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γ-LA-Supplementation to IVC for IVP Bovine Embryos

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ABSTRACT : The present study aimed to examine the effects of γ-linolenic acid (GLA) supplementation to *in vitro* culture (IVC) medium on *in vitro* developmental competence, freezability and morphology of *in vitro* matured and fertilized bovine embryos. *In vitro* produced (IVP) bovine zygotes were cultured in IVC medium supplemented with 0 (negative control), 15, 31, 62, 125, 250, 500 or 1,000 ppm GLA, 250 ppm linoleic acid albumin (LAA) and without any supplement as a control. Day 6 blastocysts derived from culture control were cultured in IVC medium containing either 62, 250 GLA or 250 LAA for 24 h, and at Day 7 were subjected to freezing or morphological examination by electron microscope. GLA 15 showed a tendency to have a higher cleavage rate at Day 2 (70.3%) than other groups. The hatching rate at Day 9 in LAA (38.2%) was significantly higher than the control and all treatment groups ($p<0.05$), while the blastocyst rate in LAA (32.4%) did not differ from those of 15 (30.5%), 31 (27.1%), and 62 GLA (33.1%) or the control (35.1%). GLA in concentrations of 125, 250, 500, and 1,000 ppm had significantly detrimental effect on the blastocyst rate compared to 15, 31 and 62 ppm GLA, LAA, and control groups ($p<0.05$). In contrast, the highest post-thaw survival rate (100%) was observed in the control group ($p<0.01$). Large lipid droplets were observed in the cytoplasm of trophoblastic cells, even in the control, but were abundant in GLA groups. Taking the results of the study into consideration, the addition of GLA to the culture medium for IVP bovine embryos at the dose of 15 ppm increased the developmental competence of zygotes and enhanced the cleavage rate up to Day 2. However, blastulation rate and post-thaw survival were not increased when GLA was added to the culture media. (**Key Words :** γ-Linolenic Acid, Freezability, Post-thaw Survival, *In vitro* Produced Bovine Embryo, Trophoblastic Cells)

INTRODUCTION

For decades, *in vitro* produced (IVP) preimplantation embryos of domestic animals have been widely used for research and commercial purposes in many countries (Trounson et al., 1994; Hasler et al., 1995; Rehman et al., 2001; Smeaton et al., 2003). However, in the case of cow embryos, at most 20 to 40% of *in vitro* matured and fertilized oocytes reach the blastocyst stage (Brackett and Zuelke, 1993; Imai et al., 2002; Wheeler et al., 2006). Numerous reports have clarified the important role of the *in vitro* maturation (IVM) (Van De Leemput et al., 1999), and *in vitro* culture (IVC) environment following fertilization to the quality, quantity and freezability of IVP embryos (Rizos et al., 2001; Rizos et al., 2002a; Mucci et al., 2006). IVP embryos were morphologically (Abe et al., 1999; Crosier et al., 2001), and physiologically (Krisher et al., 1999; Rizos et al., 2003), different from embryos recovered from live animals. The proportion of *in vitro* matured/fertilized oocytes cultured *in vivo* can reach the blastocyst stage at a higher rate than that of *in vitro* matured, fertilized and cultured oocytes (Lonergan et al., 2003). Therefore, researchers attempt to improve the IVC environment by supplementing a variety of additives such as amino acids (Wirtu et al., 2004), hormones (Vitt et al., 1998; Kreeger et al., 2005), carbohydrates (Wirtu et al., 2004) and fatty acids (Hochi et al., 1999; Yahia et al., 1999), and to change the culture conditions (temperature, gas concentration, and so forth) (Pratt and George, 1989; Ravindranatha et al., 2003; Squires, 2003). On the other hand, an efficient cryopreservation method is indispensable for the optimum utilization of IVP embryos, since IVP bovine embryos are more sensitive to chilling and freezing than *in vivo* produced bovine embryos (Leibo and Loskutoff, 1993). Therefore, many researches have been trying to improve the qualilty of embryo (Lim et al., 2006), to enhance the

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freezability of embryos, to elect best embryonic stage for freezing (Park et al., 2009), to identify less toxic cryoprotectants, and to optimize cryopreservation procedures (Kaidi et al., 1999).

Addition of linoleic acid combined with albumin (LAA) to embryo culture medium improves the developmental and survival rates after freezing of IVP bovine embryos (Hochi et al., 1999a; Hochi et al., 1999b; Imai et al., 1997). Linoleic acid (LA) is found in cooking oils and processed foods and converts to γ-linolenic acid (GLA) in the body by the enzyme-mediated action of Δ6-desaturase. GLA is then metabolized to dihomo-γ-linolenic acid (DGLA) and arachidonic acid. Arachidonic acid is a precursor of prostaglandin $F_{2\alpha}$ and prostaglandin E_2, which are most closely associated with reproduction. However, in general, stressful conditions (Kojima et al., 1997), and several other factors could suppress the function of Δ6-desaturase. Therefore, the addition of GLA, which is converted by Δ6-desaturase from LA, could obtain a more beneficial effect on the freezability of IVP bovine embryos by enhancing the permeability and fluidity of their cell membranes than the addition of LAA, if the beneficial effect of LAA on post-thaw survival derives from the action of LA as an enhancer of arachidonic cascade.

The aim of the present study is to examine the effects of the addition of GLA to the IVC media on *in vitro* development, post-thaw survival and electron microscopy morphology of IVP bovine embryos, compared with LAA. In the present study, in order to determine the appropriate dose, GLA was added to the IVC media at various doses.

MATERIALS AND METHODS

Oocyte collection and *in vitro* maturation (IVM)

Bovine ovaries were collected from slaughtered Japanese Black cows and heifers at a local abattoir and transported to the laboratory within 4 h in the physiological saline at room temperature. Cumulus-oocyte complexes (COCs) were aspirated from 3 to 8 mm follicles using a 21-gauge needle attached to a 10 ml-syringe. Only those oocytes surrounded by several layers of unexpanded cumulus cells and homogeneous ooplasms were selected and washed twice with TCM199 (Invitrogen Japan K.K., Tokyo, Japan) added 5 mM $NaHCO_3$, 1 mg/ml polyvinylalchol, 100 U/ml penicillin and 100 μg/ml streptomycin (Meiji Seika Co., Ltd., Tokyo, Japan). COCs were washed twice with TCM199 supplemented with 10% fetal bovine serum (FBS; Hyclone Laboratories, Logan, Utah, USA) and 20 μg/ml estradiol (Sigma-Aldrich Co., St. Louis, MO, USA) and antibiotics as maturation medium, and then were cultured in groups of 30 COCs in a 100 μl droplet of the maturation medium in a 35 mm culture dish (Falcon 1008, Becton-Dickinson Ltd., NJ, USA) for 20 to 24 h at 38.5°C with saturated humidity under 5% CO_2 in air.

In vitro fertilization (IVF)

COCs were cultured with frozen-thawed semen from a Japanese Black sire bull (Kubota et al., 1998). Frozen-thawed semen was washed twice by centrifugation in modified Brackett-Oliphant medium (m-BO) (Kubota et al., 1998), supplemented with 10 mM caffeine and 4 mg/ml bovine serum albumin (BSA; Sigma-Aldrich Co., St. Louis, MO, USA) and then resuspended in a fertilization medium (m-BO supplemented with 10 μg/ml heparin and 4 mg/ml BSA). The concentration of spermatozoa was adjusted to 1×10^7 spermatozoa/ml. Approximately 25 COCs were transferred to a 100 μl droplet of the fertilization medium for 6 h at 38.5°C with saturated humidity under 5% CO_2 in air.

In vitro culture (IVC)

After 6 h, presumptive zygotes were washed twice with CR1aa medium added 3 mg/ml BSA (CR1aa-BSA) and randomly allocated into 10 groups. Three μl of GLA (Idemitsu Kosan Co., Ltd., Tokyo, Japan) was dissolved in 1 ml of 99.8% methanol (Wako Pure Chemical Industries, Ltd., Osaka, Japan) and each concentration of 0 (negative control) was adjusted to 15, 31, 62, 125, 250, 500 and 1,000 ppm with CR1aa-BSA. CR1aa-BSA without methanol as control and 250 ppm LAA (Sigma, USA) were also prepared. Therefore, a total of 10 media were examined at serial concentrations of 0, 15, 31, 62, 125, 250, 500 and 1,000 ppm for GLA, LAA and control. After 48 h in IVC, cumulus cells were removed from embryos by gentle pipetting, and the rates of cleaved embryos were recorded. The cleaved embryos were cultured in CR1aa containing 5% FBS (for control) and supplemented with either GLA at the same eight concentrations described above or LAA for another 7 days and the developmental rates to blastocyst stage were recorded at days 7 and 9. All IVC was undertaken at 38.5°C with saturated humidity under 5% CO_2, 5% O_2 and 90% N_2. The developmental stages of the embryos and the numbers were recorded during a period of 10 days.

Freezing and thawing

To examine the effects of transient culture in the presence of GLA on post-thaw survival, embryos in the control were reallocated to 5 groups at day 6 and cultured in CR1aa-5% FBS medium supplemented with either 62 ppm GLA or 250 ppm LAA in a 100 μl droplet for 24 h, and then were subjected to freezing at day 7. Also, day 7 blastocysts which cultured in IVC medium supplemented with either 62 ppm GLA, 250 ppm LAA for 7 days or control were

subjected to freezing. Some blastocysts and expanded blastocysts from control, 62 ppm GLA, and 250 ppm LAA for 24 h or 7 days were examined for viability after freezing and thawing. Embryo freezing and thawing procedures were followed as described in a previous report (Kubota et al., 1998). Briefly, 5% (v/v) ethylene glycol, 6% (v/v) propanediol and 0.1 mol sucrose were used as cryoprotectants in the freezing solution. The embryos were loaded into 0.25-ml straws immediately after equilibration with the freezing solution. The straws were then placed into the cooling chamber of a programmable alcohol bath-freezer (ET-1N, Fujihira Industry Co., Ltd., Tokyo, Japan) and cooled from 0°C to -6.5°C at a rate of 1°C/min. The straws loaded embryos were seeded at -6.5°C, held at that temperature for 10 min, cooled to -30°C at 0.3°C/min, and finally plunged into liquid nitrogen (LN_2). Frozen embryos were stocked in LN_2 for at least 1 week. Thawing was performed by exposure in air for 5 sec and then by immersion in a 30°C water bath until the ice in the straw disappeared (about for 15 sec). Then, embryos were directly transferred to TCM199 supplemented with 100 μmol β-mercaptoethanol (Nacalai Tesque Inc., Kyoto, Japan) and then cultured in the same medium at 38.5°C with saturated humidity under 5% CO_2, 5% O_2 and 90% N_2, to examine their survival rate and hatching rate for 72 h.

Electron microscopy observation of embryos

The embryos from control, 62 ppm GLA, 250 ppm GLA and 250 ppm LAA were examined morphologically with a transmission electron microscope (JEM-1010, JEOL, Tokyo, Japan). Only embryos ranked A morphologically were subjected to the morphological examination. Embryos from GLA, LAA or control were cultured for 24 h or 7 days as described above. Embryos were prefixed with 2.5% glutaraldehyde for 45 min and fixed with 2% osmium tetraoxide for 45 min. After staining with 2% uranyl acetate for 30 min, individual embryo was dehydrated and embedded in TAAB Quetol 653 mixture. Ultrathin sections (80 nm) were stained with uranyl acetate and lead citrate prior to examination with the electron microscope.

Experiment 1: Cleavage and development to blastocyst stage after IVC

In Exp. 1, 1,136 bovine oocytes have been used to examine the effect of GLA supplementation to IVC medium on the embryo development and competence, and to determine the most appropriate dose of GLA. Presumptive zygotes after IVF were allocated to the following groups; 0, 15, 31, 62, 125, 250, 500 and 1,000 ppm for GLA, 250 ppm LAA and control with IVC medium. Data of developmental rates of the embryos were recorded during a period of 10 days.

Experiment 2: Survival after freezing and thawing

In Exp. 2, 98 blastocysts have been used to evaluate the survival rate after slow-freezing and rapid-thawing of temporary (24 h) and whole period (7 days) culture of embryos in media supplemented with either control, 62 ppm GLA or 250 ppm LAA. Blastocysts were allocated to 5 groups as follows; 62 ppm GLA for 24 h (n = 19), 62 ppm GLA for 7 days (n = 17), LAA for 24 h (n = 19), LAA for 7 days (n = 17), and control (n = 26). Data of survival and hatching rates were recorded at 72 h after rapid-thawing of the frozen embryos.

Experiment 3: Observation of embryos with a transmission electron microscope

To examine the electron microscopical pictures of blastocysts cultured in media containing either GLA 62 ppm, GLA 250 ppm, LAA 250 ppm or control for 24 h or 7 days.

Data analysis

The proportions of cleavage, blastulation, hatching of embryos out of the total treated oocytes and the proportions of post-thaw surviving and hatching out of the total cryopreserved embryos were subjected to arc sin transformation and analyzed by one-way analysis of variance. The significance of difference between groups was compared by a post hoc, Fisher's protected least significant difference test (PLSD test). Differences at a probability value (P) of less than 0.05 were considered significant.

RESULTS

Experiment 1: Cleavage and development to blastocyst stage after IVC

The cleavage rates after 48 h of IVC are shown in (Figure 1). The highest cleavage rate (70.3%) among the GLA treatment groups was observed in 15 ppm GLA. The lowest cleavage rate (51.8%) was observed in 1,000 ppm

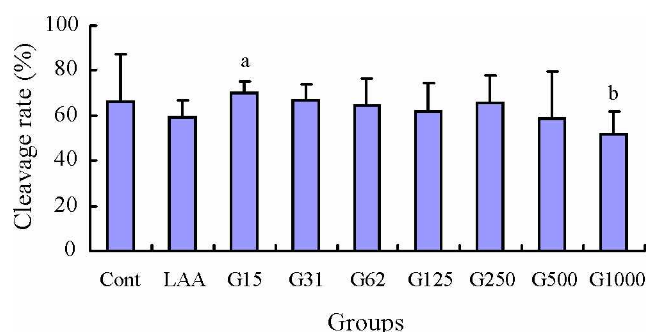

Figure 1. Cleavage rate of IVP bovine embryos cultured in various media for 48 h. Values shown are mean±SD. Letters, a and b differ significantly (p<0.05).

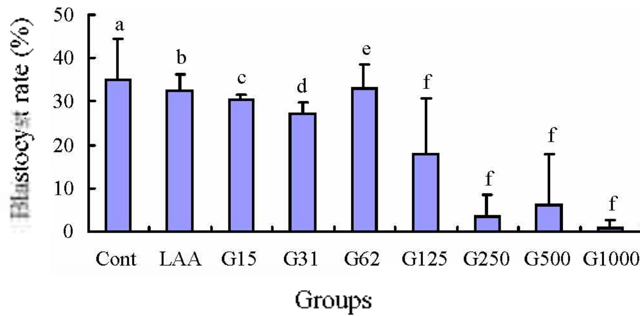

Figure 2. Blastocyst rates of IVP bovine embryos cultured in various media after 7 days of IVF. Values shown are mean±SD. Significant differences were found between (a, f), (b, f), (c, f), (d, f), and (e, f) ($p<0.01$).

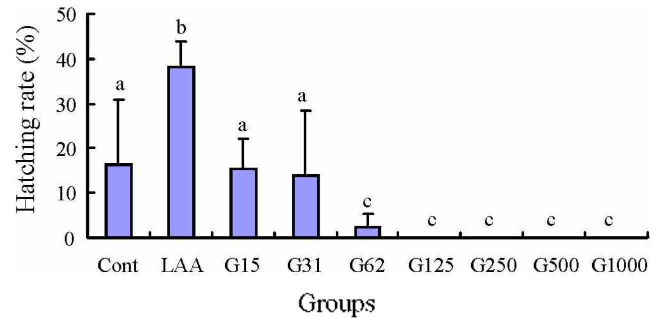

Figure 3. Hatching rates of IVP bovine embryos cultured in various media after 9 days of IVF. Values shown are mean±SD. Significant differences were found between (a, b), (a, c), and (b, c) ($p<0.05$).

GLA. There was no significant difference in the cleavage rate between control, and other treatment groups of GLA and LAA. 125 ppm GLA had a tendency to be more beneficial on developing beyond 5-cell stage embryos but not statistically significant (67.1%; $p>0.05$) among the treatment groups. (Figures 2 and 3) show developmental rates to blastocyst and hatching stages respectively. There was no statistically variation ($p>0.05$) in the blastulation rate between control (35.1%), 15 (30.5%), 31 (27.1%), 62 (33.1%) ppm in GLA and 250 ppm LAA (32.4%). The blastocyst rate on day 7 in control, 15, 31, 62 ppm GLA, and LAA was significantly higher than those of 125, 250, 500 and 1,000 ppm GLA ($p<0.05$). The hatching rate in LAA (38.2%) was significantly higher than control and all of the treatment groups ($p<0.01$). Hatching rate of 62, 125, 250, 500, and 1,000 ppm GLA was significantly lower than control. Based on our results, GLA in the concentration of 125, 250, 500, and 1,000 ppm had detrimental effect on the blastulation and hatching rate of the bovine embryos. No detrimental effect of 0 ppm GLA (negative control) on cleavage and blastulation of embryos was observed. The embryos cultured in the media containing GLA at concentrations of more than 250 ppm looked microscopically dark in comparison with control embryos.

Experiment 2: Survival after freezing and thawing

Figure 4 shows survival rate and Figure 5 shows hatching rates after freezing and thawing of embryos cultured in various media with respect to fatty acids and culture period. The post-thaw survival rate (100%; $p<0.01$) and hatching rate (84.6%; $p<0.05$) in control were highest among all of the experimental groups. Among the treatment groups, 62 ppm GLA for 24 h was numerically higher (73.7%; $p>0.05$) in post-thaw survival rate. The hatching rate in LAA for 7 days (70.6%) was significantly higher than 62 ppm GLA for 7 days (23.5%; $p<0.01$).

Experiment 3: Observation of embryos with a transmission electron microscope

Table 1 summarizes the morphological features of

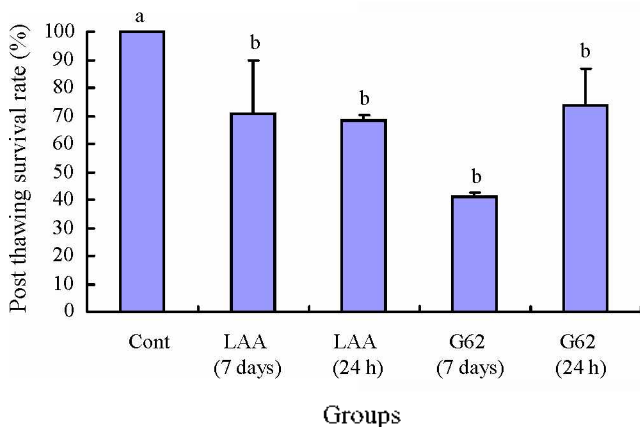

Figure 4. Survival rate after 72 h post-thawing of IVP bovine embryos that were whole period (7 days) or temporary cultured (for 24 h) in LAA 250 ppm or GLA 62 ppm. Values shown are mean±SD. Significant differences were found between (a, b) in the survival rate ($p<0.01$).

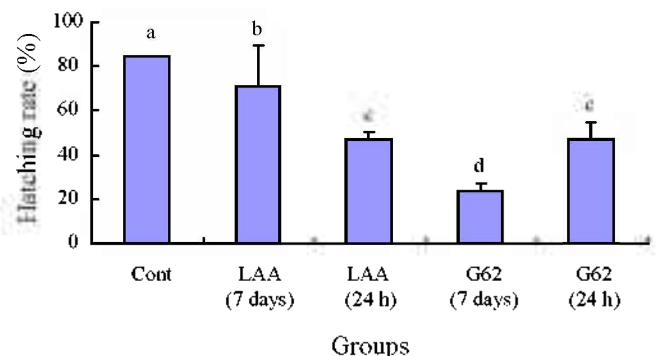

Figure 5. Hatching rate after 72 h post-thawing of IVP bovine embryos that were whole period (7 days) or temporary cultured (for 24 h) in LAA 250 ppm or GLA 62 ppm. Values shown are mean±SD. Significant differences were found between (a, b), (a, c), (a, d) ($p<0.01$), and (b, d) ($p<0.05$).

Table 1. Summarization of transmission electronic microscopy images of day 7 IVP bovine embryos cultured in various media

	Control		LAA		γLA					
			0.25 mg/ml		62 ppm		125 ppm		250 ppm	
	No.1	No.2	24 h	7 d	24 h	7 d	24 h	7 d	24 h	7 d
Zona pellucida										
Irregular shape of blastocyst	-	-	-	+	-	Slightly+	-	+	-	-
Thickness (mm)	4.1	4.1	3.7+1.2 [2]	3.1	4.3	3.8	4.5	4.2	2.9	6
Degeneration	-	-	Outer+ [2]	-	-	-	-	+	-	-
Space between the trophoblast	-	-	+	-	+	-	p+	-	-	+
Expanded blastocoel	-	-	-	+	-	-	-	+	-	-
Degenerative cells between the ZP and trophoblasts	-	-	-	-	-	-	-	-	+	-
Trophoblast										
Microvilli; long	-	-	+	p+[3]	+ 0.8	-	+ 0.7	p+	p+	p+ 0.6
Microvilli; scanty	-	-	+	-	p+	-	p+	-	-	p+
Degeneration	-	-	-	p+	p+	+	-	-	-	-
Lipid droplet; rich	+	+	+	+	+	+accumulative	+accumulative	+	+	+
Lipid droplet; large (mm)[1]	p+ 3	p+ 5.5	-	p+ 2.5	p+ 3.1	p+ 3	p+ 2	p+ 2	p+ 2	p+ 2.1
Apoptosis	-	-	-	- [4]	-	-	-	-	-	-
ICM										
Degeneration	+	+	+	+	+	+	+	+	+	+
Lipid droplet; rich	+	+	+	+	+	+	+	+	+	+
Lipid droplet; large (mm)	-	p+ 8.2[5]	p+ 3	p+ 2.5	p+	p+	p+	p+	p+	p+
Apoptosis	+	+	+	+	+	+	+	+	+	+
Degenerative nuclei	+	-	+	+	+	-	-	-	+	+
Lipid deposition in the degenerative nuclei	+	-	-	-	+	-	-	-	+	+
Interstitium of the cells; wide	+	-	+	-	-	-	+	-	-	-

p+: partially positive

[1] Large lipid droplets are also existed in the control trophoblasts. [2] Observed 2 layers, i.e., outer and inner.

[3] No interstitium. [4] Expanded blastocyst. [5] Large lipid droplets are existed in the degenerative ICM.

blastocysts observed with the electron microscope. Microvilli of trophoblastic cells obtained from LAA or any concentrations of GLA groups were comparatively longer and distributed sparsely. Consequently, some spaces were observed between the zona pellucida and trophoblastic cells. In all groups including control, small lipid droplets were observed in the cytoplasm of the ICM cells and the trophoblastic cells. Large droplets (3 to 5 μm in diameter) were observed in the cytoplasm of trophoblastic cells even in those of control. As to the size of lipid droplets, there was no difference between control, GLA and LAA groups. However, in GLA groups, an accumulation of large lipid droplets was observed in the cytoplasm of the trophoblastic cells. Apoptosis of some cells in ICM was observed in both control and GLA groups. Deposition of lipid droplets was prominent in the area of the degenerative nuclei of ICM cells. Degenerative cells were observed between the zona pellucida and trophoblastic cells in 250 ppm GLA for 24 h.

DISCUSSION

The hypothesis of the present study was that addition of GLA, which is converted by Δ6-desaturase from LA, could produce more beneficial effect on the freezability of IVP bovine embryos by possibly enhancing the permeability and fluidity of their cell membranes than the addition of LAA, if the beneficial effect of LAA on post-thaw survival is derived from the action of LA as an enhancer of arachidonic cascade. In humans, some studies showed growth failure in infants who lacked sufficient Δ6-desaturase activity (Caldwell et al., 1972), and/or its vital co-workers-vitamin C, vitamin B6, vitamin B3, zinc and magnesium. Δ6-desaturase is responsible for converting LA to GLA as described earlier. Moreover, dietary GLA and arachidonic

acid have superior biopotency to LA (Chapkin, 1998).

Several researches conducted previously showed no effect of LAA on the embryo development and cleavage rate (Hochi et al., 1999a; Imai et al., 1997; Tominaga et al., 2000). In the present study, bovine embryos cultured in IVC media containing various concentrations of GLA showed varied results with respect to developmental competence. A higher cleavage rate was obtained in 15 ppm GLA (70.3%; $p>0.05$) after 48 h, but it was not significant compared to control (66.1%; $p>0.05$) and 250 ppm LAA (59.0%; $p>0.05$). The previous studies referred to the 250 ppm LAA as the best concentration for improving the post-thaw survival rate but has no effect on the development rate, while in the present study we showed that supplementation of 15 ppm and 62 ppm GLA were superior (but not statistically significant) in cleavage and blastocyst rates to LAA. In the present study we found that the optimum concentration of GLA was 62 ppm, based on the blastulation rate. Among the treatment groups, however, the highest hatching rate on Day 9 was observed in LAA (38.2%; $p<0.01$) followed by control (16.4%), while the lowest hatching rate was in 62 ppm GLA (2.4%).

Several other studies showed that the composition of IVC medium caused morphological changes of embryos (Abe et al., 1999), resulting in variation of developmental rate and post-thaw survival rate (Imai et al., 1997; Imai et al., 2002). LAA supplementation improves the survival rates of *in vitro* produced bovine 16-cell embryos (Hochi et al., 1999a; Hochi et al., 1999b; Tominaga et al., 2000), blastocysts and expanded blastocysts after freezing and thawing (Imai et al., 1997). In the present study the post-thaw survival rate had a tendency to be higher in 62 ppm GLA for 24 h (73.7%) compared to LAA for 24 h (68.4%) and LAA for 7 days (70.6%) while the best survival rate was observed in the control (100%). In previous studies, the post-thaw survival rate of IVP bovine embryos was 68% in LAA for 7 days (Mucci et al., 2006).

An inverse correlation was found between embryo lipid contents and post-thaw survival rate in several studies (Diez et al., 1996; Diez et al., 2001; Mucci et al., 2006). Since bovine embryos cultured in serum-containing medium showed abundant accumulation of lipid content in cytoplasm and significantly lower post-thaw survival (Abe et al., 1999; Rizos et al., 2003). In contrast, a medium without serum, which showed lower blastulation rate and lower lipid contents than a medium containing serum, caused higher post-thaw survival of IVP bovine blastocysts (Rizos et al., 2003; Mucci et al., 2006). The species-specific difference of oocytes' fatty acids content represents an important factor in cryopreservation sensitivities (McEvoy et al., 2000; Rizos et al., 2002b). The sensitivities may be due mainly to PUFA contents in their oocytes (McEvoy et al., 2000). Since pig oocyte has three- to four-fold greater linoleic acid content than ruminant oocytes (McEvoy et al., 2000), and it shows lower post-thaw survival. It may be due to the high abundance of fatty acids in the form of triglyceride in porcine oocytes comparing to bovine and ovine oocytes (McEvoy et al., 2000). One study suggested improvement in the maturation and cryopreservation by alteration of fatty acid constitution of bovine oocytes (Kim et al., 2000). Other studies found more successful cryopreservation after delipidation of porcine embryos (Abe et al., 2002), and bovine embryos (Diez et al., 2002). In the present study all embryos, even embryos in control, cultured in IVC medium in spite of kinds or amounts of fatty acids supplemented were observed to have lipid droplets in their cytoplasm of ICM and trophoblastic cells, especially embryos in GLA showed accumulation of large lipid droplets in the cytoplasm of the ICM cells. We found that the lowest post-thaw survival and hatching rate was obtained in GLA for 7 days in the present study. These findings support the results of previous studies showing inverse correlation between the accumulation of lipids and post-thaw survival. In the present study, we suggest that low post-thaw survival might result from accumulation of the lipids in embryos cultured in IVC media containing GLA, which could explain the darkness of blastocysts cultured in concentrations of over 250 ppm GLA.

Administration of dietary fatty acids in the form of plant or fish oils to pregnant sows resulted in a higher survival rate of piglets in the treatment group than in the control group (Perez Rigau et al., 1995). A paper published previously by the co-author of the present paper showed that gilts supplemented with dietary GLA during mid-summer produced increasing numbers of corpora lutea, recovered embryos and embryos with normal morphology than other gilts supplemented with other dietary linoleic acid or α-linolenic acid (Kojima et al., 1997). Also, ewes supplemented with ω-3 PUFA enriched diet produced an increased number of high quality oocytes, and the integrity of the oocytes following chilling was improved (Zeron et al., 2002). On the other hand, the balance between ω-3 and ω-6 PUFA appeared to be important for normal growth and development, stimulation of skin and hair growth, regulation of metabolism, and maintenance of reproductive processes (Squires, 2003). The imbalance between ω-3 and ω-6 PUFA can alter the structural properties of cell membranes, such as fluidity and permeability (Squires, 2003), since ω-3 and ω-6 PUFA compete for same enzymes in order to produce their final product. It was shown in humans that the conversion of deuterated α-linolenic acid of ω-3 PUFA and LA of ω-6 PUFA to longer-chain metabolites was reduced by 40-54% when dietary intake of LA was increased from 15 to 30 g/d as a result of competition between ω-3 and ω-6 PUFA for desaturase and

elongase enzymes (Emken et al., 1994). Therefore, the balance between ω-3 and ω-6 PUFA in case of PUFA supplementation to IVC media should be considered.

In conclusion, the results of the present study indicate that addition of GLA to IVC medium for IVP bovine embryos in the dose of 15 ppm increases the developmental competence of presumptive zygotes and enhances the cleavage rate up to 48 h. However, the blastulation rate and the post-thaw survival did not increased when GLA added to the culture media. Further studies are needed to investigate the optimum utilization of PUFA including GLA as beneficial supplementation to improve *in vitro* development and freezability of IVP bovine embryos, based on understanding the action mechanism of PUFA supplemented to IVC medium for IVP bovine embryos.

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