Efficient production of embryos by in vitro fertilization and somatic cell nuclear transfer

体外受精および体細胞核移植胚の効率的生産

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ABSTRACT

Several mammalian species including ungulates have disappeared or are threatened by extinction. Much emphasis is currently given to the use of assisted reproductive techniques (ART) as a potential tool for saving endangered species as done in humans. However, the application of reproductive biotechnologies for endangered animals is not performed on a daily basis and the available gametes are markedly fewer in endangered animals. Therefore, development not only of ART according to individual animal species but also in ways that improve the quality of the gametes itself is necessary for the conservation of endangered animals using ART.

The first series of experiments was conducted to ascertain the effects of epigallocatechin gallate (EGCG), a polyphenol in green tea with antioxidant activity, during *in-vitro* fertilization (IVF) on the sperm quality and penetration ability into porcine oocytes. When frozen-thawed spermatozoa were incubated in IVF medium supplemented with 0 (control), 1, 50, and 100 μM EGCG for 1, 3, and 5 h, supplementation with 50 and 100 μM EGCG improved motility, but not viability, of the spermatozoa compared with controls. When frozen-thawed spermatozoa were co-incubated with *in vitro* matured (IVM) oocytes in IVF medium supplemented with 50 and 100 μM EGCG for 5 h, supplementation of EGCG showed positive effects on sperm penetration rates. When frozen-thawed spermatozoa from 6 boars were co-incubated with IVM oocytes in IVF medium supplemented with 50 μM EGCG, the effect of EGCG on sperm penetration and development of oocytes after fertilization was found to differ among individual boars. Our results indicate that the motility and penetration ability of boar spermatozoa are improved by co-incubation with 50 μM

EGCG, but the effects vary among individual boars.

The second series of experiments was conducted to evaluate the nuclear maturation of bovine oocytes that had been pre-cultured with various concentrations (0, 50, 100, and 200 μM) of roscovitine before IVM and to examine the development of somatic cell nuclear transfer (SCNT) embryos derived from oocytes pre-cultured with roscovitine. Before IVM, 72% of oocytes that had been cultured without roscovitine (control) reached the metaphase II (MII) stage, whereas culture with roscovitine decreased the rates of oocytes reaching MII (11%–27%). After IVM, the maturation rate of oocytes that had been pre-cultured with 200 μM roscovitine was significantly higher than that of control oocytes (79% vs. 58%). Moreover, significantly more oocytes extruded the first polar body in the 50-μM roscovitine group than in the control group (64% vs. 51%). The rate of blastocyst formation of the reconstructed embryos that derived from oocytes pre-cultured with 50 μM roscovitine was significantly higher than that from the control oocytes (14% vs. 6%). Results of this study indicate that the addition of roscovitine to culture medium delays the completion of meiotic maturation of bovine oocytes, and indicate that the cytoplasm derived from oocytes that have been pre-cultured under meiotic inhibition can support the development of SCNT embryos.

The third series of experiments was conducted to investigate the influence of recipient cytoplasm on the development of SCNT embryos, using recipient oocytes and donor cells obtained from cats, cows, and pigs. When cat cumulus cells were transferred into cow, pig, and cat oocytes, the percentages of fusion and cleavage in the cow-cat and pig-cat interspecies groups were similar to those in the cat-cat intraspecies group. No significant differences were found in the percentages of fusion and cleavage between the interspecies (cow-cat and pig-cat groups) and intraspecies SCNT (cow-cow and

pig-pig groups) embryos in each recipient oocyte species. However, no interspecies SCNT embryo developed to the morula and blastocyst stage. The percentages of fusion and cleavage in cow-cat SCNT embryos were significantly higher than those in pig-cat SCNT embryos. The results suggest that bovine and porcine cytoplasm are useful to support the early embryonic development of interspecies SCNT with a cat donor nucleus. However, interspecies SCNT embryos were unable to develop to the late embryonic stage.

Progress in ART for endangered animals is expected to contribute to halting of the reduction and extinction of endangered animals. Along with further development, earnest investments in basic science and in the reproductive biology of endangered animals are urgently needed. Moreover, experiments on individual species should be continued.

GENERAL INTRODUCTION

Extinction of mammalian species is an irreversible part of the natural process of evolution, but it is now occurring at a much higher rate than speciation because of human activities such as habitat destruction, overhunting, and competition with introduced herbivores (IUCN, World Conservation Union; http://www.iucnredlist.org/). The direct consequence of such a phenomenon is progressive contraction of biodiversity worldwide. Paradoxically, this problem does not involve wild species only, but also domestic ones because local or typical breeds are often being replaced by a limited commercial breeds with more productive genotypes. Preservation of their habitats is the most desirable strategy to prevent extinction of these species. However, these efforts are sometimes insufficient for the propagation of small populations and for maintaining adequate genetic diversity. Such conservation programs for endangered mammalian species might not be advantageous over modern reproductive biotechnologies or assisted reproductive techniques (ART) including artificial insemination (AI), embryo transfer (ET), *in vitro* fertilization (IVF), gamete/embryo micromanipulation, semen/embryo sexing, and genome resource banking (GRB). The development of ART in recent years has been dramatic, but it has not yet been investigated well in endangered animals' gametes. Because available gametes are markedly fewer in endangered animals, we must develop not only ART according to the individual animal species but also means that improve the quality of gametes themselves.

Based on these conceptions, we first specifically examined to improve spermatozoa quality. The manipulation of gametes and embryos in an *in vitro* environment when performing ART induces the risk of exposure of these cells to

supraphysiological levels of reactive oxygen species (ROS) (Guerin et al., 2001). ROS in *in vitro* culture medium might engender damages to proteins, lipids and nucleic acid components, resulting in mitochondrial alteration (Guerin et al., 2001), sperm axonemal protein phosphorylation (Aitken et al., 1993), 2-cell blocks of embryos (Nasr-Esfahani and Johnson, 1992) and reduced embryo development (Blondin et al., 1997; Watson et al., 1994). Addition of antioxidants such as glutamine and hypotaurine to *in vitro* culture medium is known to be able to reduce ROS and to increase developmental competence of porcine embryos. Epigallocatechin gallate (EGCG), the principal polyphenol in green tea (*Camellia sinensis*), is known to have higher levels of antioxidant activity than either vitamin C or vitamin E (Zhao et al., 1989). However, the literature describing the influence of EGCG supplementation during IVF on the quality and penetrability of boar spermatozoa is scarce. Therefore, in the first series of this study (Article 1), the effects of EGCG on the quality and penetrability of frozen-thawed boar spermatozoa and the developmental competence of oocytes fertilized with the spermatozoa were investigated.

When oocytes are collected from the ovaries, they must be subjected immediately to *in vitro* maturation (IVM) culture medium for maintenance of oocyte viability. If oocytes could be cultured without losing their viability for a few days before onset of IVM culture, then the temporal storage of oocytes at the germinal vesicle (GV) of the early meiotic stage before IVM culture might accommodate a more convenient schedule for subsequent oocyte manipulation. Roscovitine, a specific inhibitor of M-phase promoting factor, can maintain the GV stage of bovine oocytes for 24 h (Mermillod et al., 2000). Roscovitine has been reported as having less of a negative

effect on the developmental competence of bovine oocytes after IVF compared with other inhibitors such as cycloheximide and 6-dimethylminopurine (Mermillod et al., 2000). However, in my literature survey, the effect of roscovitine has not been investigated yet on the development of somatic cell nuclear transfer (SCNT) embryos derived from bovine oocytes that those examining IVF. Therefore, in the second series of this study (Article 2), the effects of various concentrations of roscovitine on the meiotic competence of oocytes pre-cultured for 24 h before IVM were examined.

When compared with spermatozoa, oocyte collection is more difficult because the reproductive stage must be considered. Moreover, obtaining the oocytes from a surgically cut ovary. Additionally, because the storage of immature or maturing oocytes remains unsatisfactory in almost all species to date, it might be difficult to prepare oocytes for subsequent IVF even if one were able to prepare the spermatozoa. In interspecies SCNT (iSCNT), donor cells are transplanted into a recipient enucleated oocyte of a different species/family/order/class obtained from accessible and abundant species (Loi et al., 2011). The resulting embryos are then transferred into the uterus of a suitable foster mother, normally the oocyte donor, for development of offspring. Successful development of iSCNT embryos to the blastocyst stage has been reported in cats (Thongphakdee et al., 2008), gaur (Mastromonaco et al., 2007), human (Chang et al., 2004), and cattle (Uhm et al., 2007) using rabbit, bovine, or pig oocytes as recipient cytoplasts. However, little information is available about the use of bovine and porcine oocyte cytoplasm for interspecies SCNT in feline species. Therefore, in the third of the series of these reports (Article 3), the embryonic developmental competence of iSCNT embryos derived from the domestic cat (*Felis catus*) into oocyte cytoplasm obtained

from cows (*Bos taurus*) and pigs (*Sus scrofa*) was examined to investigate the effect of recipient cytoplasm on the development of iSCNT embryos.

Article 1

EFFECTS OF (-)-EPIGALLOCATECHIN GALLATE ON THE MOTILITY AND PENETRABILITY OF FROZEN-THAWED BOAR SPERMATOZOA INCUBATED IN THE FERTILIZATION MEDIUM

ABSTRACT

Epigallocatechin gallate (EGCG) is the major polyphenol in green tea (*Camellia sinensis*) and is known for its antioxidant effects. The objective of the present study was to examine the effects of EGCG during *in vitro* fertilization (IVF) on the sperm quality and penetrability into oocytes. In the first experiment, the effects of concentration and incubation period of EGCG on the motility and penetrability of boar spermatozoa were examined. When frozen-thawed spermatozoa were incubated in IVF medium supplemented with 0 (control), 1, 50, and 100 μM EGCG for 1, 3, and 5 h, supplementation with 50 and 100 μM EGCG improved motility of the spermatozoa ($p < 0.05$), but not viability, as compared with the control group. When the spermatozoa were co-incubated with *in vitro*-matured (IVM) oocytes in IVF medium supplemented with 50 and 100 μM EGCG for 5 h, it showed that supplementation of EGCG had positive effects on sperm penetration rates. In the second experiment, the effects of supplementation of EGCG in IVF medium on penetrability of sperm from different boars and developmental competence of the fertilized oocytes were evaluated. When frozen-thawed spermatozoa from 6 boars were co-incubated with IVM oocytes in IVF medium supplemented with 50 μM EGCG, the effect of EGCG on sperm penetration and development of oocytes after fertilization was found to differ within individual boars. Our results indicate that motility and penetration ability of boar spermatozoa are improved by co-incubation with 50 μM EGCG, but the effects vary depending on the boar.

INTRODUCTION

When oocytes are fertilized *in vitro* with spermatozoa, they are necessarily exposed to light and higher concentrations of oxygen than those fertilized *in vivo*. This can lead to increased production of reactive oxygen species (ROS) such as superoxide anions (O_2^-), hydroxyl radicals (. OH), and hydrogen peroxide (H_2O_2) (Ho et al., 1996). Many *in vitro* studies have shown negative effects of ROS on cellular functions, such as damage to proteins, lipids, and nucleic acid components, resulting in mitochondrial alterations (Guerin et al., 2001), sperm axonemal protein phosphorylation (Aitken et al., 1993), 2-cell block of embryos (Nasr-Esfahani and Johnson, 1992), and reduced embryo development (Blondin et al., 1997; Watson et al., 1994). ROS are also one of the reasons for developmental block of porcine embryos cultured in vitro. Suzuki et al. (Suzuki et al., 2007) reported that addition of glutamine and hypotaurine to in vitro culture medium can reduce intercellular H_2O_2 and increase developmental competence of porcine embryos. Antioxidants such as beta-mercaptoethanol and vitamin E are known to have similar effects (Kitagawa et al., 2004).

Green tea (*Camellia sinensis*) contains many types of catechins and polyphenolic compounds. These compounds include (-)-epicatechin (EC), (-)-epigallocatechin (EGC), (-)-epicatechin gallate (ECG), and (-)-epigallocatechin gallate (EGCG). The difference among these catechins is that EGCG has both pyrogallol and gallate-ester moieties at the 2- and 3-positions of its ring, whereas EGC and ECG have only the pyrogallol moiety and the gallate-ester moiety (Baek et al., 2005). EGCG accounts for more than 65% of the total catechin content and more than 10% of the extract dry weight of green tea (Goto et al., 1996). Many in vivo and in vitro

studies have shown that catechin and EGCG have beneficial effects, such as an anti-carcinogenic effect (Ahmad et al., 1997), prevention of spontaneous mutations (Mure and Rossman, 2001), reduction of chromosomal damage caused by ROS (Roy et al., 2003; Sugisawa and Umegaki, 2002), inhibition of lipogenesis (Miura et al., 2000), suppression of diet-induced obesity (Murase et al., 2002), and antimicrobial activities (Gordon and Wareham, 2010). Catechins also have a remarkable antioxidative effect (Dufresne and Farnworth, 2001) and can inhibit the effect of ROS superoxide, hydrogen peroxide, hydroxyl radicals, and nitric oxide produced by various chemicals (Schroeder et al., 2003). Green tea catechins are reported to have a higher level of antioxidant activity than that of vitamins C and E (Zhao et al., 1989). However, there is little information on the effect of EGCG, a major polyphenolic compound found in green tea, on the quality and penetrability of boar sperm.

The objective of the present study was to examine the effects of EGCG on the quality and penetrability of frozen-thawed boar spermatozoa and the developmental competence of oocytes fertilized with the spermatozoa.

MATERIAL AND METHODS

Preparation of porcine spermatozoa

Frozen-thawed semen was used in this study. Sperm-rich fractions of ejaculates were obtained from 6 Large White boars and frozen according to the method described by Yuge et al. (Yuge et al., 2003) with minor modifications. Briefly, a suspension of spermatozoa in a 50-mL glass tube was placed in a refrigerator at 15°C for 3 h and then centrifuged at 800 × *g* for 10 min. Precipitated spermatozoa were diluted with the first extender, designated as the NSF-I extender, which consisted of 8.8% (w/v) lactose (Wako Pure Chemical Industries, Osaka, Japan), 200 μg/mL ampicillin (Kyoritsu Seiyaku, Tokyo, Japan), and 20% (v/v) egg yolk in distilled water. The diluted spermatozoa were equilibrated in a water bath at 4°C for 2 h. After equilibration for 2 h, the second extender (NSF-II; NSF-I extender supplemented with 6% [v/v] glycerol and 1.48% [v/v] EQUEX STM [Miyazaki Kagaku, Tokyo, Japan]) was added with the half of NSF-I extender. The spermatozoa were then equilibrated at 4°C for an additional 5 min. At the end of the equilibration period, the same volume of NSF-II extender was added at 4°C. The sperm concentration was adjusted to 2×10^8 cells/mL. The spermatozoa were immediately loaded into 0.25-mL French straws (no. AAA201; IMV, L'Aigle, France). The spermatozoa were frozen by placing the straw (4 cm in height from the surface of liquid nitrogen) on a styrofoam plate in liquid nitrogen vapour for 20 min and subsequently storing it in liquid nitrogen. On the day of examination, the straw was immediately submerged into a 30°C water bath for 30 s for thawing.

Oocyte collection and in vitro maturation

Porcine ovaries were obtained from a slaughterhouse and transported to the laboratory in 0.9% physiological saline at 35°C within 3 h of slaughter. Cumulus-oocyte complexes (COCs) were aspirated from follicles (diameter, 3–6 mm) by using an 18-gauge needle and then transferred into a disposable 5-mL syringe. They were collected in modified phosphate-buffered saline (m-PBS; Nihon Zenyaku, Fukushima, Japan) supplemented with 100 IU/mL penicillin G potassium (Meiji, Tokyo, Japan) and 0.1 mg/mL streptomycin sulphate (Meiji). Only COCs with uniform ooplasm and compact cumulus cells were used in this experiment. The in vitro maturation (IVM) of oocytes was carried out according to the method described by Kikuchi et al. (Kikuchi et al., 2002). In brief, COCs were cultured for 22 h in a maturation medium, a modified North Carolina State University (NCSU)-37 solution supplemented with 10% (v/v) porcine follicular fluid, 0.6 mM cysteine (Sigma-Aldrich, St. Louis, MO, USA), 1 mM dibutyryl cyclic AMP (dbcAMP; Sigma-Aldrich), 50 μM β-mercaptoethanol (Wako Pure Chemical Industries), 10 IU/mL equine chorionic gonadotropin (Kyoritsu Seiyaku), 10 IU/mL human chorionic gonadotropin (Kyoritsu Seiyaku), and 50 μg/mL gentamicin (Sigma-Aldrich). They were subsequently cultured in NCSU-37 without dbcAMP and hormones for 22 h. All cultures were performed in a 38.5°C humidified incubator containing 5% CO_2.

In vitro fertilization and embryo culture

In vitro fertilization (IVF) was carried out according to the method described by Kikuchi et al. (Kikuchi et al., 2002) with minor modifications. Spermatozoa were thawed and pre-incubated for 15 min at 38.5°C in tissue culture medium 199 with Earle's salts (Invitrogen, Carlsbad, CA, USA) adjusted to pH 7.8. A portion (10 μL) of

pre-incubated spermatozoa was introduced into 90 μL of fertilization medium containing 10–20 matured oocytes. The fertilization medium consisted of 90 mM NaCl, 12 mM KCl, 25 mM $NaHCO_3$, 0.5 mM NaH_2PO_4, 0.5 mM $MgSO_4$, 10 mM sodium lactate, 3 mg/mL bovine serum albumin (BSA; fatty acid-free, Sigma-Aldrich), 5 mM caffeine (Sigma-Aldrich), and 50 μg/mL gentamicin (Sigma-Aldrich). The final sperm concentration was adjusted to 1×10^6/mL. The oocytes were co-incubated with spermatozoa for 5 h. The inseminated oocytes were then denuded from the cumulus cells and attached spermatozoa by mechanical pipetting, and subsequently transferred to a culture medium. Putative zygotes were cultured in NCSU-37 solution (Petters and Wells, 1993) without glucose but supplemented with 4 mg/mL BSA, 0.17 mM sodium pyruvate, 2.73 mM sodium lactate, 50 μM β-mercaptoethanol, and 50 μg/mL gentamicin. At 72 h after insemination, all embryos were transferred into fresh culture medium: original NCSU-37 containing 5.55 mM D-glucose and supplemented with 4 mg/mL BSA, 50 μM β-mercaptoethanol, and 50 μg/mL gentamicin. The embryos were cultured for an additional 5 days to evaluate their ability to develop to the blastocyst stage.

Assessment of sperm motility and viability

After the incubation in the fertilization medium, each sample was placed on a warm glass chamber for assessment of motility. Briefly, a portion (approximately 10 μL) of the sperm suspension was transferred to the warm chamber (MUR-500; Matsunami, Osaka, Japan) and placed on a warm plate at 38.5°C. The motility of spermatozoa was immediately examined under a phase contrast microscope. Sperm motility was expressed as a motility index that was calculated according to the method

described by Okamura et al. (Okamura et al., 1985) with minor modifications:

$$\text{Sperm motility index} = \frac{100\,w + 75\,x + 50\,y + 25\,z}{100}$$

wherein w is the percentage of spermatozoa with rapid progressive motion to all spermatozoa in the assay mixture; x is the percentage of spermatozoa with slow progressive motion; y is the percentage of rotative or non-progressive motion; and z is the percentage of faint or pendulum-like motile spermatozoa.

Sperm viability was assessed by a live/dead stain combination (SYBR-14/propidium iodide [PI], Fertilight Kit; Molecular Probes, Eugene, OR, USA) with minor modification (Blanco et al., 2000). Briefly, an aliquot (5 μL) of semen was mixed with a 50-μL solution containing 5 μL PI (diluted 1:100 in distilled water) and 1 μL SYBR-14 (diluted 1:100 in DMSO). Assessments of 100 spermatozoa were made in duplicate aliquots for every sample and evaluated under a fluorescent microscope (400×) (Optiphot-2; Nikon, Tokyo, Japan) with 480-nm wavelength excitation filters.

Assessment of fertilization and embryo development

The presumptive zygotes were mounted on a glass slide 16 h after IVF and fixed with acetic acid:ethanol (1:3 v/v) for 48–72 h. The fixed zygotes were stained with acetic orcein (1% orcein in 45% acetic acid) and examined under a phase contrast microscope. Oocytes containing both female and male pronuclei were considered as fertilized and categorized as normal or polyspermic according to the number of swollen sperm heads and male pronuclei in the cytoplasm.

To evaluate their ability to develop to the cleavage and blastocyst stages, on day 8 (day 0 = insemination), all embryos were fixed and permeabilized for 15 min at

room temperature in PBS containing 3.7% (w/v) paraformaldehyde and 1% (v/v) Triton X-100 (Sigma-Aldrich) and then placed in PBS containing 0.3% (w/v) polyvinylpyrrolidone for 15 min at room temperature. Embryos were then placed in a drop of mounting medium on a slide. The mounting medium consisted of 90% (v/v) glycerol containing 1.9 μM Hoechst 33342 (Sigma-Aldrich). The embryos were subsequently overlaid with a cover slip supported by 4 droplets of vaseline/paraffin and incubated for 1 night at 4°C. The embryos were examined under a fluorescence microscope with a 355-nm wavelength excitation filter. Embryos with a clear blastocoele or with more than 32 cells were defined as blastocysts. The numbers of cleaved embryos and blastocysts were recorded.

Experimental design

Experiment 1

Effect of EGCG concentration on sperm quality

To examine the effects of the concentration of EGCG (E4143, Sigma-Aldrich) on the motility and viability of spermatozoa, frozen-thawed spermatozoa from a Large White boar (age, 1.5 years) were incubated in fertilization medium supplemented with 0 (control), 1, 50, and 100 μM of EGCG for 1, 3, and 5 h at 38.5°C in a humidified atmosphere of 5% CO_2 in air. After each incubation, the motility and viability of spermatozoa were examined as described above.

Effect of EGCG concentration on sperm penetrability

The effect of EGCG concentration on the penetrability of porcine spermatozoa

was evaluated. After 44 h of maturation culture, the COCs were co-incubated with the spermatozoa in fertilization medium supplemented with 0 (control), 1, 50, and 100 μM of EGCG for 5 h. The oocytes were fixed 16 h after the initiation of co-incubation and stained to evaluate fertilization as described above.

Experiment 2

Effect of EGCG on quality of spermatozoa from different boars

To examine whether the effect of EGCG on sperm quality differs among individual boar sperm, we analysed both the motility and viability of frozen-thawed spermatozoa from 6 Large White boars (ages, 1–2 years), including a boar (boar B) used in Experiment 1. In Experiment 1, 50 μM of EGCG showed the highest effect on the quality and penetrability of spermatozoa, and the effects of EGCG on both the motility and viability of spermatozoa were observed until 3 h of incubation. In this experiment, therefore, frozen-thawed spermatozoa were incubated for 3 h in fertilization medium supplemented with 50 μM EGCG.

Effect of EGCG on sperm penetrability and embryo development after IVF using spermatozoa from different boars

To examine whether the effect of EGCG on sperm penetrability and embryodevelopment after IVF differs among individual boar sperm, the COCs were co-incubated with 6 different boar spermatozoa in fertilization medium supplemented with 50 μM EGCG for 5 h. After co-incubation, some oocytes were fixed and stained to assess the fertilization as described above. The remaining oocytes were cultured for 8 days, fixed, and stained to evaluate their ability to develop to the cleavage and

blastocyst stages as described above.

Statistical analysis

Data are expressed as mean ± SEM. All percentage data and sperm motility indices were subjected to arcsine transformation prior to analysis of variance (ANOVA) for repeated measures. In Experiment 1, the transformed data were tested by ANOVA with followed by Scheffe's contrasts using the StatView program (Abacus Concepts, Inc., Berkeley, CA, USA). In Experiment 2, all percentage data and sperm motility indices were analyzed by ANOVA using the general linear models (GLM) procedure of SAS (SAS for Windows, version 9.1, SAS Institute Japan, Tokyo, Japan). The statistical model included the type of boars, treatments, and the two-way interactions. When significant interactions were not observed between boars and treatments, they were excluded from the model. Probability values (p) of 0.05 or less were considered to be significant.

RESULTS

Experiment 1

Effect of EGCG concentration on sperm quality

The motility of frozen-thawed spermatozoa increased with an increased EGCG concentration irrespective of the incubation time (Fig. 1A). The sperm motility index of the spermatozoa co-incubated with 100 μM EGCG (2.55 ± 0.47) were significantly higher ($p < 0.05$) than that of the control group without EGCG (0.85 ± 0.29), even after 5 h of incubation. The survival rate of the spermatozoa co-incubated with 100 μM EGCG significantly decreased, even after 1 h of incubation, when compared with the control group (10.8% ± 1.1% vs. 16.4% ± 1.2%, respectively) (Fig. 1B). However, there were no significant differences in the survival rates among the groups after 5 h of incubation.

Effect of EGCG concentration on sperm penetrability

The penetration rates of spermatozoa co-incubated with 50 and 100 μM EGCG (50.0% ± 2.7%, and 46.7% ± 4.4%, respectively) were significantly higher ($p < 0.05$) than those of the control group (21.3% ± 2.0%) (Fig. 2A). There were no significant effects of EGCG on the monospermic fertilization rates, whereas the polyspermic fertilization rate of spermatozoa co-incubated with 100 μM EGCG increased when compared with the control group (Fig. 2B and C).

Experiment 2

Effect of EGCG on quality of spermatozoa from different boars

The motility of spermatozoa from 6 Large White boars was evaluated. The sperm motility index of boar B,was significantly higher ($p < 0.05$) when the sperm were co-incubated with 50 μM EGCG than when they were not co-incubated with EGCG (5.53 ± 0.38 vs. 2.85 ± 0.21). However, in the other boars, there were no significant effects of EGCG on sperm motility. There were no effects of EGCG on the survival rate of the spermatozoa, irrespective of the boar (Fig. 3A and B).

Effect of EGCG on sperm penetration ability and embryo development after IVF using spermatozoa from different boars

When evaluating the rates of sperm penetration, a significant boar x treatment interaction was observed ($p < 0.01$) after analysis by ANOVA. Co-incubation with 50 μM EGCG significantly increased the rates of sperm penetration in boars A, B, E, and F, but there were no apparent effect of EGCG on sperm penetration in the other 2 boars (boar C and D) (Fig. 4A). No significant boar x treatment interactions were observed between the rates of monospermic and polyspermic fertilization, cleavage, and blastocyst formation. There were no significant effects of EGCG on the rates of monospermic and polyspermic fertilization except for the monospermic fertilization rate in boar E (Fig. 4B and C). There were no significant effects of EGCG on the rates of cleavage, irrespective of the boar (Fig. 5A). Co-incubation with EGCG significantly increased the rates of blastocyst formation in boars D and E, but there were no apparent effects of EGCG on blastocyst formation in the other 4 boars (Fig. 5 B).

DISCUSSION

It has been demonstrated that addition of antioxidants to the in vitro culture medium for porcine embryos can improve the developmental competence of the embryo (Kitagawa et al., 2004; Suzuki et al., 2007). However, there is little information on the effect of EGCG supplementation during IVF on sperm quality and developmental competence of porcine oocytes after IVF. In this study, the motility of spermatozoa increased as the EGCG concentrations increased, irrespective of the incubation time. Mammalian spermatozoa largely expend energy for motility by generation of intracellular ATP (Storey, 2008). During the process of ATP production in mitochondria, ROS, especially superoxide anions (O_2^-), are formed. ROS are known to exert physical and chemical damage on sperm motility (Baumber et al., 2000). Chen et al. (Chen et al., 2003) reported that tea catechins significantly increased cell viability, decreased intracellular Ca^{2+} levels and ROS formation, and improved mitochondrial membrane potential in cells. EGCG is the most abundant catechin in tea and has the maximum antioxidative potency among catechins (Frei and Higdon, 2003). Therefore, our results indicate that EGCG might protect spermatozoa from oxidative stress and improve ATP production, leading to improvement in sperm motility. However, when the spermatozoa were incubated with 100 μM EGCG for 1 h, the survival rate significantly decreased as compared with that of spermatozoa incubated without EGCG. EGCG reportedly has 2 different actions: an antioxidant action at lower concentrations and a pro-oxidant action at higher concentrations (Chen et al., 2003; Kusakabe and Kamiguchi, 2004). EGCG can protect cell membranes from lipid peroxidation (Chen et al., 2003), whereas it acts as a pro-oxidant and damages the DNA and membranes of

sperm (Kusakabe and Kamiguchi, 2004). Therefore, a high concentration of EGCG supplementation may change the plasma membrane integrity and may cause a decrease in sperm viability.

It has been suggested that sperm motility is related to the ability of sperm cells to penetrate oocytes (Kikuchi et al., 1998). In the first experiment, we observed that the motility of frozen-thawed spermatozoa increased as the EGCG concentration increased. Moreover, the penetration rates of spermatozoa co-incubated with 50 and 100 μM EGCG increased as compared with that of the control group. These results indicate that the increase in sperm motility by the addition of EGCG resulted in an increase in sperm penetration into oocytes. However, the addition of 100 μM EGCG had negative effects on the rates of polyspermic fertilization. The increase in sperm penetration by the addition of 100 μM EGCG seems to be due in part to the increase in polyspermic fertilization. In this group, porcine oocytes were also exposed to high concentrations of EGCG during IVF. It has been demonstrated that EGCG at low concentrations has no negative effects on oocyte maturation and fertilization (Spinaci et al., 2008). However, EGCG at high concentrations induces chromosomal damage of cells and reduces cell-cycle progression (Sugisawa and Umegaki, 2002; Tanaka, 2000), indicating that EGCG might induce oocyte damage. Therefore, EGCG at high concentrations might influence both spermatozoa and oocytes, resulting in an increase in the polyspermic fertilization rate.

In this study, co-incubation with 50 μM EGCG had a positive effect on spermatozoa from boar B, showing an increase in the motility index. Moreover, EGCG supplementation increased the rates of sperm penetration in the 4 boars, but the effects of EGCG on the motility and penetration of sperm were dependent on individual boars.

It is widely accepted that penetration and polyspermy rates in pig IVF are affected by large variations among individual males (Wang et al., 1991). These considerable differences among boars suggest that all males do not equally respond to IVF conditions with EGCG. In other words, EGCG may be an effective supplement for improvement in the motility and penetration of spermatozoa for one boar, but not for another. On the other hand, co-incubation with EGCG improved blastocyst formation in 2 boars, but apparent effects of EGCG were not found in the other 4 boars. Even in boar B, there were no differences in the rates of cleavage and blastocyst formation between co-incubation with and without EGCG. Our results indicate that EGCG supplementation increases the motility and penetration of spermatozoa, but does not improve the blastocyst formation after fertilization.

In conclusion, our results indicate that addition of 50 μM EGCG improves the motility and penetrability of boar spermatozoa, but the effects of EGCG varied in individual boars. Moreover, co-incubation with EGCG during IVF has no apparent effect on the development of porcine oocytes.

Article 2

EFFECTS OF ROSCOVITINE PRETREATMENT ON THE MEIOTIC MATURATION OF BOVINE OOCYTES AND THEIR SUBSEQUENT DEVELOPMENT AFTER SOMATIC CELL NUCLEAR TRANSFER

ABSTRACT

Roscovitine, a specific inhibitor of kinase activity of M-phase promoting factor, was used to inhibit the completion of meiotic maturation of bovine oocytes. The objectives of this study were to evaluate the nuclear maturation of bovine oocytes pre-cultured with various concentrations (0, 50, 100, and 200 μM) of roscovitine before in vitro maturation (IVM) and to examine the development of somatic cell nuclear transfer (SCNT) embryos derived from the oocytes pre-cultured with roscovitine. Before IVM, 72% of oocytes that were cultured without roscovitine (control) had reached the metaphase II (MII) stage, whereas culture with a variety of roscovitine concentrations decreased the rates of oocytes reaching the MII stage (11%–27%). After IVM, the maturation rate of oocytes pre-cultured with 200 μM roscovitine was significantly higher than that of control oocytes (79% vs. 58%). Moreover, significantly more oocytes extruded the first polar body in the 50-μM roscovitine group than in the control group (64% vs. 51%). The rate of blastocyst formation of reconstructed embryos derived from oocytes pre-cultured with 50 μM roscovitine was significantly higher than that from the control oocytes (14% vs. 6%). In conclusion, the addition of roscovitine to maturation medium delays the completion of meiotic maturation of bovine oocytes, and the cytoplasm derived from oocytes pre-cultured under meiotic inhibition can support the development of SCNT embryos.

INTRODUCTION

Since the cloned sheep "Dolly", live births in other species including mouse, cattle, goat, pig, rabbit, cat, horse and rat have been produced by somatic cell nuclear transfer (SCNT) techniques. SCNT techniques are valuable for wild animal conservation and production of transgenic animals. In general, bovine ovaries are collected at the slaughter house and brought to the laboratory. The oocytes are collected from the ovarian follicles and used for experiments. In the cloning technique, the matured oocytes with the first polar body are enucleated and used as a recipient oocyte for reconstructing embryos with donor cells. If oocytes could be cultured without reducing their viability for 1-2 days before the onset of in vitro maturation (IVM) culture, the temporal storage of oocytes at the germinal vesicle (GV) or an early meiotic stage before IVM culture may allow a more convenient schedule for subsequent oocyte manipulations. However, in the present situation in many laboratories, bovine oocytes must be collected immediately after transportation of ovaries to the laboratory and subjected to IVM culture for maintenance of the viability of oocytes.

Roscovitine is a purine analog that blocks M-phase promoting factor (MPF) activation, and can maintain the GV stage of bovine oocytes for 24 h (Mermillod *et al.*, 2000). It has been shown that early embryonic development is not compromised even when oocytes are pre-cultured with roscovitine for 24 h before IVM and in vitro fertilization (IVF) (Mermillod *et al.*, 2000). Moreover, roscovitine treatment before IVM culture does not affect the establishment of pregnancy (Kasinathan *et al.*, 2001) or fetal development (Ponderato *et al.*, 2002), and subsequent birth of live animals (Coy *et al.*, 2005). In other studies, similar approaches have already provided results of culture

of bovine oocytes maintained at the GV stage for 24 h by inhibition of protein synthesis with cycloheximide or of phosphorylation with 6-dimethylaminopurine (Avery et al., 1998; Lonergan et al., 1997). However, roscovitine has been suggested to have less detrimental effects on the developmental competence of bovine oocytes after IVF compared with other inhibitors such as cycloheximide or 6-dimethylaminopurine (Mermillod *et al.*, 2000). Because the effect of roscovitine has been limited to be reported on IVF of bovine oocytes, less information is available concerning SCNT for bovine oocytes. The understanding of the inhibitory events involved in meiotic resumption of oocytes would offer meaningful information on the efficiency of SCNT procedures.

In the present study, we examined the effects of various concentrations of roscovitine on the meiotic competence of oocytes pre-cultured for 24 h before IVM. Moreover, we investigated the developmental competences of SCNT embryos derived from oocytes pre-cultured with various concentrations of roscovitine.

MATERIAL AND METHODS

Oocyte recovery and in vitro maturation

Bovine ovaries were obtained from a slaughterhouse and transported to the laboratory in 0.9% (w/v) physiological saline at 35°C within 3 h of slaughter. Cumulus-oocyte complexes (COCs) were aspirated from follicles (2–5mm in diameter) using a 5-mL syringe fitted with an 18-gauge needle. They were collected in modified phosphate-buffered saline (m-PBS; Nihonzenyaku, Fukushima, Japan) supplemented with 100 IU/mL penicillin G potassium (Meiji, Tokyo, Japan) and 0.1 mg/mL streptomycin sulfate (Meiji). Only COCs with uniform ooplasm and compact cumulus cells were used in this experiment. To examine the effects of roscovitine on the maintenance of meiotic competence of COCs, the collected oocytes were incubated in tissue culture medium (TCM) 199 with Earle's salts (Invitrogen, Carlsbad, CA, USA) supplemented with 0 (control), 50, 100, and 200 μM of roscovitine (Sigma, St. Louis, MO, USA), 10% (v/v) fetal bovine serum (FBS; Invitrogen), 2 mM L-glutamine (Sigma), 0.1 mM β-mercaptoethanol (Wako Pure Chemical Industries, Osaka, Japan), and 50 μg/mL gentamicin (Sigma) for 24 h under humidified 5% CO_2 in air at 38.5°C. After 24 h of incubation, the COCs were washed twice in maturation medium that consisted of TCM 199 supplemented with 0.02 AU/mL FSH (Kawasaki Mitaka K.K., Kawasaki, Japan), 5% FBS and 50 μg/mL gentamicin, and then cultured for 21 h at 38.5°C in humidified 5% CO_2 in air.

Assessment of oocyte nuclear status

The meiotic stage of oocytes was evaluated before and after maturation culture,

in which the COCs had been incubated in medium with each concentration of roscovitine. The oocytes were completely denuded in TCM199 supplemented with 0.1% (w/v) hyaluronidase (Sigma). Irrespective of the morphology of the oocytes, all oocytes were fixed, and permeabilized for 15 min at room temperature in Dulbecco's phosphate-buffered saline (PBS; Invitrogen) supplemented with 3.7% (w/v) paraformaldehyde and 1% (v/v) Triton-X100 (Sigma), and then placed in PBS containing 0.3% (w/v) polyvinylpyrrolidone for 15 min at room temperature. The oocytes were transferred into the small drop comprising PBS supplemented with 90% (v/v) glycerol (Wako Pure Chemical Industries) and 1.9 μM bis-benzimide (Hoechst 33342; Sigma) on a glass slide. Subsequently, the oocytes were overlaid with a coverslip supported by 4 droplets of vaseline/paraffin and incubated for one night at 4°C. The oocytes were examined using a fluorescence microscope with a 355 nm wavelength excitation filter. The meiotic stage of IVM oocytes was classified according to chromatin configuration as "germinal vesicle", "condensed chromatin", "metaphase I", or "metaphase II". Those with diffusely stained cytoplasm characteristic of non-viable cells, and those in which chromatin was unidentifiable or not visible, were considered to be degenerated.

Nuclear transfer

Cumulus cells, which were the source of donor nuclei, were removed from the COCs by vortexing, and were cultured in α-MEM (Invitrogen) supplemented with 10% FBS, 1% MEM non-essential amino acid solution (Invitrogen) and 50 μg/mL gentamicin for 7 days at 37.0°C under 5% CO_2 in air. Once the cumulus cells reached complete confluence, the cell monolayer was washed twice with α-MEM and then

incubated in 0.25% (w/v) trypsin-EDTA (Sigma) for 3 min at 37.0°C. After trypsinization, 3 mL of washing medium (α-MEM supplemented with 10% FBS) was added to neutralize trypsin activity. The cells were pelleted by centrifugation at 500 x *g* for 5 min, resuspended in the washing medium, and then remained in this medium until SCNT manipulation.

At 21 h after initiation of IVM, COCs were mechanically denuded in TCM 199 medium containing 0.1% (w/v) hyaluronidase (Sigma). Oocytes were separated according to the first polar body extrusion and then the number of oocytes with the first polar body was recorded. Prior to enucleation, oocytes were incubated for 10 min in 3 μg/mL Hoechst 33342 (Sigma) diluted in manipulation medium (PBS supplemented with 5% FBS and 5 μg/mL cytochalasin B [Sigma]). Oocytes were then washed, transferred to a drop of manipulation medium and enucleated at room temperature after minimal exposure to low-light filtered fluorescence. Oocytes were secured with a holding pipette and rotated as needed into a position suitable for enucleation. The zona pellucida above the first polar body was cut with a glass needle. A small volume (about 5%-10%) of cytoplasm underneath the first polar body was squeezed out. Complete enucleation was confirmed by the existence of metaphase plate after staining the squeezed out cytoplasm with Hoechst 33342. After enucleation of each individual oocyte, the donor cell (diameter: 14–16 μm) was then placed into the perivitelline space, adjacent to the plasma membrane of the oocyte. Karyoplast/oocyte couplets were transferred to modified synthetic oviduct fluid (mSOF) medium (Kwun *et al.*, 2003) supplemented with 0.4% (w/v) bovine serum albumin (BSA) and 50 μg/mL gentamicin (mSOF/BSA) and incubated for at least 30 min prior to fusion and activation.

The couplets were equilibrated for 3 min in the Zimmerman cell fusion

medium (Wolfe and Kraemer, 1992), transferred into a drop of Zimmerman cell fusion medium, and then manually aligned between the two electrode needles connected to the micromanipulator (MO-202D; Narishige, Tokyo, Japan). A single simultaneous fusion and activation by an electrical pulse of 2.3 kV/cm for 30 μsec was applied to the couplets using an electro cell fusion (LF101, Nepagene, Chiba, Japan). After fusion, the couplets were incubated in mSOF/BSA for 20 min before chemical activation. The successfully fused couplets were activated in mSOF/BSA supplemented with 10 μg/mL cycloheximide (Sigma) and incubated for up to 5 h. Following cycloheximide treatment, the couplets were washed, transferred into mSOF/BSA medium, and then cultured for 72 h at 38.5°C in humidified 5% CO_2 and 5% O_2. After 72 h of culture, only cleaved embryos were further co-cultured with bovine cumulus cells in mSOF supplemented with 5% FBS at 38.5°C in humidified 5% CO_2 for an additional 5 days to evaluate their ability to develop to the blastocyst stage.

Statistical analysis

Five to eight replicate trials were carried out. Data are expressed as mean ± SEM. The percentages of oocytes reaching each stage of meiosis before and after IVM, embryos cleaved, and embryos developed to the blastocyst stage were subjected to arcsin transformation prior to the analysis of variance (ANOVA). The transformed data were tested by ANOVA followed by a post hoc Fisher's protected least significant difference (PLSD) test using the Statview program (Abacus Concepts, Inc., Berkeley, CA). Probability values (P) of 0.05 or less were considered to be significant.

RESULTS

Meiotic status of oocytes before and after in vitro maturation

Before IVM culture (Fig.6A), significantly more oocytes that were pre-cultured without roscovitine had reached the MII stage (72.4%) as compared with other oocytes pre-cultured with roscovitine (10.7%–26.9%) ($P<0.05$). After IVM culture (Fig.6B), when the oocytes were pre-cultured with 200 μM roscovitine, the maturation rate of oocytes was significantly higher ($P<0.05$) than that of oocytes pre-cultured without roscovitine (79.4% vs. 57.9%). However, no significant differences in the rates of oocytes reaching the MII stage were observed among the three groups with the addition of roscovitine. Significantly more oocytes with a visible polar body were observed in the 50-μM roscovitine group (63.8%) than in the control group (50.8%) ($P<0.05$). However, an increased concentration of roscovitine had no positive effects on the percentage of oocytes with a visible polar body (Fig. 7).

Development of SCNT embryos

There were no significant differences in the cleavage rates among the groups (Table 1). The rate of development to blastocysts of reconstructed embryos derived from oocytes pre-cultured with 50 μM roscovitine was significantly higher ($P<0.05$) than that from control oocytes (14.2% vs. 5.8%). There were no significant differences in the cell numbers of blastocysts among the groups.

DISCUSSION

The results demonstrated that the addition of roscovitine to culture medium delays the completion of meiotic maturation of bovine oocytes and maintains the ability of cytoplasmic maturation of oocytes during/after pre-incubation for 24 h, resulting in a higher blastocyst formation of the reconstructed embryos derived from oocytes pre-cultured with 50 μM roscovitine before IVM.

Attempts to develop in vitro culture systems to maintain bovine or porcine oocytes at the GV stage using different meiotic inhibitors have been reported (Avery et al., 1998; Dode and Adona, 2001; Faerge et al., 2001; Liu et al., 1998; Lonergan et al., 1997). If the oocytes could be maintained at the GV stage or early meiotic stage during the inhibitory period without negative effects on subsequent development to the blastocyst stage, the maturation schedules in a laboratory can be prolonged and more flexible (Choi et al., 2006; Coy et al., 2004). Therefore, this method, involving a two-step culture system (pre-culture in a medium with inhibitor and main culture in IVM medium), could be applied for in vitro embryo production of bovine embryos using not only IVF methods (Coy *et al.*, 2005) but also SCNT programs (Motlik *et al.*, 2000).

In the present study, we found that roscovitine delays the completion of meiotic maturation of bovine oocytes during incubation, but 11%–27% of oocytes had already reached the MII stage at the end of the pre-culture period. It has been shown that the inhibitory effect of roscovitine varies according to the concentration, but exposure to concentrations of roscovitine greater than 50 μM can prevent meiotic resumption in ~60% of oocytes (Albarracin *et al.*, 2005). It is well known that, when an oocyte is

removed from its follicular environment, it spontaneously resumes meiosis (Mermillod and Marchal, 1999). During oocyte collection, we collected and selected COCs, which were placed in m-PBS without any inhibitors after follicle aspiration. It seemes that some oocytes have already resumed meiosis before the pre-culture treatment with roscovitine. Therefore, some MII-stage oocytes observed in the present study might result from spontaneous meiotic resumption of oocytes before the treatment.

It has been shown that after pre-incubation with roscovitine, the oocytes were resumed from the meiotic arrest by transferring them into the IVM medium (Adona *et al.*, 2008). In the present study, similarly, we found that the maturation rates of oocytes pre-cultured with roscovitine increased from <27% at the onset of IVM culture to 66%–79% at the end of IVM culture. The maturation rates of oocytes observed in the present study were similar to those in other studies in which the rates of bovine oocytes reached the MII stage after incubation with roscovitine and subsequent maturation culture were 60%–90% (Albarracin et al., 2005; Donnay et al., 2004; Mermillod et al., 2000). Moreover, the incubation of oocytes with 200 μM roscovitine enhanced the meiotic competence of oocytes compared with control oocytes (79% vs. 58%). These results indicate that the meiotic inhibition of the oocytes treated with roscovitine is completely reversible, even when the oocytes did not develop further beyond the MI stage during inhibition culture.

At the end of maturation culture, more oocytes with a visible polar body were observed in the 50-μM roscovitine group (64%) than the control group (51%). Our results are consistent with the result of Lagutina *et al.* (Lagutina et al., 2002) who reported that about 70% of bovine oocytes pre-incubated with roscovitine extruded their polar bodies after 11 h of maturation culture. It has been suggested that some factors

acting upstream of MPF activation may accumulate progressively during roscovitine inhibition and that their presence may allow a faster course of early stages of meiotic resumption, decreasing the whole maturation time (Vigneron *et al.*, 2004). Therefore, incubation with roscovitine before IVM culture may increase the extrusion rates of oocytes.

Roscovitine has been shown to be capable of reversibly inhibiting meiotic resumption in bovine oocytes for 24 h without negative effects on subsequent development of IVF embryos to the blastocyst stage (Mermillod *et al.*, 2000). In contrast, Donnay *et al.* (Donnay et al., 2004) reported that pre-incubation treatment with roscovitine prevented the progression of meiosis in bovine oocytes but that this treatment led to a drastic decrease in the rate of embryo development after IVF. In the present study, similarly, the developmental rate of reconstructed embryos derived from oocytes pre-cultured with roscovitine (7%–14%) was significantly lower than that (blastocyst/fused couplets; 48/151, 33.5 ± 7.7%) obtained from fresh oocytes without pre-culture treatment (data not shown). Lonergan *et al.* (Lonergan et al., 2003) reported that morphological changes occurred in immature and in vitro matured bovine oocytes following exposure to roscovitine; pre-maturation treatment with roscovitine caused swelling of the mitochondrial cristae, degeneration of the cortical granules, and convolution of the nuclear membrane in the oocytes. Therefore, the decrease in the development rate of reconstructed embryos derived from pre-cultured oocytes might result in part from the cytoskeleton alterations caused by exposure to roscovitine. However, I found that the development of reconstructed blastocysts derived from oocytes pre-cultured with 50 μM roscovitine was significantly higher than that from control oocytes pre-cultured without rescovitine (14% vs. 6%). It has been reported that

enhancing the cytoplasmic maturation of recipient oocytes resulted in an improvement of SCNT outcome (Wongsrikeao *et al.*, 2007). Moreover, successful and reliable oocyte maturation (both cytoplasmic and nuclear maturation) improves the efficiency of preimplantation embryonic development as well as fetal development (Sagirkaya *et al.*, 2007). Therefore, pre-incubation of oocytes with roscovitine might maintain or improve cytoplasmic maturation, resulting in an increase in the development of reconstructed embryos. Moreover, our result indicates that the addition of 50 μM roscovitine as a meiotic inhibitor is effective for the development of SCNT embryos.

In conclusion, the addition of roscovitine to culture medium can delay the meiotic resumption of oocytes, and their cytoplasm can support the development of SCNT embryos even when the oocytes are pre-cultured for one day before the onset of IVM culture, allowing a more convenient schedule for subsequent oocyte manipulations.

Article 3

IN VITRO DEVELOPMENT OF CAT INTERSPECIES NUCLEAR TRANSFER USING PIG'S AND COW'S CYTOPLASM

ABSTRACT

This study was conducted to investigate the influence of recipient cytoplasm on the development of somatic cell nuclear transfer (SCNT) embryos, using recipient oocytes and donor cells that were obtained from cats, cows, and pigs. Bovine and porcine oocytes were collected from ovaries obtained at a slaughterhouse, and cat oocytes were collected from ovaries obtained at local veterinary clinics following ovariohysterectomy. Cumulus cells from oocytes of each species were used as donors. When cat cumulus cells were transferred into cow, pig, and cat oocytes, the percentages of fusion and cleavage in the cow-cat and pig-cat interspecies groups were similar to those in the cat-cat intraspecies group. There were no significant differences in the percentages of fusion and cleavage between the interspecies (cow-cat and pig-cat groups) and intraspecies (cow-cow and pig-pig groups) SCNT embryos in each recipient oocyte species. However, none of the interspecies SCNT embryos developed to the morula and blastocyst stages. The percentages of fusion and cleavage were significantly higher ($P<0.05$) in cow-cat SCNT embryos than those in pig-cat SCNT embryos. In conclusion, bovine and porcine cytoplasm can be used to support the early embryonic development of interspecies SCNT with cat donor nucleus. However, the interspecies SCNT embryos could not develop to the late embryonic stage such as the morula or blastocyst stages.

INTRODUCTION

Interspecies somatic cell nuclear transfer (SCNT) is an invaluable tool for studying nucleous-cytoplasm interactions, and may provide an alternative for cloning endangered animals, whose oocytes are difficult to obtain. Utilization of oocytes as recipient cytoplasts obtained from accessible and abundant species would greatly benefit ongoing research on reprogramming of stem cell sciences. It has been shown that bovine, sheep, and rabbit oocyte cytoplasm supports *in vitro* development of embryos produced by interspecies nuclear transfer of somatic cells from various unrelated mammalian species; however, no pregnancy has gone to full-term after transfer of interspecies SCNT embryos to surrogate animals (2002; 1999b; 1999b). It has been suggested that the ability of interspecies SCNT embryos to develop to the blastocyst stage decreases as the taxonomic distance between the donor and recipient species increases (2007). However, information remains limited on the effects of genetic differences between donors and recipients upon the developmental competence of interspecies SCNT embryos. Moreover, to our knowledge, little information is available concerning the use of bovine and porcine oocyte cytoplasm for interspecies SCNT in feline species.

The domestic cat provides a valuable model for reproductive studies of non-domestic felid species. *In vitro* fertilization and nuclear transfer techniques in domestic cats may eventually enable rescue of genetic materials from endangered felid species (Farstad, 2000). Since interspecies SCNT might become an alternative method for producing SCNT embryos, the establishment of an interspecies cat SCNT embryo model may be useful for conservation strategies of endangered animals.

The objective of this Article 3 was to assess embryonic development following nuclear transfer of somatic cells derived from the domestic cat (*Felis catus*) into oocyte cytoplasm obtained from cows (*Bos taurus*) and pigs (*Sus scrofa*), in order to investigate the influence of recipient cytoplasm upon the development of interspecies SCNT embryos. Moreover, we compared the development of intraspecies and interspecies SCNT embryos to examine nucleous-cytoplasm interactions.

MATERIAL AND METHODS

Recovery of bovine, porcine, and cat oocytes.

Bovine and porcine ovaries were obtained from a slaughterhouse and transported to the laboratory in 0.9% (w/v) physiological saline at 35°C within 3 h of slaughter. Cumulus-oocyte complexes (COCs) were aspirated from follicles (≤5 mm in diameter) using a 5-mL syringe fitted with an 18-gauge needle. COCs were collected in modified phosphate buffered saline (m-PBS; Nihonzenyaku, Japan) supplemented with 100 IU/mL of penicillin G potassium (Meiji, Japan) and 0.1 mg/mL of streptomycin sulfate (Meiji). Cat ovaries were obtained from local veterinary clinics following routine ovariohysterectomy and kept in physiological saline at room temperature before oocyte recovery. Each ovary was sliced repeatedly with a scalpel blade to release COCs into a 90-mm culture dish containing m-PBS supplemented with 100 IU/mL of penicillin G potassium and 0.1 mg/mL of streptomycin sulfate.

In vitro maturation of oocytes.

Bovine oocytes were matured according to procedures previously described by Mori *et al.* (2002). Only COCs with uniform ooplasm and compact cumulus cells were used in this experiment. The COCs were cultured for 21 h in maturation medium that consisted of TCM 199 with Earle's salts (Invitrogen, USA) supplemented with 0.02 AU/mL of follicle stimulating hormone (FSH; Kawasaki Mitaka K.K.), 5% foetal bovine serum (FBS), and 50 μg/mL of gentamicin (Sigma, USA). Porcine oocytes were matured according to procedures previously described by Wongsrikeao *et al.* (2006). The porcine COCs were cultured for 22 h in maturation medium that consisted of a

modified North Carolina State University (NCSU)-37 solution (1993) supplemented with 0.6 mM cysteine, 1 mM dibutyryl cyclic AMP (dbcAMP; Sigma), 10 IU/mL of equine chorionic gonadotropin (eCG; Kawasaki Mitaka K.K.), 10 IU/mL of human chorionic gonadotropin (hCG; Kawasaki Mitaka K.K.), 50 μg/mL of gentamicin, and 10% (v/v) porcine follicular fluid. Porcine COCs were then cultured for an additional 22 h in the maturation medium without hormones and dbcAMP. The maturation cultures for bovine and porcine oocytes were maintained at 38.5°C in an atmosphere of 5% CO_2 and 95% air with high humidity. Cat oocytes were matured according to procedures previously described by Karja *et al.* (Karja et al., 2002). Briefly, cat COCs were cultured in a maturation medium consisting of TCM 199 with Earle's salts supplemented with 4 mg/mL of bovine serum albumin (BSA; Sigma), 0.1 IU/mL of human menopausal gonadotropin (Teikoku Zoki, Japan), 10 IU/mL of hCG, 1 μg/mL of 17β-oestradiol (Sigma), and 50 μg/mL of gentamicin. The maturation cultures for cat oocytes were maintained at 38.0°C for 24 h in an atmosphere of 5% CO_2 and 95% air with high humidity.

Preparation of donor cells.

Cumulus cells, which were the source of donor nuclei, were removed from bovine, porcine, and cat COCs by vortexing, and cultured in α-MEM (Invitrogen) supplemented with 10% FBS, 1% MEM non-essential amino acid solution (Invitrogen), and 50 μg/mL of gentamicin for 7 d at 37.0°C in a 5% CO_2 atmosphere. Once the cumulus cells reached complete confluence, the cell monolayer was washed twice with α-MEM and then incubated in 0.25% (w/v) trypsin-EDTA (Sigma) for 3 min at 37.0°C. After trypsinisation, washing medium (α-MEM supplemented with 10% FBS) was

added to neutralise trypsin activity. The cells were pelleted by centrifugation at 500× *g* for 5 min, resuspended in the washing medium, and then maintained in this medium until SCNT manipulation.

Nuclear transfer.

After maturation culture, bovine, porcine, and cat COCs were mechanically denuded in a TCM199 medium or NCSU-37 solution, which contained 0.1% (w/v) hyaluronidase (Sigma). Oocytes with the first polar body extrusion were collected and then incubated for 10 min in 3 μg/mL of Hoechst 33342 (Sigma) diluted in manipulation medium (PBS supplemented with 5% FBS and 5 μg/mL of cytochalasin B [Sigma]). Oocytes were then washed, transferred to a drop of manipulation medium, and enucleated at room temperature after minimal exposure to low-light filtered fluorescence. Oocytes were secured with a holding pipette and rotated as needed into a position suitable for enucleation. The zona pellucida above the first polar body was cut with a glass needle, and a small volume (approximately 5%–10%) of cytoplasm underneath the first polar body was squeezed out. Complete enucleation was confirmed by staining the squeezed-out cytoplasm. After enucleation of each individual oocyte, the donor cell was then placed into the perivitelline space adjacent to the plasma membrane of the oocyte. Couplets were equilibrated for 3 min in Zimmerman cell fusion medium (1992), transferred into a drop of Zimmerman cell fusion medium, and then manually aligned between two electrode needles connected to a micromanipulator (MO-202D; Narishige, Japan). Couplets with cat, porcine, and bovine ooplasm were fused and activated simultaneously with a single DC pulse of 1.5 (Karja et al., 2006), 2.0 (Lee et al., 2003), and 2.3 kV/cm (Wongsrikeao et al., 2007) for 30 μsec, respectively, using an

electro cell fusion generator (LF101; Nepagene, Japan). The successfully fused couplets were activated in each culture medium supplemented with 10 μg/mL of cycloheximide (Sigma) and incubated for 5 h.

In vitro culture of SCNT embryos.

Following cycloheximide treatment, the couplets reconstructed with bovine cytoplasm were cultured in a modified synthetic oviduct fluid (mSOF) medium (2003) supplemented with 4 mg/mL of BSA and 50 μg/mL of gentamicin for 72 h at 38.5°C in a humidified atmosphere of 5% CO_2 and 5% O_2. After 72 h of culture, only cleaved embryos were further co-cultured with bovine cumulus cells in mSOF supplemented with 5% FBS at 38.5°C in a humidified 5% CO_2 atmosphere for an additional 5 d to evaluate their ability to develop to the blastocyst stage. The couplets reconstructed with porcine cytoplasm were cultured in NCSU-37 solution supplemented with 4 mg/mL of BSA, 0.17 mM sodium pyruvate, 2.73 mM sodium lactate, and 50 μg/mL of gentamicin for 72 h at 38.5°C in a humidified 5% CO_2 atmosphere. After 72 h of culture, only cleaved embryos were transferred into fresh culture medium; NCSU-37 supplemented with 4 mg/mL of BSA, 5.55 mM D-glucose, and 50 μg/mL of gentamicin. The cleaved embryos were cultured for an additional 5 d to evaluate their ability to develop to the blastocyst stage. The couplets reconstructed with cat cytoplasm were cultured in modified Earle's balanced salt solution (MK-1) (Kanda et al., 1998)) supplemented with 4 mg/mL of BSA for 72 h at 38.0°C in a humidified 5% CO_2 atmosphere. After 72 h of culture, only cleaved embryos were transferred into fresh MK-1 supplemented with 5% FBS and 50 μg/mL of gentamicin. The cleaved embryos were cultured for an additional 5 days to evaluate their ability to develop to the blastocyst stage.

Statistical analysis.

Five to eight replicate trials were performed. Data are expressed as means ± SEMs. The percentages of fused embryos, embryos cleaved, and embryos that developed to the morula and blastocyst stage were subjected to arc sin transformation before analysis of variance (ANOVA). The transformed data were tested by ANOVA followed by Fisher's protected least significant difference (PLSD) test, using the StatView programme (Abacus Concepts, Inc., USA). Differences with a probability value (P) of 0.05 or less were considered statistically significant.

RESULTS

The percentage of cleavage to the >4-cell stage in the cow-cat interspecies group was significantly higher ($P<0.05$) than that in the cat-cat intraspecies group, but there were no differences in the percentages of fusion and total cleavage between the cow-cat and pig-cat interspecies groups and the cat-cat intraspecies group (Table 2). When the embryos were reconstructed with ooplasm derived from the same recipient, there were no significant differences in the percentage of fusion and cleavage between the intraspecies and interspecies SCNT embryos. However, none of the interspecies SCNT embryos developed to the morula and blastocyst stage. The percentages of fusion and total cleavage were significantly higher ($P<0.05$) in the cow-cat interspecies group than that in the pig-cat interspecies group. Similarly, significantly more intraspecies SCNT embryos in the cow-cow group cleaved and developed to the blastocyst stage compared with the pig-pig group ($P<0.05$).

DISCUSSION

Previous studies have shown that oocyte cytoplasm from cow (1999b), sheep (1999b), and rabbit (2002) oocytes are able to support early development of interspecies SCNT embryos from somatic cell nuclei of sheep, pig, monkey, rat, and giant panda. The presented study showed that the percentages of fusion and cleavage of interspecies cow-cat and pig-cat embryos were similar to those of the intraspecies cat-cat embryos. These findings indicate that the developmental characteristics up to the cleavage stage of interspecies cat SCNT embryos were similar to those of intraspecies cat SCNT embryos, irrespective of the recipient ooplasm species. However, none of the interspecies SCNT embryos developed to the blastocyst stage. It has been suggested that mtDNA transferred into recipient cytoplasm by nuclear transfer may influence the developmental ability of embryos (Tecirlioglu et al., 2006). The mitochondrial heteroplasmy may be involved in incompatibilities between the nucleus and the cytoplasm inhibiting development of cloned embryos (Thongphakdee et al., 2008). Moreover, it has been suggested that the maternal-zygotic transition occurs at earlier embryonic stages and may be associated with developmental blocks (1990b). The early embryonic development block has been suggested to be related to embryo species, culture medium, and culture conditions (Yang et al., 2003a). It is well known that embryos from different mammalian species require species-specific embryo culture conditions. However, it is unclear whether the culture medium for interspecies SCNT embryos should be matched to the culture medium for the donor cells or for the recipient oocytes. In the presented study, the culture media used for embryos reconstructed with cat, porcine, and bovine enucleated oocytes were MK-1, NCSU-37,

and mSOF, respectively. These media have been widely used to culture *in vitro* fertilized embryos in each species. We observed that the percentages of fusion and cleavage were similar between the intraspecies and interspecies SCNT units when compared in each recipient oocyte species (cow and pig). Although it remains unclear whether the culture medium used in the presented study is optimal for interspecies SCNT embryos, our results indicate that the culture medium used according to each recipient oocyte species can support early development of interspecies SCNT embryos up to the cleavage stage as effectively as intraspecies SCNT embryos.

Wen *et al.* (Wen et al., 2005) demonstrated that rabbit cytoplasm can support development up to the blastocyst stage of embryos reconstructed with cat fibroblasts. Moreover, it has been shown that porcine cytoplasm is able to dedifferentiate somatic cells from Siberian tiger and supports early development of the embryos up to the blastocyst stage (Hashem et al., 2007). Although many studies have been conducted on interspecies SCNT using bovine and porcine oocytes as the recipient cytoplasm, little information is available concerning the influence of bovine and porcine cytoplasm on the development of interspecies cat SCNT embryos. When bovine and porcine oocytes were matured and then activated by the combination of 7% ethanol and cycloheximide treatment, the percentage of pronucleus formation was significantly higher in bovine oocytes than in porcine oocytes (91.7% vs. 64.1%; data not shown). Moreover, we found that the cleavage rate of cow-cat SCNT embryos increased when compared to pig-cat SCNT embryos. With regard to our intraspecies SCNT results, more cow-cow SCNT embryos cleaved and developed to the blastocyst stage compared to pig-pig SCNT embryos. This result indicates that bovine cytoplasm not only supports the development of reconstructed nuclei from various species to early cleavage stages, but

also may be superior to porcine cytoplasm for the production of interspecies SCNT embryos.

In conclusion, bovine and porcine cytoplasm can be used to support the early embryonic development of interspecies SCNT with cat donor nucleus. However, the interspecies SCNT embryos could not develop to the late embryonic stage such as morula and blastocyst stages. Further studies including the modification of embryo culture and protocol of fusion and activation are needed for the improvement of developmental competence of interspecies cat SCNT embryos.

OVERALL DISCUSSION AND FUTURE DIRECTIONS

Because animal species have decreased in number dramatically, reproductive biologists and zoologists have begun consideration of reproductive technologies including artificial insemination, embryo transfer/sexing, *in vitro* fertilization, gamete/embryo micromanipulation, semen sexing, genome resource banking, and somatic cell nuclear transfer (cloning) as tools to expand animal populations. However, current reproductive biotechnologies are species-specific or are inefficient for many endangered animals because of insufficient knowledge related to basic reproduction, such as the estrous cycle, seasonality, structural anatomy, gamete physiology, and sites for semen deposition or embryo transfer of non-domestic species. To improve the embryo development after ART treatment, the quality of the gamete (spermatozoa and oocyte) itself must be improved.

In Article 1, to examine the effects of EGCG during the IVF, sperm quality and penetrability into oocytes were investigated. It has been already demonstrated that antioxidant supplementation such as glutathione and hypotaurine to the *in vitro* culture medium of porcine embryos can increase the embryo development rate (Suzuki and Yoshioka, 2005). In my study, the motility of spermatozoa increased concomitantly with increased EGCG concentrations, irrespective of the incubation time. Mammalian spermatozoa expend energy, generated as intracellular ATP, mainly to support motility (Storey, 2008). Spermatozoa produce spontaneously various reactive oxygen species (ROS), including the super oxide (SO) anion, hydrogen peroxide (HP), and nitric oxide. High concentrations of the ROS cause sperm pathology (ATP depletion) leading to

insufficient axonemal phosphorylation, lipid peroxidation, and loss of motility and viability (Baumber et al., 2000). Against ROS attack, sperm cells are well equipped with a powerful defense system of antioxidants. Antioxidants are the main defense factors against oxidative stress induced by free radicals. EGCG accounts for more than 65% of total catechin contents and has the highest antioxidant activity potency among catechins (Goto et al., 1996). Catechins show prooxidant activity in high concentrations, induce DNA cleavage, and accelerate the peroxidative property of unsaturated fatty acid (Kusakabe and Kamiguchi, 2004). Therefore, our results show that EGCG with low concentration might protect spermatozoa from oxidative stress and improve ATP production, leading to improved sperm motility. However, high concentrations of EGCG might change the plasma integrity and engender decrease of sperm viability.

The sperm penetration rate has been known to increase as the sperm motility increases. In this study, the penetration rate of spermatozoa co-incubated with 50 μM and 100 μM EGCG increased as compared with the control group. The addition of 100 μM EGCG had negative effects on the rates of polyspermic fertilization. High concentrations of EGCG engender chromosomal damage of cells and reduce cell cycle progression, indicating that EGCG induces oocyte damage (Sugisawa and Umegaki, 2002; Tanaka, 2000). Therefore, supplementation of high concentrations of EGCG influenced both spermatozoa and oocytes, thereby increasing the polyspermic fertilization rate. Results of the first series of experiments indicate that the addition of 50 μM EGCG improves the motility and penetrability of boar spermatozoa.

In Article 2, the effects of various concentrations of roscovitine on the meiotic competence of oocytes pre-cultured for 24 h before IVM were reported. Several reports

have described that supplementation of meiotic inhibitors to the *in vitro* culture system were able to maintain bovine or porcine oocytes at the GV stage (Avery et al., 1998; Dode and Adona, 2001; Faerge et al., 2001; Liu et al., 1998; Lonergan et al., 1997). Retention of an oocyte's ability to synthesize and store molecules, which is necessary for subsequent embryonic development during the inhibitory period, can be prolonged and can produce more flexible maturation schedules in the laboratory (Choi et al., 2006; Coy et al., 2005).

The present study revealed that roscovitine inhibits the resumption of meiotic maturation of bovine oocytes during incubation but 11–27% of oocytes had already reached the MII stage at the end of pre-culture period. When a meiotically competent oocyte is removed from its follicular environment, it spontaneously resumes meiosis (Mermillod et al., 2000). Because oocytes were placed in m-PBS with no inhibitors after follicle aspiration, some oocytes might have already started meiotic maturation before pre-culture treatment with roscovitine.

The maturation rate of bovine oocytes that reached the MII stage after incubation with roscovitine was similar to that found in the present study, and resembles those in other studies (Albarracin et al., 2005; Donnay et al., 2004; Mermillod et al., 2000). Furthermore, supplementation of 200 μM roscovitine facilitates the meiotic competence of oocytes compared with the control groups. These results indicate that the meiotic resumption inhibition by roscovitine is completely reversible even if the oocytes did not develop further than the MII stage during the inhibition culture.

After the IVM culture, the oocytes cultured with 50 μM roscovitine extruded more polar bodies than oocytes of the control group. Vigneron et al. (2004) reported that some factors acting upstream that affect MPF activation might accumulate progressively

during roscovitine inhibition and that their presence might enable a faster course of early stages of meiotic resumption, decreasing in turn the whole maturation time. Therefore, incubation with roscovitine before IVM culture might increase the extrusion rates of bovine oocytes.

In this study, the development rate of reconstructed embryos derived from oocytes pre-cultured with roscovitine was significantly lower than that obtained from fresh oocytes without pre-culture treatment. Mermillod et al. (2000) showed that roscovitine can produce meiotic inhibition without decreasing their resulting developmental potential in bovine oocytes. In contrast, Donnay et al. (2004) reported that roscovitine incubation inhibits the meiotic resumption in bovine oocytes, but that this treatment decreased the embryo development after IVF. Reportedly, morphological changes such as swelling of mitochondrial cristae, degeneration of cortical granules, and convolution of the nuclear membrane occurs in bovine oocytes incubated with roscovitine (Lonergan et al., 1997). Therefore, the development rate of reconstructed embryos derived from pre-cultured oocytes with roscovitine might decrease because of cytoskeleton alterations attributable to roscovitine exposure.

Reportedly the promotion of cytoplasmic maturation of recipient ooplasm improves SCNT embryo development (Wongsrikeao et al., 2007). Furthermore, consistently successful and reliable oocyte maturation (both cytoplasmic and nuclear maturation) is expected to improve the efficiency of preimplantation embryonic development as well as fetal development dramatically (Sagirkaya et al., 2007). Therefore, the addition of roscovitine to the culture medium can delay the meiotic resumption of oocytes. Consequently, cytoplasm can support the development of SCNT embryos even when the oocytes are pre-cultured for 24 h before IVM culture.

In Article 3, the influence of recipient cytoplasm on the development of SCNT embryos was examined when the used recipient oocytes and donor cells that were obtained from cats, cows, and pigs was examined. Several reports have described that oocyte cytoplasm of cow (Dominko et al., 1999), sheep (White et al., 1999), and rabbit (Chen et al., 2002) can support early development of iSCNT embryos derived from somatic cells of sheep, pig, monkey, rat, and giant panda. In the present study, the fusion and cleavage rates of interspecies cow-cat and pig-cat embryos were similar to the rates of intraspecies cat-cat embryos. However, no interspecies embryo developed to the blastocyst stage.

Tecirlioglu et al. (2006) reported that mtDNA in the recipient ooplasm might affect the developmental competence of embryos. Maternal-zygotic transition occurs at earlier embryonic stages associated with developmental blocks (Telford et al., 1990). Moreover, the development of cloned embryos is inhibited by mitochondrial heteroplasmy involving incompatibilities between the nucleus and the cytoplasm (Thongphakdee et al., 2006).

Species-specific culture conditions are well known to be necessary for embryos from different mammalian animals. Moreover, the early embryo development block is related to embryo species, the culture medium, and culture conditions (Yang et al., 2003). In the present study, the culture medium used for embryos reconstructed with cat, porcine, and bovine enucleated oocytes were, respectively, MK-1, NCSU-37, and mSOF. The percentages of fusion and cleavage were similar between intraspecies and interspecies SCNT embryos when compared in each recipient oocyte species (cow and pig). These results show that the culture medium used in each recipient oocyte species

supported the early development of interspecies SCNT embryos as intraspecies SCNT embryos.

Many studies have examined the developmental competence of iSCNT using bovine and porcine oocyte as recipient cytoplasm, but little information is available in relation to the effect of bovine and porcine cytoplasm on the development of cat iSCNT embryos. In this study, the cleavage rate of cow-cat iSCNT embryos was higher than that of pig-cow iSCNT embryos. Moreover, in intraspecies SCNT embryos, more cow-cow SCNT embryos developed to the blastocyst stage than pig-pig SCNT embryos did. These results indicate that bovine cytoplasm not only supports the development of SCNT embryos reconstructed with somatic cell of various species; it might also be superior to porcine cytoplasm for production of iSCNT embryos. Therefore, bovine cytoplasm is useful to support the early embryonic development of iSCNT embryos with cat donor nucleus.

As ART develops, a suitable gamete preservation system is necessary to maintain genetic diversity. If both spermatozoa and oocytes are preserved effectively, then it will be possible to perform IVF/ICSI procedures and subsequent ET when conditions such as recipient animals are complete. Consequently, the gamete preservation system will enable us to increase opportunities for suitable ET. It is also expected to engender increased production of live offspring. However, progress in ART for endangered animals will continue at a slow pace because of limited resources, and also because the management and conservation of endangered animals is biologically quite complex. We expect that this investigation presents some possibilities for assisting conservation programs in the protection of some species of endangered animals.

TABLES AND FIGURES

Fig. 1. Effects of concentration (0, 1, 50 and 100 μM) of (-)-epigallocatechin gallate (EGCG) on motility (A) and viability (B) of porcine frozen-thawed spermatozoa after incubation for various times. Mean ± SEM are presented. Five replicated trials were carried out. Bars with different letters within each incubation time differ significantly ($p < 0.05$).

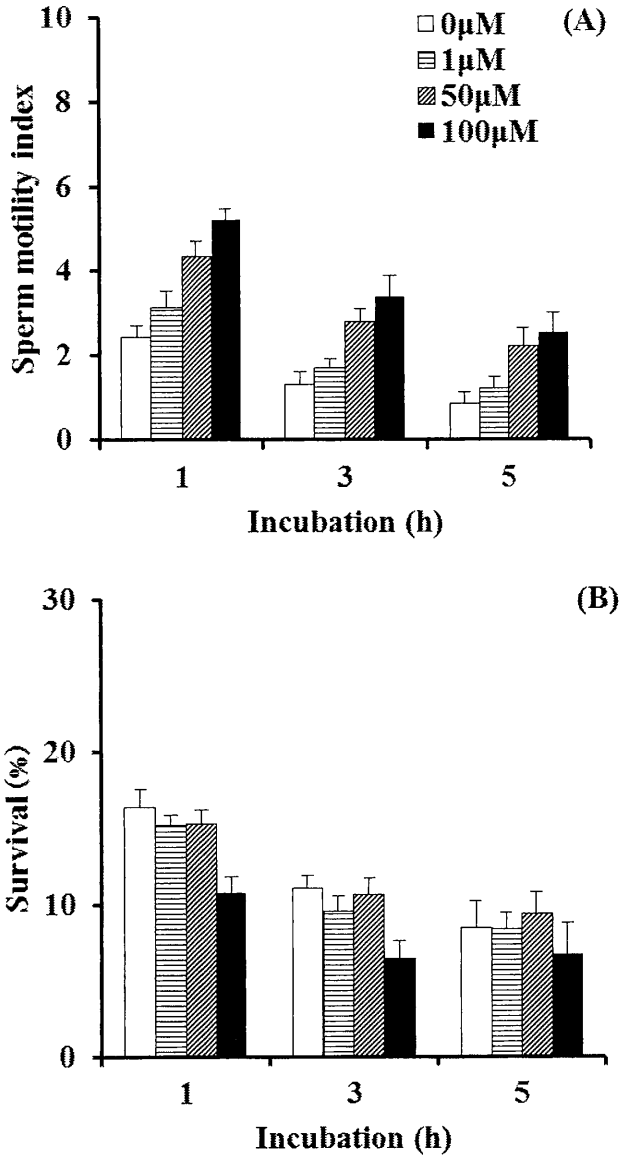
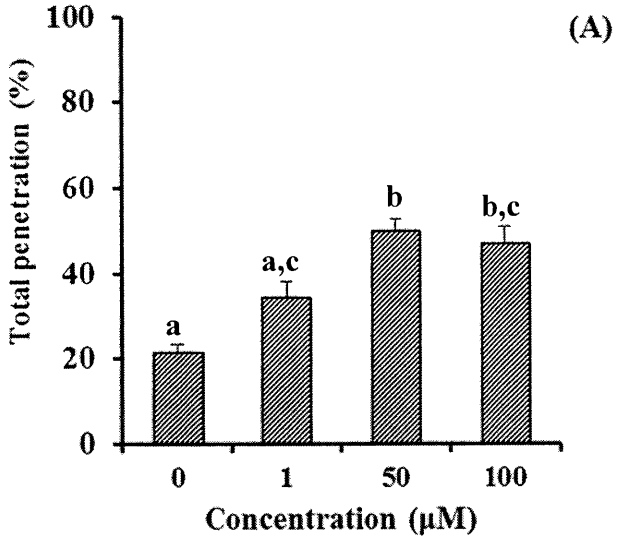
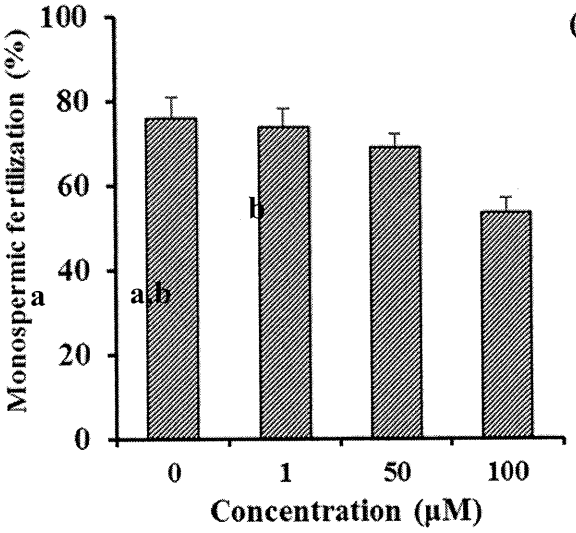


Fig. 2. Effects of concentration (0, 1, 50 and 100 μM) of (-)-epigallocatechin gallate (EGCG) on penetration (A), monospermic fertilization (B) and polyspermic fertilization (C) of porcine spermatozoa after co-incubation with oocytes. The oocytes were co-incubated with frozen-thawed spermatozoa in fertilization medium supplemented with 0, 1, 50 and 100 μM EGCG for 5 h. Mean ± SEM are presented. Five to six replicated trials were carried out. Bars with different letters differ significantly ($p < 0.05$).



a


(B)
Monospermic fertilization (%)
100
80
60
40
20
0
a
a,b
b
0
1
50
100
Concentration (μM)

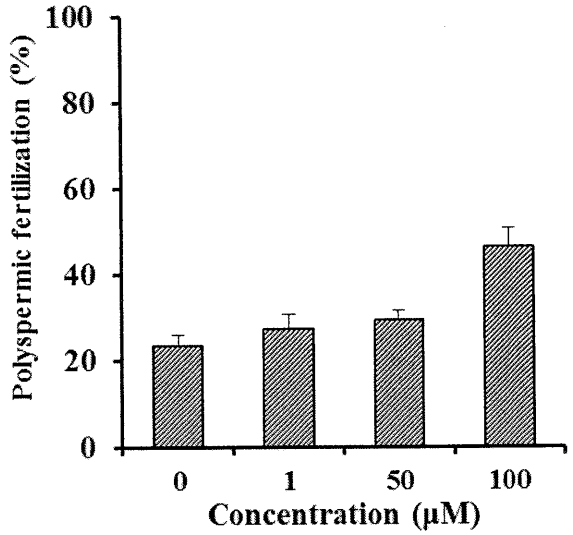

(C)
Polyspermic fertilization (%)
100
80
60
40
20
0
0
1
50
100
Concentration (μM)

Fig. 3. Effects of 50 μM (-)-epigallocatechin gallate (+ EGCG,) on motility (A) and viability (B) of frozen.thawed spermatozoa from six different boars after 3 h of incubation. As a control group, effects of the absense of EGCG (– EGCG) were also examined. Mean ± SEM are presented. Four to six replicated trials were carried out. Bars with different letters differ significantly ($p < 0.05$).

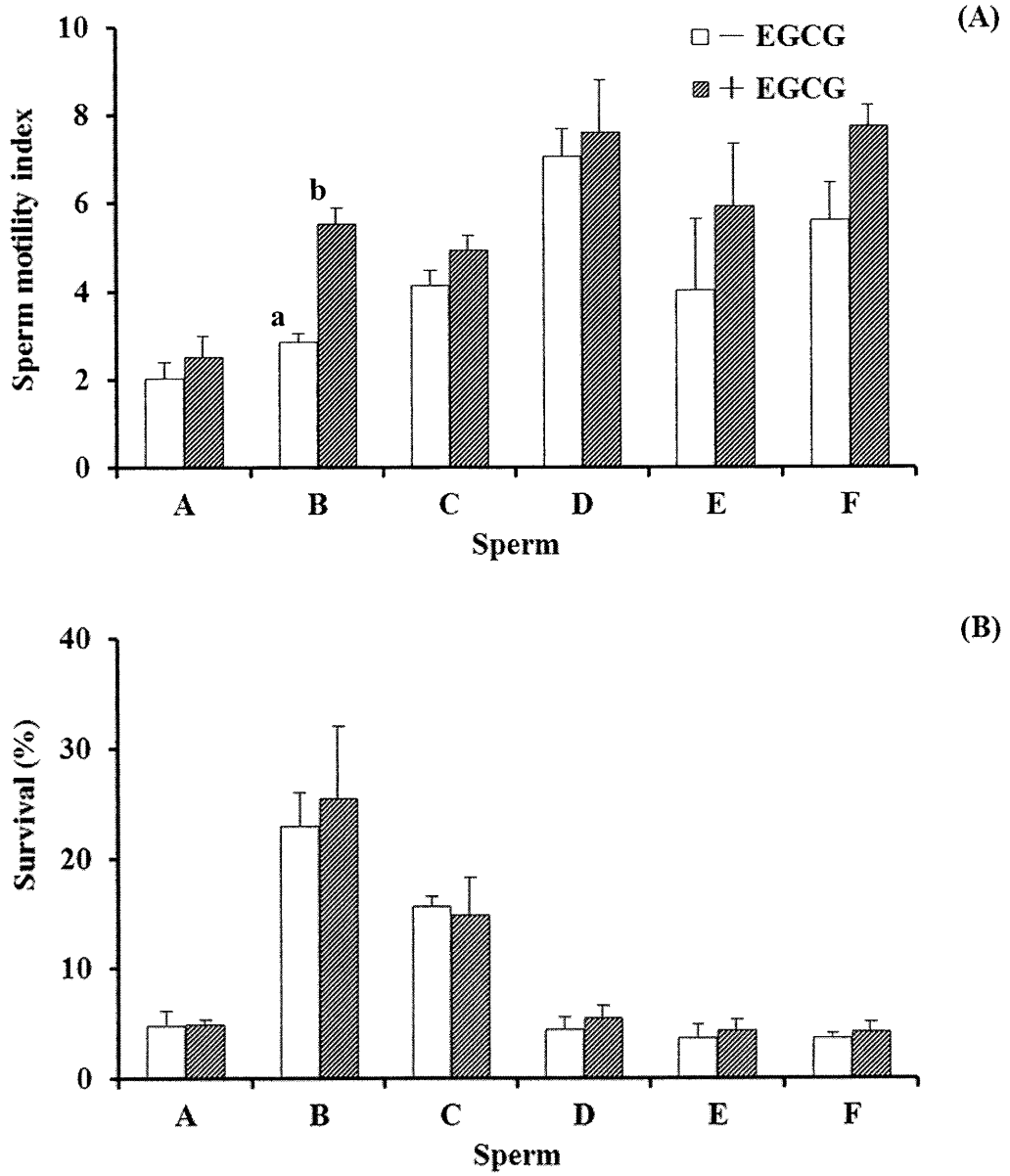
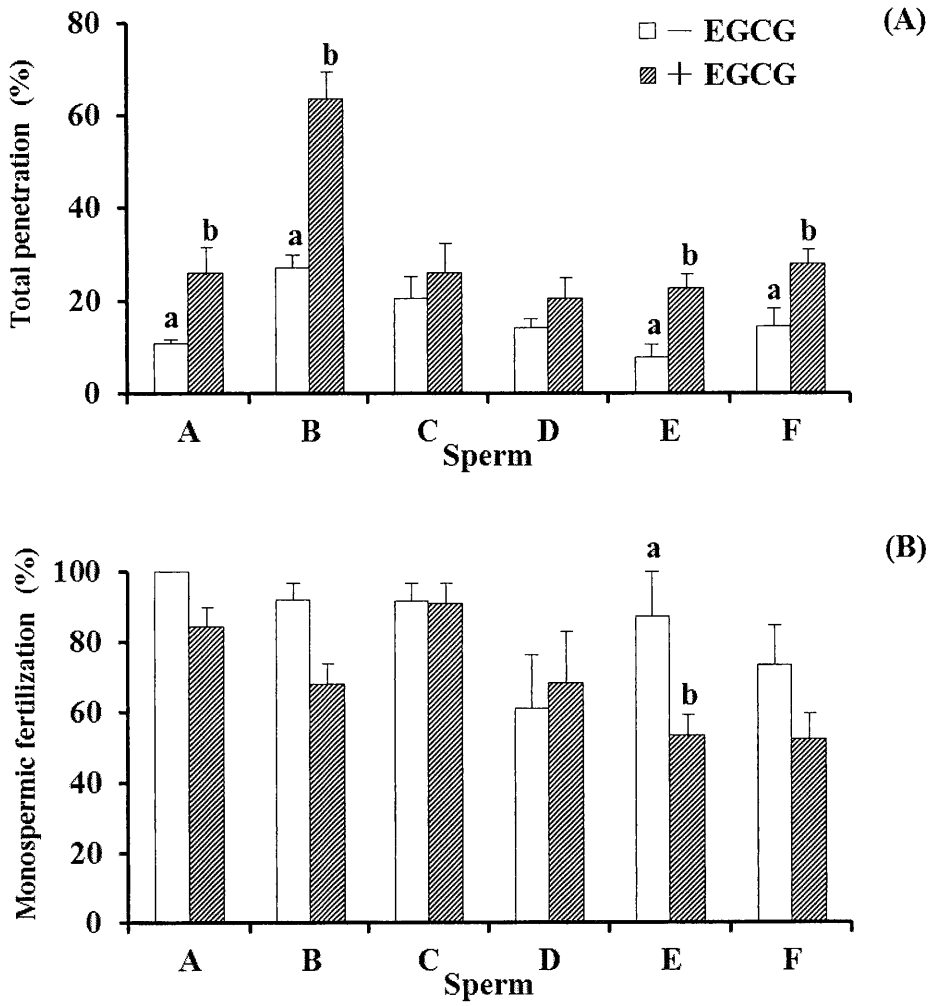


Fig. 4. Effects of 50 μM (-)-epigallocatechin gallate (EGCG,) (+ EGCG) on penetration (A), monospermic fertilization (B) and polyspermic fertilization (C) of frozen-thawed spermatozoa from six different boars after co-incubation with oocytes. As a control group, effects of the absense of EGCG (– EGCG) were also examined. Mean ± SEM are presented. Five to six replicated trials were carried out. Bars with different letters within each boar differ significantly ($p < 0.05$).



(C)

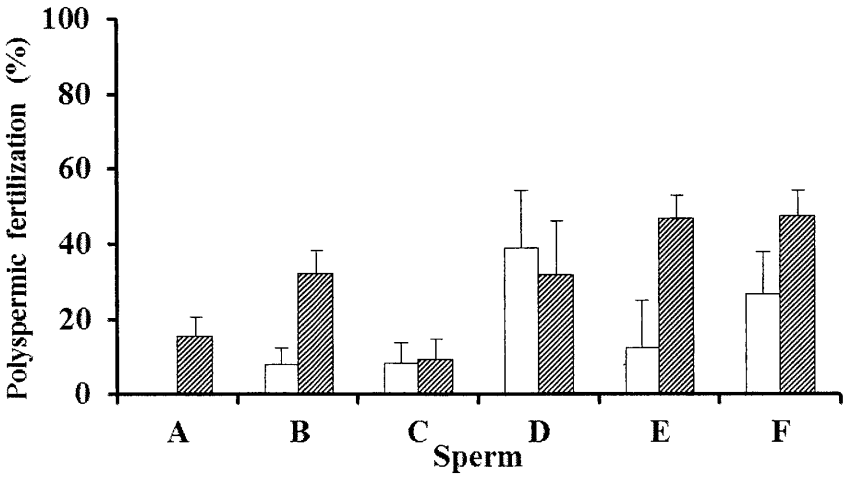


Fig. 5. Effects of 50 μM (-)-epigallocatechin gallate (EGCG,) (+ EGCG) on rates of cleavage (A) and blastocyst formation (B) of oocytes fertilized with frozen.thawed spermatozoa from six different boars. As a control group, effects of the absense of EGCG (– EGCG) were also examined. Mean ± SEM are presented. Six to seven replicated trials were carried out. Bars with different letters within each boar differ significantly ($p < 0.05$).

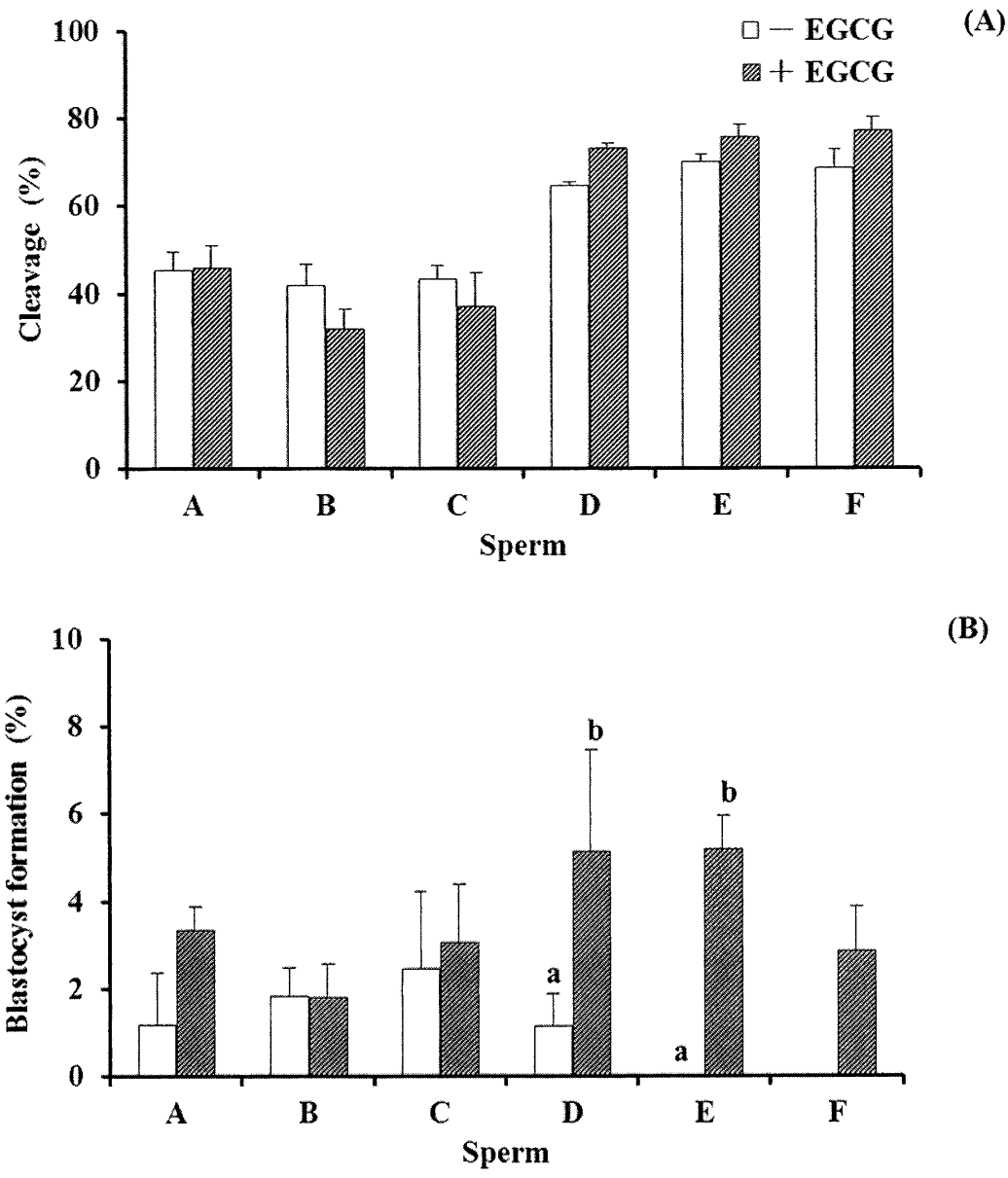


Fig. 6. The rates of oocytes reaching the MII stage before (A) and after (B) in vitro maturation culture following pre-culture with various concentrations (0, 50, 100 and 200 μM) of roscovitine for 24 h. Mean ± SEM are presented. Numbers within parentheses indicate the total number of oocytes examined. Bars with different letters differ significantly ($p<0.05$).

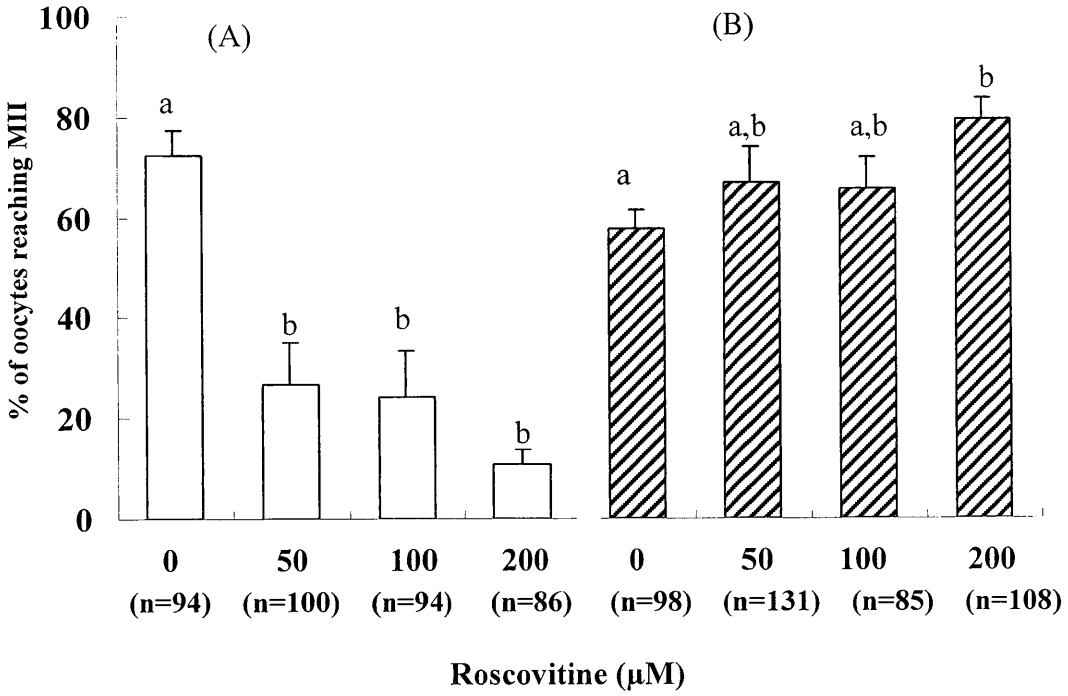


Fig. 7. The rates of oocytes with extruded of the first polar body after in vitro maturation culture for 21 h following pre-culture with various concentrations (0, 50, 100 and 200 μM) of roscovitine for 24 h.. Mean ± SEM are presented. Numbers within parentheses indicate the total number of oocytes examined. Bars with different letters differ significantly ($p<0.05$).

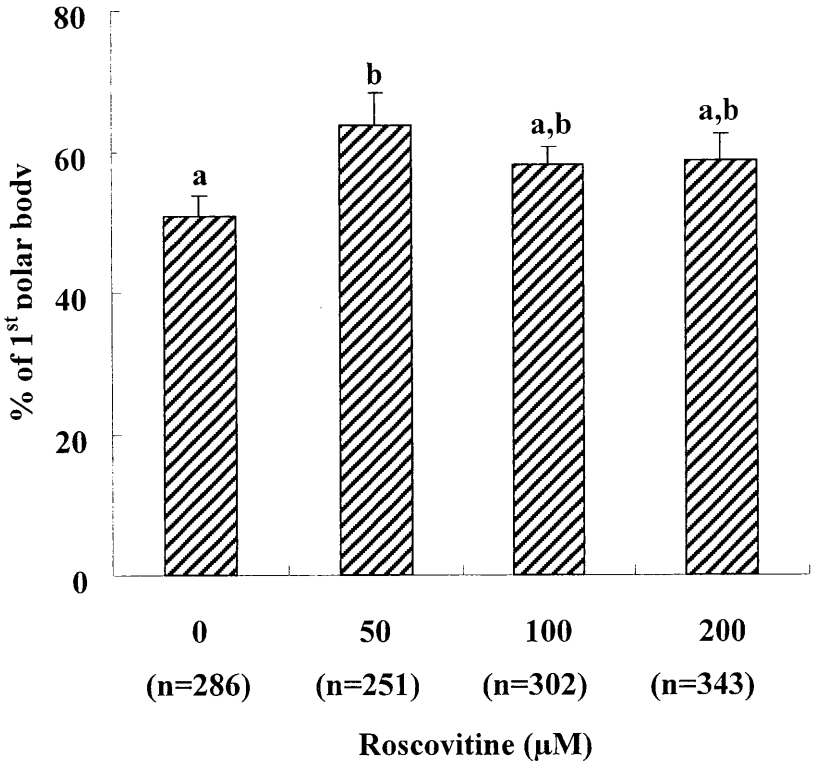


Table 1. Development of somatic cell nuclear transferred embryos derived from oocytes pre-cultured for 24 h with various concentrations of roscovitine before in vitro maturation

Roscovitine concentrations (μM)	No. of fused couplets	No. (Mean ± SEM) of embryos		Mean cell No. ± SEM of blastocysts
		Cleaved	Developed to blastocysts	
0	116	66 (56.6 ± 6.1)	8 (5.8 ± 1.7)[a]	74.6 ± 7.1
50	74	35 (52.7 ± 10.1)	10 (14.2 ± 5.4)[b]	79.9 ± 7.8
100	139	68 (46.1 ± 6.0)	10 (6.8 ± 3.0)[a,b]	106.9 ± 22.5
200	145	77 (52.7 ± 7.0)	13 (7.7 ± 2.2)[a,b]	88.2 ± 9.7

[a–b]Values with different superscript letters differ significantly ($p<0.05$).

Table 2. *In vitro* Development of intraspecies and interspecies nuclear transferred embryos

Donor nucleus species	Recipient oocyte Species	Number of couples	Number of fused oocytes (%)*	Number of cleaved embryos (%)**			Number of developed embryos (%)**	
				2 cell	>4 cell	Total	morula	blastocyst
Cat	Cow	83	64 (77.1)^{a,c}	17 (26.6)	35 (54.7)^{a,b}	52 (81.3)^{a}	0 (0)^{a}	0 (0)^{a}
Cow	Cow	177	151 (85.3)^{a}	28 (18.5)	81 (53.6)^{a}	109 (72.2)^{a}	64 (42.4)^{b}	48 (31.8)^{b}
Cat	Pig	114	60 (52.6)^{b}	16 (26.7)	19 (31.7)^{b,c}	35 (58.3)^{b}	0 (0)^{a}	0 (0)^{a}
Pig	Pig	155	87 (56.1)^{b}	18 (20.7)	31 (35.6)^{b,c}	49 (56.3)^{b}	20 (23.0)^{a,b}	4 (4.6)^{a}
Cat	Cat	74	48 (64.9)^{b,c}	11 (22.9)	13 (27.1)^{c}	24 (50.0)^{a,b}	13 (27.1)^{a,b}	9 (18.8)^{a,b}

*Fused oocyte (%), fused oocytes/couples; **cleaved or developed embryos (%), number of cleaved or developed embryos/number of fused oocytes.

$^{a\text{-}c}$ Mean values in the same column with different superscripts are significantly different ($p<0.05$).

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