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Original article

The effect of oleic and linoleic acid addition to the culture media on bovine embryonic development following vitrification

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Abstract

In this study, the effects of oleic (18:1 cis-9-octadecenoic acid) and linoleic (18:2 (n-6), 9,12-octadecadienoic acid) acids added to the embryo culture media for bovine embryonic development after vitrification were investigated in cattle. Following maturation and fertilization, the oocytes were placed in Charles Rosencrans (CR1aa) culture drops containing 10, 100, 500, and 1000 μM of oleic or linoleic acids. On day 7 or 8 of the culture, the blastocysts and expanded blastocysts were vitrified and warmed to evaluate the viability and development. High doses of oleic acid (1000 μM) in the culture media increased the viability of embryos after vitrification. Similarly, linoleic acid at 1000 μM increased the viability compared to the other linoleic acid doses. It was observed that the addition of essential fatty acids improved the development of embryos. Increasing the concentration of linoleic and oleic acid concentrations in the media proportionally advanced the embryonic development and hatching capability after vitrification/warming. Specifically, the addition of high doses of oleic acid had dramatic effects on the embryonic development after vitrification/warming probably due to the increased lipid storage. In conclusion, the present results suggest that the ratio of unsaturated fatty acids in the culture media affects significantly the embryonic development *in vitro*.

Key words: embryo, linoleic acid, oleic acid, vitrification

Introduction

The capability of embryos after the vitrification method demonstrates the success of assisted reproduction (Lane et al. 2002). The culture media, freezing protocols, and stress factors affect significantly the viability of embryos produced in *in vitro* (Massip et al. 1995, Lane et al. 2002). Osmotic stress due to dehydration, toxic effects of cryoprotectants, and damage

in the cell membrane are important stress factors (Lane et al. 2002).

One reason for poor resistance of *in vitro* produced embryos to freezing is the components such as fetal calf serum (FCS) included in the culture medium (Rizos et al. 2003, Pereira et al. 2007). The FCS in the culture medium increases the intracellular lipid stocks of the embryo and the higher lipid level enhances the susceptibility of the embryo to the freezing damage (Alminana

and Cuello 2015, Meneghel et al. 2017). The fatty acids are known to have important roles in embryonic development (McKeegan and Sturmey 2011). Long chain fatty acids, commonly found in the mammalian oocytes, are rich in energy. Unsaturated fatty acids such as oleic acid may affect cell membrane fluidity and the viability of oocytes/embryos after freezing (Zhang et al. 2012). Although oleic acid in cell cultures promotes the accumulation of triglycerides, it also acts as a protective agent against the lipotoxicity of triglycerides (Listenberger et al. 2003).

In the vitrification method, the embryos are treated with a high concentration of solutions and the glass-like solidification of ice without crystallization occurs in solutions with rapidly increasing viscosity (Kizil et al. 2007). A major disadvantage of vitrification is the requirement of high concentration of cryoprotectants and associated embryo toxicity (Pugh et al. 2000). Previous studies have shown that fatty acids have positive effects on normal oocyte maturation, fertilization, and embryonic development (Khandoker and Tsujii 1999, Kim et al. 2001).

Effects of polyunsaturated fatty acids differ depending on the cell types (Wang et al. 2009). In the cultured endothelial cells, linoleic acid increased the amount of intracellular calcium while producing superoxide and nitric oxide products (Saraswathi et al. 2004). However, linoleic acid was reported to protect cells against oxidative stress in insulin-secreting cell lines and primary normal human fibroblasts (Beeharry et al. 2003, Beeharry et al. 2004). The addition of trans-10, cis-12 octadecadienoic acid (t10, c12 CLA), a derivative of conjugated linoleic acid, to the culture media of bovine embryos has been shown to increase the viability of embryos after freezing-thawing (Pereira et al. 2007). After the addition of linoleic acid to the serum-containing culture media after freezing-thawing, an increase in the development rates of the morula stage embryos was observed (Hochi et al. 1999). Aardema et al. (2011) examined the effects of three different fatty acids (saturated palmitic, stearic acid, and unsaturated oleic acid) on the development of bovine oocytes. Palmitic and stearic acids had dose-dependent inhibitory effects on the amount of fats stored in lipid droplets and showed a detrimental effect on oocyte development. In contrast, they reported that oleic acid significantly increased lipid deposition in lipid droplets and increased oocyte growth. Remarkably, the negative effects of palmitic and stearic acids were eliminated in the presence of oleic acid. These results showed that the saturated and unsaturated fatty acid ratios and amounts were suitable for lipid storage in the maturing oocyte and this was related to the developmental competence of maturing oocytes. Esterification of fatty

acids and storage of lipid droplets also protect oocyte from fatty acid-induced lipotoxicity (Listenberger et al. 2003). The fatty acid composition of high- and low-quality oocytes is different: high-quality oocytes contain more oleic, linoleic and arachidonic acid (Kim et al. 2001).

There is a lack of investigation regarding the effects of fatty acids, especially oleic acid, on the stage of development of bovine embryos *in vitro* after vitrification. The aim of this study was to evaluate the effects of oleic and linoleic acid addition into the embryo culture medium on the bovine embryo development *in vitro* after vitrification.

Materials and Methods

Permission for the study was received from the Hayvancılık Merkez Araştırma Enstitüsü Animal Experiments Local Ethics Committee (2011/56) before the experimental process. All materials used in this study were obtained from Sigma Chemical Co. (St. Louis, MO, ABD) and Gibco (Grand Island, NY, ABD).

Oocyte collection and maturation

The ovaries for oocyte collection were obtained from a slaughterhouse in Çubuk, Ankara. The location of the slaughterhouse was 40°14' North 33°01' East and 985 m above the sea level. The ovaries were transferred to the laboratory in 0.9% NaCl containing 125 mg/L kanamycin sulphate. Follicles at sizes of 2-8 mm were aspirated and washed in Dulbecco modified phosphate buffer solution (D-PBS). Maturation of oocytes were performed in tissue culture medium (TCM-199) containing 10% FCS + 2 µg/mL FSH. A total of 20 oocytes was placed in 100 µl TCM199, covered with mineral oil, and incubated for 22 hours at 5% CO₂, 95% humidity, and 38.5°C.

In vitro fertilization (IVF)

Fertilization of oocytes was performed in Bracket & Oliphant (BO) medium using Holstein sperm thawed with direct sperm washing method. In order to avoid differences in *in vitro* fertilization, sperm collected from a single bull and with equivalent motility were used. BO medium was used in the capacitation of sperm. In order to obtain the capacity and intensity of spermatozoa, the fertilization medium was prepared according to the method proposed by Saito et al. (1994). Four drops of 100 µl BO solution were prepared in 35 mm dishes and each drop was covered with mineral oil. A total of 20 oocytes was placed in 100 µl BO solution. 25000 spermatozoa for a mature cumulus-

Table 1. Total viability number of bovine embryos after vitrification in the groups.

Culture medium (Total)	Vitrified warmed embryo	Viable embryo ratio %	Hatching embryo rate %
Linoleic	125	72.00(90) ^b	20.80(26) ^b
Oleic	180	83.89(151) ^a	31.67(57) ^a
Control	27	70.37(19) ^b	18.52(5) ^b

Within a column, means without a common superscript differ ($p < 0.05$, Kruskal-Wallis Test).

Different letters within a column are significantly different between treatments

Table 2. Viability after vitrification in bovine embryos in the control and treatment groups.

Culture medium	Vitrified warmed embryo	Re-expansion 24 hours after warming	Viable embryo ratio %	Hatching embryo 72. hour after thawing	Hatching embryo rate %
Linoleic 10	33	23	69.70	7	21.21
Linoleic 100	25	17	68.00	5	20.00
Linoleic 500	30	22	73.33	6	20.00
Linoleic 1000	37	28	75.68	8	21.62
Oleic 10	38	30	78.95	11	28.95
Oleic 100	44	37	84.09	13	29.55
Oleic 500	46	39	84.78	14	30.43
Oleic 1000	52	45	86.54	19	36.54
Control	27	19	70.37	5	18.52

* Chi-Square Test ($p > 0.05$)

-oocyte complex were added for fertilization and incubated for 5-6 hours.

***In vitro* development (IVD)**

Following *in vitro* fertilization, the cumulus cells surrounding the oocytes were removed by pipetting. The oocytes were then taken to the culture drop of CR1aa. The fertilized oocytes were allocated into three groups: 1) Linoleic acid treatment (10, 100, 500 and 1000 μM), 2) Oleic acid treatment (10, 100, 500 and 1000 μM), 3) control group with no treatment, and placed in an incubator (5% CO_2 , 95% humidity, 38.5°C). The embryonic development was monitored at 48 hours and day 7 and 8.

Vitrification of embryos

Embryos were vitrified according to the method of Saito et al. (1994). *In vitro* production (IVP) embryos and vitrification solutions were equilibrated at room temperature (25°C). In the blastocyst and expanded blastocyst stages, embryos of 7 and 8 days were washed twice in PBS solution containing 20% calf serum (CS) and then, were taken into the vitrification medium. For the purpose of vitrification, the embryos were incubated in vitrification solution 1 (VS1: 0.1M sucrose + 0.1M xylose + 1% PEG + 10% glycerol) and

VS2 (0.2M sucrose + 0.2M xylose + 2% PEG + 10% glycerol + 10% ethylene glycol) 5 minutes and followed by 1 minute in VS3 (0.3M sucrose + 0.3M xylose + 3% PEG + 20% glycerol + 20% ethylene glycol). The embryos were then placed in 0.25 ml straws (one embryo per straw) and the straws were put directly in liquid nitrogen.

Warming procedure

The straws with the embryos were removed from the liquid nitrogen and kept in air for about 6 seconds and then at 20°C water for about 10 seconds until fully dissolved. The embryos were taken out of the straws and incubated at 30°C in 0.5 M sucrose for 5 min and 0.25 M sucrose for 5 min for devitrification. Devitrified embryos were then transferred into PBS solution containing 20% CS and washed in 3 different droplets. The embryos transferred to the CR1aa medium were washed 3 times. The embryos were taken into CR1aa culture drops and were controlled for development at 48 and 72 hours after warming in an incubator at 38.5°C containing 5% CO_2 and 95% humidity. Re-expansion, development of embryos and hatching of blastocysts were recorded at 48 and 72 h post-warming.

Statistical analysis

Comparisons among dose effect (oleic, linoleic) on post-warming embryo survival were performed using the Chi-square test. Kruskal Wallis test was used to compare the groups. The statistical significance level was accepted as 5% and the SPSS statistical package program was used for the calculations.

Results

Total linoleic viability ratio post-warming was determined as 72%. Total oleic viability ratio after warming was determined to be 83.89%. The viability ratio after warming of control group was determined to be 70.37%. Detailed results for viability and hatching of embryos in each group by concentrations of the fatty acids used and total results for each group were presented in Tables 1 and 2. When the results of the different doses of each group were compared, it was observed that the ratio of embryos that maintained viability in the oleic acid group was higher than the linoleic and control group and this was statistically significant ($p < 0.05$). The same ratio in the total linoleic acid group was higher than that in the control group (Table 1). Hatching ratio of the embryos after warming was higher for oleic acid group compared to the other groups ($p < 0.05$) and the linoleic acid group presented higher results than those found in the control group. Compared to the different doses of oleic and linoleic acid the statistical difference was not important. It was observed that linoleic and especially oleic acid gave better results as the dose increased (Table 2).

Discussion

This study was performed to evaluate the response of the supplementation of oleic and linoleic acids at different doses on the embryo development and viability after vitrification. Freezing of oocytes and embryos increases susceptibility to oxidative stress, thus reduces the chances of survival of embryos after warming (Nedambale et al. 2006). Especially, *in vitro* produced bovine embryos are more susceptible to freezing (Al-Katanani et al. 2002, Rizos et al. 2003, Gomez et al. 2008). Antioxidants added to the embryo culture media are expected to increase the viability of embryos after warming. This study is the first to investigate the effect of oleic acid in the culture media on the viability of *in vitro* produced and vitrified bovine embryos after warming. In this study, after the vitrification/warming of bovine blastocysts, high survival and hatching rates were obtained on days 7 and 8 *in vitro*. Vitrification

is a simple, fast and economical method for long-term storage of bovine embryos (Gupta et al. 2016). Pereira et al. (2007), using conjugated linoleic acid, reported that embryos frozen by the slow-cooling procedure significantly improved their ability to maintain post-thawing viability and to re-expand following cryopreservation. However, the mechanism by which linoleic acid improves embryo survival after freezing has not been elucidated. One explanation for that could be the specific effects of 10t, 12c CLA that reduce the synthesis and uptake of fatty acids by adipocytes (Pereira et al. 2007).

The present study also reports that oleic and linoleic acid in the culture media increased embryonic viability after vitrification. Modifications of fatty acid profile induced by 10t, 12c CLA during the embryo culture may also contribute to the increased embryo resistance against cryopreservation (Pereira et al. 2007). The ratios of re-expansion and hatching obtained in this study were found to be higher than those obtained without the addition of any antioxidants to the culture media by Gomez et al. (2008). Specifically, the results from the oleic acid groups were thought to be the positive effect of this fatty acid since other studies by Shirazi et al. (2009) using TCM-199 media, as in this study presented, lower ratios of survival and hatching without oleic acid. Bovine embryos produced by the IVF method are usually very sensitive to cryo-damage due to the intracellular and membrane disturbances caused by the change of water and cryoprotectant substances between the cell and the extracellular environment after vitrification (Dinnyes et al. 1999).

Generally, polyunsaturated fatty acids and especially linoleic acid support a number of key developmental processes in the embryo. They act as precursors of eicosanoids and regulate endocytosis/exocytosis, ion-channel modulation, DNA polymerase inhibition, and gene expression (Haggarty et al. 2006). The proportion of unsaturated fatty acids in the embryo is positively correlated with the chance of embryo survival (Haggarty et al. 2006). In the present study, especially high doses of oleic acid increased the viability and hatching ratios of embryos after vitrification/warming process. Although 500 and 1000 μM doses of linoleic acid increased the viability rate, low doses were not as effective. This is similar to a previous report by Darwich et al. (2010) who employed unsaturated fatty acids such as linoleic acid, linolenic acid, and docosahexaenoic acid. Except for 100 μM of linolenic acid and 10 and 100 μM of docosahexaenoic acid, fatty acid addition to the culture media had no effect on the embryonic development after warming (Darwich et al. 2010). Our results were in accordance with those of Pereira et al. (2007) where high doses of oleic and

linoleic acid supported embryo development. Similarly, it also supports the previous studies that oocytes obtained from PUFA-fed sheep were of better quality and more resistant to freezing (Zeron et al. 2002).

Kim et al. (2001) found that in fresh and frozen-thawed immature bovine oocytes, the quality of oocytes increased as their concentrations of oleic, linoleic and arachidonic acids increased. In our study, it was seen that the viability ratio of the embryos increased after vitrification as the amount of oleic and linoleic acid increased. In *in vitro* produced embryos, there is a dense accumulation of lipids, especially in the membrane (Abe et al. 2002). Perhaps, the most important factor affecting the viability of embryos in the freezing process is the presence and distribution of lipids. Excessive lipid accumulation was suggested as the main cause for reduced cryotolerance of IVP embryos (Abe et al. 2002). The specific mechanism through which lipid accumulation in the IVP embryos influenced cryotolerance is unknown; however, peroxidation of the lipids might account for this decrease (Seidel 2006). Aardema et al. (2011) noted that effective storage of the esterified fatty acids in the lipid droplets may prevent an increase in the lipotoxic effects that may result from fatty acids present in the cell. This is similar to our observation that the high oleic acid concentration causes storage of neutral lipids and does not affect the developmental efficacy of freeze-thawed embryos.

As a result, it was observed that essential fatty acids made significant contributions to embryo development after vitrification/warming. As the amount of linoleic acid and oleic acid in the media increased, the embryonic development and hatching ability after warming were enhanced. High doses of oleic acid had particularly remarkable effects on the embryo development, which is likely to result from an increase in the lipid storage. Therefore, it can be concluded that the ratio of unsaturated fatty acid found in the culture medium affected positively the embryonic developmental capacity.

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