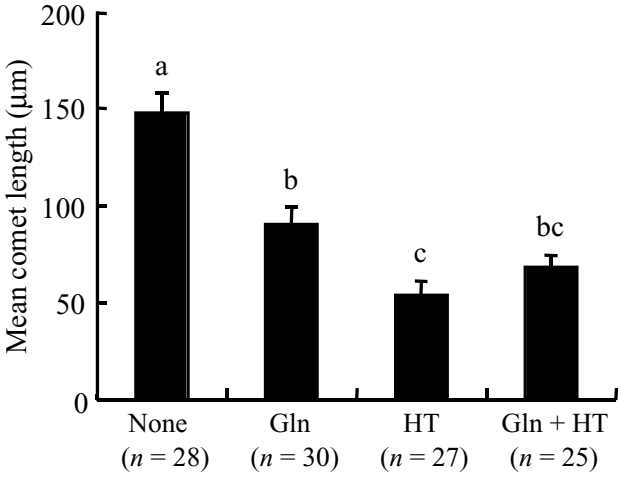

Figure 6 Effects of Gln and HT in PZM-5 from day 2 to day 3 on DNA damage in day-3 cleaved embryos. Data represent the mean and SEM. Different letters above the bars denote significant differences ($p < 0.05$). Numbers in parentheses indicate the total number of embryos in three replicates.

Discussion

The results of the present study demonstrate that the presence of Gln and/or HT in PZM from day 2 to day 3 after IVF promotes development of porcine embryos in conjunction with the improved intracellular oxidative stratus, as shown by the decrease in intracellular H_2O_2 content and extent of DNA damage. However, the presence of Gln and HT had no effect on the number of cells and the intracellular GSH levels of day-3 cleaved embryos.

In the present study, the presence of Gln only, or both Gln and HT from day 2 to day 3 increased embryo development to the blastocyst stage. Furthermore, the total number of cells was greater in blastocysts cultured in the presence of Gln and HT than that with no addition. Previously, we found that the addition of Gln and HT throughout the culture period from day 0 to day 5 promoted the development of porcine embryos cultured in chemically defined PZM (Suzuki & Yoshioka, 2006). In this study, the presence of Gln from day 2 to day 3 only resulted in enhancement of subsequent blastocyst formation, while coexistence with HT increased the total number of cells in blastocysts. Conversely, the total number of cells in day-3 cleaved embryos did not differ among treatments. Therefore, Gln and HT seem to improve the embryo quality functionally rather than morphologically for 24 h from day 2 to day 3.

The presence of Gln and/or HT from day 2 to day 3 reduced the intracellular H_2O_2 content. The developmental competence of porcine (Kitagawa *et al.*, 2004) and bovine (Takahashi *et al.*, 2000) IVF embryos cultured under 20% O_2, which increases the H_2O_2 content, was lower than that of those cultured under 5% O_2. Our study showed that high levels of H_2O_2 in embryos cultured in the absence of Gln and HT is associated with a reduction in embryo development even under the lower O_2 condition (5%). Higher levels of OH^- derived from H_2O_2 can induce lipid peroxidation (Slater, 1984; Marnett, 2000). Peroxidation of fatty acids has been reported to inhibit the function of cells, potentially inducing cell death (Spiteller, 2001). PZM is a completely defined medium that contains no protein or serum sources (Yoshioka *et al.*, 2002). Therefore it is possible that embryos are more sensitive to oxidative stress derived from internal conditions or external conditions without antioxidative components such as BSA or other factors in serum.

Gln is a precursor for glutamate and, in particular, GSH synthesis (Babu *et al.*, 2001; Matés *et al.*, 2002). GSH is the principle non-enzymatic defense system against ROS in embryos and has beneficial effects on embryo development (Abeydeera *et al.*, 1999; Guérin *et al.*, 2001). However, the presence or absence of Gln had no relationship with intracellular GSH levels as shown in Fig. 2, although Gln reduced the intracellular H_2O_2 content and increased the blastocyst yield. Thus, Gln might act as a defense mechanism against ROS except the GSH system. Nitric oxide (NO) is important for mouse preimplantation embryo development and is thought to limit oxygen consumption in preimplantation embryos at the level of mitochondrial cytochrome oxidase (Manser *et al.*, 2004). NO is produced from L-arginine, which is consumed in significant quantities by human preimplantation embryos (Houghton *et al.*, 2002), indicating that NO production might be essential in early development. Gln may be a useful precursor for arginine, with citrulline acting as an intermediate in this pathway

https://doi.org/10.1017/S0967199407004273 Published online by Cambridge University Press

(Murphy & Newsholme, 1997). Manser *et al.* (2004) reported that conditions where production of NO by the embryo is likely to be lower (i.e. in medium with no glutamine or arginine) result in embryos with a higher oxygen consumption and lower cell number. Therefore, it is possible that the decreased H_2O_2 content observed in the presence of Gln is related to the limiting effect of NO for oxygen consumption.

ROS cause DNA strand breaks (Halliwell & Aruoma, 1991). OH^- derived from H_2O_2 is highly reactive, can modify purines and pyrimidines and cause strand breaks resulting in DNA damage (Mello Filho & Meneghini, 1984). The presence of Gln and/or HT in the medium from day 2 to day 3 reduced DNA damage of day-3 embryos in the present study. HT has a greater anti-oxidant effect and provides better protection against DNA damage than taurine in the calf thymus (Messina & Dawson, 2000). Since lipid peroxidation products have also been shown to promote the formation of DNA adducts (Burcham, 1998), the inhibitory effect of HT on lipid peroxidation (Alvarez & Storey, 1983) might have contributed to the reduction of DNA damage in this study. Conversely, Gln also inhibited the extent of DNA damage regardless of GSH synthesis. Moreover, the extent of DNA damage also seems to have been reduced due to the reducing effects of Gln on the intracellular H_2O_2 content of the embryos. Gln also prevents apoptosis through metabolic pathways independent of GSH production in human colonic epithelial cells (Evans *et al.*, 2003). Further, Gln has also been shown to upregulate nuclear factor kappa B activation, an event that may inhibit apoptosis via induction of Bcl-X_L and caspase-8/FLICE inhibiting protein (Irmler *et al.*, 1997) or by preventing the formation of apoptosomes (Bos *et al.*, 1987). Additional studies to clarify the role of these or other pathways in the regulation of DNA damage by glutamine are therefore required.

Zygotic gene activation (ZGA), which corresponds to the transition from maternal to embryonic control of embryo development, is a critical step characterized by a developmental block or slowing down of cleavage under *in vitro* culture conditions (Johnson & Nasr-Esfahani, 1994). When oxidative stress occurs before and during ZGA, embryonic development is arrested at the 2-cell stage, in association with a rise in ROS in mice (Nasr-Esfahani *et al.*, 1990). In pig embryos, ZGA occurs at the 4-cell stage (Tománek *et al.*, 1989; Schoenbeck *et al.*, 1992; Anderson *et al.*, 1999). In the culture with PZM-5, porcine embryos were able to develop beyond the 4-cell stage on day 3. Therefore, embryos cultured in the absence of Gln and HT from day 2 to day 3, causing higher levels of H_2O_2 and increases in the extent of DNA damage, might show perturbed normal induction of gene expression essential for further development.

In the previous study, the addition of both Gln and HT throughout the culture period from day 0 to day 5 enhanced blastocyst yields (35.5–39.6%) per cleaved embryos compared with Gln only (6.1%) or HT only (3.5%) (Suzuki & Yoshioka, 2006). In the present study, the absence of Gln even from day 2 to day 3 suppressed embryo development to the blastocyst stage. Conversely, the presence of HT only did not enhance the subsequent embryo development, although it showed lower DNA damages of day-3 embryos compared with Gln only. The reason why the preventive effect of HT against DNA damage was not reflected in the blastocyst yield of porcine embryos is unclear. The enhancement of embryo development by Gln might not be brought about only by 60% reduction in DNA damage on day-3 embryos assessed by comet length. Further analysis of Gln and HT, including the period that they are most effective for embryo development, will be required to elucidate the actions of Gln and HT.

In conclusion, the presence of Gln and HT from day 2 to day 3 was shown to enhance *in vitro* development of porcine embryos to the blastocyst stage. This high developmental competence of porcine embryos cultured was thought to have occurred, in part, due to the decreased accumulation of H_2O_2 and the prevention of DNA damage in day-3 cleaved embryos, providing a suitable environment for embryos by protecting them from oxidative stress during *in vitro* development.

Acknowledgements

We thank Dr K. Iga, National Agricultural Research Center for Tohoku Region, for technical advice concerning the GSH assays and Dr M. Ozawa, National Institute of Agrobiological Sciences, for useful advice regarding the method for analysis of the H_2O_2 measurements. This study was supported by a grant from the Ministry of Agriculture, Forestry and Fisheries of Japan.

References

Abeydeera, L.R., Wang, W.H., Cantley, T.C., Prather, R.S. & Day, B.N. (1999). Glutathione content and embryo development after *in vitro* fertilisation of pig oocytes matured in the presence of a thiol compound and various concentrations of cysteine. *Zygote* **7**, 203–10.

Aitken, R.J., Clarkson, J.S. & Fishel, S. (1989). Generation of reactive oxygen species, lipid peroxidation and human sperm function. *Biol. Reprod.* **40**, 183–97.

Alvarez, J.G. & Storey, B.T. (1983). Taurine, hypotaurine, epinephrine and albumin inhibit lipid peroxidation in rabbit spermatozoa and protect against loss of motility. *Biol. Reprod.* **29**, 548–55.

Anderson, J.E., Matteri, R.L., Abeydeera, L.R., Day, B.N. & Prather, R.S. (1999). Cyclin B1 transcript quantification over the maternal to zygotic transition in both in vivo- and in vitro-derived 4-cell porcine embryos. *Biol. Reprod*. **61**, 1460–7.

Babu, R., Eaton, S., Drake, D.P., Spitz, L. & Pierro, A. (2001). Glutamine and glutathione counteract the inhibitory effects of mediators of sepsis in neonatal hepatocytes. *J. Pediatr. Surg*. **36**, 282–6.

Bos, J.L., Fearon, E.R., Hamilton, S.R., Verlaan de, V.M., van Boom, J.H., Van Der Eb, A.J. & Vogelstein, B. (1987). Prevalence of *Ras* gene mutations in human colorectal cancers. *Nature* **327**, 293–7.

Boza, J.J., Moennoz, D., Bournot, C.E., Blum, S., Zbinden, I., Finot, P.A. & Ballevre, O. (2000). Role of glutamine on the de novo purine nucleotide synthesis in Caco-2 cells. *Eur. J. Nutr*. **39**, 38–46.

Burcham, P.C. (1998). Genotoxic lipid peroxidation products: their DNA damaging properties and role in formation of endogenous DNA adducts. *Mutagenesis* **13**, 287–305.

Calvin, H.I., Grosshans, K. & Blake, E.J. (1986). Estimation and manipulation of glutathione levels in prepuberal mouse ovaries and ova: relevance to sperm nucleus transformation in the fertilized egg. *Gamete Res.* **14**, 265–75.

Dumoulin, J.C.M., Evers, J.L.H., Bras, M., Pieters, M.H.E.C. & Geraedts, J.P.M. (1992). Positive effect of taurine on preimplantation development of mouse embryos in vitro. *J. Reprod. Fertil*. **94**, 373–80.

Evans, M.E., Jones, D.P. & Ziegler, T.R. (2003). Glutamine prevents cytokine-induced apoptosis in human colonic epithelial cells. *J. Nutr*. **133**, 3065–71.

Fox, R.E., Hopkins, I.B., Cabacungan, E.T. & Tildon, J.T. (1996). The role of glutamine and other alternate substrates as energy sources in the fetal rat lung type II cell. *Pediatr. Res.* **40**, 135–41.

Funahashi, H., Cantley, T.C., Stumpf, T.T., Terlouw, S.L. & Day, B.N. (1994). Use of low salt culture medium for in vitro maturation of porcine oocytes is associated with elevated oocyte glutathione levels and enhanced male pronuclear formation after in vitro fertilization. *Biol. Reprod.* **51**, 633–9.

Guérin, P., El Mouatassim, S. & Ménézo, Y. (2001). Oxidative stress and protection against reactive oxygen species in the pre-implantation embryo and its surroundings. *Hum. Reprod. Update* **7**, 175–89 (review).

Guyader-Joly, C., Guérin, P., Renard, J.P., Guillaud, J., Ponchon, S. & Ménézo, Y. (1998). Precursors of taurine in female genital tracts: effects on developmental capacity of bovine embryo produced in vitro. *Amino Acids* **15**, 27–42.

Halliwell, B. & Aruoma, O.I. (1991). DNA damage by oxygen-derived species. Its mechanism and measurement in mammalian systems. *FEBS Letts* **281**, 9–19 (review).

Houghton, F.D., Hawkhead, J.A., Humpherson, P.G., Hogg, J.E., Balen, A.H., Rutherford, A.J. & Leese, H.J. (2002). Non-invasive amino acid turnover predicts human embryo developmental capacity. *Hum. Reprod*. **17**, 999–1005.

Irmler, M., Thome, M., Hahne, M., Schneider, P., Hofmann, K., Steiner, V., Bodmer, J.L., Schroter, M., Burns, K., Mattmann, C., Rimoldi, D., French, L.E. & Tscopp, J. (1997). Inhibition of death receptor signals by cellular FLIP. *Nature* **388**, 190–5.

Johnson, M.H. & Nasr Esfahani, M.H. (1994). Radical solutions and cultural problems: could free oxygen radicals be responsible for the impaired development of preimplantation mammalian embryos in vitro? *Bioessays* **16**, 31–8 (review).

Kitagawa, Y., Suzuki, k., Yoneda, A. & Watanabe, T. (2004). Effects of oxygen concentration and antioxidants on the in vitro developmental ability, production of reactive oxygen species (ROS) and DNA fragmentation in porcine embryos. *Theriogenology* **62**, 1186–97.

Kosower, N.S. & Kosower, E.M. (1978). The glutathione status of cells. *Int. Rev. Cytol*. **54**, 109–60.

Manser, R.C., Leese, H.J. & Houghton, F.D. (2004). Effect of inhibiting nitric oxide production on mouse preimplantation embryo development and metabolism. *Biol. Reprod*. **71**, 528–33.

Marnett, L.J. (2000). Oxyradicals and DNA damage. *Carcinogenesis* **21**, 361–70.

Matés, J.M., Pérez-Gòmez, C., Núñez de Castro, I., Asenjo, M. & Márquez, J. (2002). Glutamine and its relationship with intracellular redox status, oxidative stress and cell proliferation/death. *Int. J. Biochem. Cell. Biol*. **34**, 439–58.

Mello-Filho, A.C. & Meneghini, R. (1984). In vivo formation of single-strand breaks in DNA by hydrogen peroxide is mediated by the Haber-Weiss reaction. *Biochim. Biophys. Acta*. **781**, 56–63.

Messina, S.A. & Dawson, R. (2000). Attenuation of oxidative damage to DNA by taurine and taurine analogues. *Adv. Exp. Med. Biol*. **483**, 355–67.

Miller, J.G.O. & Schultz, G.A. (1987). Amino acids content of preimplantation rabbit embryos and fluids of the reproductive tract. *Biol. Reprod*. **36**, 125–9.

Murphy, C. & Newsholme, P. (1997). Glutamine as a possible precursor of L-arginine and thus nitric oxide synthesis in murine macrophages. *Biochem. Soc. Trans*. **25**, 404S.

Nasr-Esfahani, M.H., Aitken, J.R. & Johnson, M.H. (1990). Hydrogen peroxide levels in mouse oocytes and early cleavage stage embryos developed in vitro or in vivo. *Development* **109**, 501–7.

Petters, R.M., Johnson, B.H., Reed, M.L. & Archibong, A.E. (1990). Glucose, glutamine and inorganic phosphate in early development of the pig embryo in vitro. *J. Reprod. Fertil*. **89**, 269–75.

Reed, M.L., Illera, M.J. & Petters, R.M. (1992). In vitro culture of pig embryos. *Theriogenology* 37, 95–109.

Sakatani, M., Kobayashi, S. & Takahashi, M. (2004). Effects of heat shock on in vitro development and intracellular oxidative state of bovine preimplantation embryos. *Mol. Reprod. Dev*. **67**, 77–82.

Schoenbeck, R.A., Peters, M.S., Rickords, L.F., Stumpf, T.T. & Prather, R.S. (1992). Characterization of deoxyribonucleic acid synthesis and the transition from maternal to embryonic control in the 4-cell porcine embryo. *Biol. Reprod*. **47**, 1118–25.

Slater, T.F. (1984). Free-radical mechanisms in tissue injury. *Biochem. J*. **222**, 1–15.

Spiteller, G. (2001). Peroxidation of linoleic acid and its relation to aging and age dependent diseases. *Mech. Ageing. Dev*. 122, 617–57.

Suzuki, C. & Yoshioka, K. (2006). Effects of amino acid supplements and replacement of polyvinyl alcohol with

bovine serum albumin in porcine zygote medium. *Reprod. Fertil. Dev*. **18**, 789–95.

Takahashi, M., Keicho, K., Takahashi, H., Ogawa, H., Schultz, R.M. & Okano, A. (2000). Effect of oxidative stress on development and DNA damage in in-vitro cultured bovine embryos by comet assay. *Theriogenology* **54**, 137–45.

Tománek, M., Kopecny, V. & Kanka, J. (1989). Genome reactivation in developing early pig embryos: an ultrastructural and autoradiographic analysis. *Anat. Embryol*. **180**, 309–16.

Yang, H.W., Hwang, K.J., Kwon, H.C., Kim, H.S., Choi, K.W. & Oh, K.S. (1998). Detection of reactive oxygen species (ROS) and apoptosis in human fragmented embryos. *Hum. Reprod*. **13**, 998–1002.

Yoshioka, K., Suzuki, C., Tanaka, A., Anas, I.M.K. & Iwamura, S. (2002). Birth of piglets derived from porcine zygotes cultured in a chemically defined medium. *Biol. Reprod*. **66**, 112–9.

Yoshioka, K., Suzuki, C., Itoh, S., Kikuchi, K., Iwamura, S. & Rodriguez Martinez, H. (2003). Production of piglets derived from in vitro-produced blastocysts fertilized and cultured in chemically defined media: Effects of theophylline, adenosine and cysteine during in vitro fertilization. *Biol. Reprod*. **69**, 2092–9.