

nuclear maturation using the HD monoculture method, in the presence of H₂O₂-induced oxidative stress.

Materials and Methods

In vitro maturation of oocytes

Ovaries were collected from prepubertal gilts at a local slaughterhouse, transported to the laboratory in a container within 2 h of removal and placed in saline water warmed to 37°C. The follicular fluid and porcine oocytes were aspirated from antral follicles (diameter: 3–6 mm) with a 10-ml syringe attached to an 18-gauge needle. Compact cumulus–oocyte complexes (COCs) with uniform ooplasm were selected in phosphate-buffered saline (PBS; Nissui Pharmaceutical, Ueno, Tokyo, Japan) supplemented with 0.1% polyvinyl alcohol (PVA; Sigma Chemical, St Louis, MO, USA). After washing three times in 0.1% PBS–PVA, the COCs were cultured in 10 µl (10 µl of medium/1 COC) of NCSU-23 medium (Petters & Wells 1993) supplemented with 50 µM β-mercaptoethanol (Sigma), 0.6 mM cysteine (Sigma), 0.5% insulin (Gibco-BRL, Cergy Pontoise, France; Cat. 18125), 10% (v/v) porcine follicular fluid, 10 IU pregnant mare serum gonadotropin (PMSG; Serotropin; Teikokuzouki, Tokyo, Japan), 10 IU human chorionic gonadotropin (hCG; Puberogen; San-kyo, Tokyo, Japan) and 1 mM dibutyryl cyclic AMP (Sigma) for the first 22 h of maturation at 38.5°C in 5% CO₂ in air. Denuded oocytes (DOs) were prepared from all of the incubated COCs by removing cumulus cells by gentle vortexing in PB1 medium (Quinn et al. 1982) that contained 0.1% hyaluronidase (Sigma). DOs were then cultured for 22 h in medium without hormones and dbcAMP. Each droplet of medium was overlaid with liquid paraffin (Nacalai Tesque, Kyoto, Japan).

The hanging drop (HD) culture method

We used the HD method for the IVM experiments. In this method, 10 µl of culture medium was placed in each well of a 96-well plate (Thermo Fisher Scientific Inc., Waltham, MA, USA), and each droplet of medium was overlaid with liquid paraffin. After 1 COC or 1 DO was placed in medium, the plate was inverted (Fig. 1).

Examination of nuclear maturation of porcine oocytes

After 44 h of incubation, DOs were mounted on glass slides and then fixed in acetic acid/ethanol (1 : 3) for

48 h. The fixed oocytes were stained with 1% aceto-orcein and observed under a phase-contrast microscope to evaluate nuclear status.

Experimental design

Experiment 1: The effect of antioxidants on oocyte nuclear maturation in *in vitro* medium. After 22 h of pre-culture, DOs were cultured at two different concentrations (0.01 and 0.1 µg/ml) of LC (Wako Pure Chemical Industries, Osaka, Japan), LF (Wako) or SF (LKT Laboratories Inc., Paul, MN, USA) for 22 h. After 44-h incubation, all oocytes were fixed. Nuclear status was analysed, and the percentage of oocytes at MII stage for all cultured oocytes was calculated.

Experiment 2: The effect of antioxidants on oocyte nuclear maturation in *in vitro* medium with H₂O₂. After pre-culture, DOs were cultured with various concentrations of LC, LF or SF prepared using Milli-Q, at final determined concentrations (0.01 and 0.1 µg/ml) and 70 µM of H₂O₂ for 22 h. After 44 h of total incubation, all oocytes were fixed. Nuclear status was analysed, and the percentages of oocytes at MII stage for all cultured oocytes were calculated.

Statistical analysis

Each experiment was replicated at least three times. Data were presented as means ± standard error (SE). Statistical tests used were analysis of variance (ANOVA) and Fisher's (PLSD) test with Statview (Abacus Concepts Inc., Berkeley, CA, USA). *p* < 0.05 were considered to be significant.

Results

Effects of antioxidant addition to *in vitro* maturation medium without H₂O₂ on nuclear maturation

The results showed that the maturation rate in LC groups (0.01 µg/ml: 54.85 ± 6.25%, 0.1 µg/ml: 53.51% ± 4.02%) was not significantly different with that of the control (53.06 ± 3.13%). However, in LF at 0.1 µg/ml (66.46 ± 6.37%), a significant increase in nuclear maturation rate was observed when compared with the control. Furthermore, when the SF was added at all doses, the nuclear maturation rate was significantly lower than the control (0.01 µg/ml: 1.75 ± 3.04%, 0.1 µg/ml: 0.00 ± 0.00%; Fig. 2).

Effects of antioxidant addition to *in vitro* maturation medium on the nuclear maturation of H₂O₂-induced impaired oocytes

The results showed that the nuclear maturation rate of H₂O₂ (41.87 ± 2.30%) treatment group was significantly lower than that of the control (65.81 ± 3.92%). When LC or LF was added at 0.1 µg/ml in IVM medium supplemented with H₂O₂, the nuclear maturation rate was statistically significant against H₂O₂ supplementation (LC: 60.30 ± 11.99%, LF: 68.77 ± 3.90%). Furthermore, the nuclear maturation rate was statistically significant by 0.01 µg/ml LF addition, and



Fig. 1. Hanging drop (HD) method

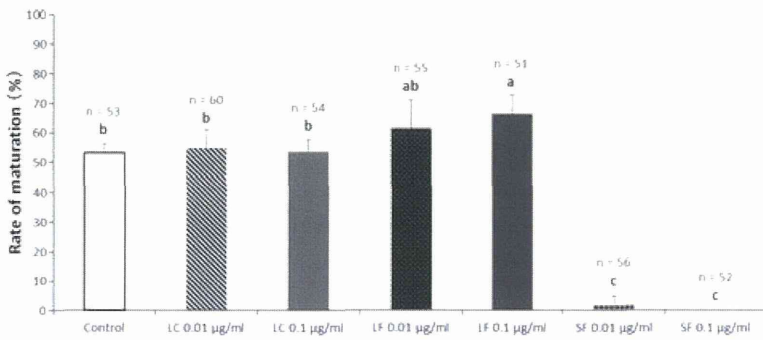


Fig. 2. Effects on nuclear maturation by antioxidant [L-carnitine (LC), lactoferrin (LF), sulforaphane (SF)] addition to *in vitro* maturation medium. Different letters indicate significant differences ($p < 0.05$) among the treatments. (n: total numbers of oocytes examined in each group)

this rate was equivalent to the control ($53.82 \pm 4.53\%$). Whereas when the SF was added at all doses, the nuclear maturation rate was significantly lower than H_2O_2 supplementation (0.01 µg/ml: $5.41 \pm 4.79\%$, 0.1 µg/ml: $2.38 \pm 4.12\%$; Fig. 3).

Discussion

It has previously been thought that the nuclear maturation rate is low in IVM by monoculture of oocytes as compared with coculture. In fact, Nishio et al. (2011) reported that the nuclear maturation rate using monoculture with CD is significantly lower than when using coculture with CD. Usually, in oocyte culture systems, more than one oocyte per culture medium drop has been used in IVM. However, in DO, the nuclear maturation rate using monoculture with HD was equivalent to coculture with CD ($65.81 \pm 3.92\%$ vs $51.62 \pm 1.93\%$) (Tao et al. 2010). Vanhoutte et al. (2009) and Xu et al. (2011) reported that this result depends on the HD method and it is possible to maintain three-dimensional structure by HD and culture in a state closer to the actual *in vivo* state. There are few reports of monoculture using HD in porcine oocyte IVM until now, and it is expected that obtaining stable nuclear maturation rate using the HD method in monoculture with porcine oocyte IVM is possible.

Additionally, this culture system derives high-throughput analysis efficiency. In this experiment, we could analyse the direct effect of antioxidants on single oocytes by monoculture using HD because this culture system can omit the effect of paracrine factors from other oocytes or cumulus cells and obtain the stable nuclear maturation rate in monoculture with porcine

oocyte IVM. In Experiment 1, we tried to improve the nuclear maturation rate by adding antioxidants to the normal IVM medium without adding H_2O_2 . Significant improvement in the nuclear maturation rate was not found for LC or SF. Only the addition of LF at a concentration of 0.1 µg/ml significantly improved the nuclear maturation rate. Our results also showed that the addition of LF was more effective than LC at the lower concentration because significant recovery was observed at 0.01 µg/ml.

In Experiment 2, we tried recovery of nuclear maturation by adding an antioxidant to the oocytes in the presence of H_2O_2 . The nuclear maturation rate significantly recovered when LC or LF was added at 0.1 µg/ml. While it has been reported that the significant concentration for recovery of the nuclear maturation rate was at 1.5–4.0 µg/ml using the CD method (Yazaki et al. 2013), the recovery was observed with a lower concentration using the HD method in Experiment 1. The nuclear maturation rate increased concentration dependently at 1.5–4.0 µg/ml in the report by Yazaki et al. (2013), namely it is expected that the nuclear maturation rate with CD is lower than with HD at 0.01–0.1 µg/ml. The addition of LF at a low concentration was the most effective in aiding recovery of nuclear maturation from damage caused by oxidative stress.

Furthermore, because the action mechanism of these three antioxidants is different, it was suggested that the previously mentioned action mechanism of LF is more important in porcine oocytes. Moreover, SF inhibited nuclear maturation in both Experiment 1 and Experiment 2, with more than 90% of the cultured porcine oocytes at germinal vesicle stage (data not shown).

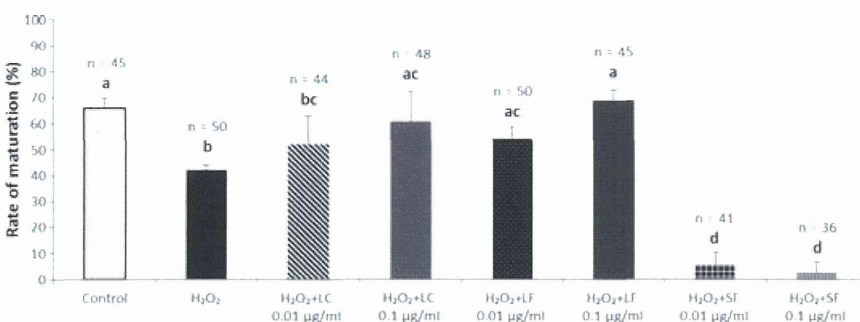


Fig. 3. Effects on nuclear maturation by antioxidant [L-carnitine (LC), lactoferrin (LF), sulforaphane (SF)] addition to *in vitro* maturation medium. Different letters indicate significant differences ($p < 0.05$) among the treatments. (n: total numbers of oocytes examined in each group)

From this, in porcine oocyte IVM, it is considered that SF inhibits nuclear maturation at a concentration of 0.01–0.1 µg/ml. Although the concentration of SF here was one-tenth the concentration that Lee et al. (2013), Ye et al. (2013), Ziaei et al. (2013) reported in other cells, this concentration was probably excessive for porcine oocytes. One possibility is that excess production of glutathione triggered toxic effects on oocytes. Although SF indirectly induces the production of glutathione (Fahey and Talalay 1999), glutathione protects oocytes from ROS, and this is evidenced by various reports, the optimal glutathione concentration has not been established. Because this is the first report investigating the effect of SF on oocytes, how SF affects the glutathione concentration is unclear. It is suggested that more specific investigation is needed.

Here, LC and LF were selected as effective antioxidants for nuclear maturation in the second half of IVM, and it was confirmed that SF, which is thought to be effective against oxidative stress, did not positively affect nuclear maturation of porcine oocytes. We

suggest that the effective antioxidant needs to be further investigated and confirm that the HD mono-culture system is useful for screening these factors in porcine oocyte IVM.

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Conflict of interest

None of the authors have any conflict of interest to declare.

Author contributions

Sadamasa Ishikawa performed all experiments. Ryosuke Machida, Kou Hiraga and Yoshihito Suda advised and supported the data analysis. Yuki Hiradate revised the manuscript. Kentaro Tanemura, who is the corresponding author, designed the experiments and drafted the manuscript.

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