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Research

Lipid and fatty acid analysis of fresh and frozen–thawed immature and *in vitro* matured bovine oocytes

J. Y. Kim[1], M. Kinoshita[2], M. Ohnishi[2] and Y. Fukui[1]*

[1]*Laboratory of Animal Genetics and Reproduction and* [2]*Laboratory of Applied Bioscience, Obihiro University of Agriculture and Veterinary Medicine, Obihiro 080-8555, Japan*

The lipid content and fatty acid composition of fresh immature and *in vitro* matured bovine oocytes cultured in media with or without serum, and also those of frozen–thawed immature oocytes were analysed. All oocytes were ranked (A or B) on the basis of their cytoplasmic quality. Fatty acid composition (mol %; w/w) in the total lipid fraction was analysed by gas chromatography. Triglyceride, total cholesterol, phospholipid (phosphocholine-containing phospholipid) and non-esterified fatty acid contents of immature and *in vitro* matured oocytes were determined using lipid analysis kits. Phosphocholine-containing phospholipid and non-esterified fatty acid contents were determined in frozen–thawed immature bovine oocytes. Palmitic acid was the most abundant fatty acid in immature oocytes (A: 35%, B: 36%), and in *in vitro* matured oocytes cultured in the medium containing serum (A: 36%, B: 35%) or polyvinyl alcohol (A: 33%, B: 36%). Oleic acid was the second most abundant fatty acid in all A ranked oocytes, whereas stearic acid was the second most abundant fatty acid in all B ranked oocytes. There were significant differences ($P < 0.05$) in linoleic and arachidonic acid fractions between A and B ranked immature oocytes. *In vitro* matured oocytes had significantly ($P < 0.05$) lower proportions of linoleic and arachidonic acids, and significantly ($P < 0.01$) lower contents of triglyceride and total cholesterol compared with those of immature oocytes. The fatty acid composition of *in vitro* matured oocytes cultured in medium containing fetal calf serum or polyvinyl alcohol was similar, but significant differences ($P < 0.01$) in triglyceride and the total cholesterol content were observed. There was a significant decrease ($P < 0.05$) in the arachidonic acid proportion in frozen–thawed immature oocytes compared with that in fresh immature oocytes. In addition, significant ($P < 0.05$) decreases in both phospholipid (15.8–10.6 pmol) and non-esterified fatty acid (11.0–4.1 pmol) were found in frozen–thawed immature oocytes. The results indicate that lipids are available for use as an energy source for maturation and that serum lipids are incorporated into the oocyte cytoplasm during *in vitro* maturation. The changes in the lipid content (mainly phospholipid) and fatty acid composition were also observed in frozen–thawed immature oocytes. The study indicates that the alteration of fatty acid composition in bovine oocytes might improve maturation and cryopreservation.

Introduction

Lipid plays a significant role in energy storage, cell structure and in modifying the physical properties and metabolic function of biological membranes. Studies on lipids in bovine oocytes and embryos have examined the fatty acid composition and triglyceride content (Ferguson and Leese, 1999; Zeron *et al.*, 1999a; McEvoy *et al.*, 2000). Other reports have indicated that intracellular lipid may have an important influence on sensitivity to freezing (Diez *et al.*, 1996; Dobrinsky, 1996; Otoi *et al.*, 1997; Sata *et al.*, 1999). However, in terms of qualitative and quantitative analysis of fatty acid and lipid content, data on bovine oocytes are still limited. In particular, it has not been established whether storage and membrane lipids influence oocyte maturation and cryopreservation.

*Correspondence
Email: fukui@obihiro.ac.jp

Oocytes of all species are sensitive to low temperatures (Leibo *et al.*, 1995). For several decades, limited success has been achieved after thawing of cryopreserved bovine oocytes. It has been reported that the cytoplasmic membrane is the major organelle sensitive to low temperature (Schmidt *et al.*, 1993; Arav *et al.*, 1996). The physical properties of this membrane are influenced by lipid composition and are regulated in response to environmental factors (temperature or diet) (Gurr and Harwood, 1991). Thus, understanding of the involvement of cytoplasmic and membrane lipids in oocyte sensitivity to low temperature and freezing damage is required to address the difficulties of development after cryopreservation of oocytes.

Serum is a common constituent of culture media. Although serum can be a useful source of growth factors and other cellular mitogenic factors, there are some disadvantages to its use. In ruminant embryos developed in a serum-supplemented medium, morphological differences, such as abundant cytoplasmic lipid droplets, have been observed (Thompson *et al.*, 1995; Abe *et al.*, 1999). Several

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studies have focused on the influence of serum in view of lipid accumulation and the changes in the fatty acid composition in oocytes and embryos (Ferguson and Leese 1999; Sata *et al.*, 1999). Familiarity with the lipids present in serum and with the mechanism for transfer of these lipids to the cells is necessary to understand the mechanism involved in the lipid nutrition of cells in culture (Spector, 1972). Furthermore, detailed studies on morphological characteristics of oocytes are required to provide workable criteria for the selection of the best quality oocytes for *in vitro* production of bovine embryos.

The aim of the present study was to measure quantitatively the lipid content and fatty acid composition in fresh immature and *in vitro* matured bovine oocytes cultured in media with or without serum, and the lipid content (mainly phospholipid) and fatty acid composition in frozen–thawed immature oocytes.

Materials and Methods

Collection of oocytes and sample preparation

Ovaries were collected from Holstein cows at a local abattoir and transported within 3 h to the laboratory in 0.9% (w/v) NaCl at 30–35°C. Cumulus–oocytes complexes (COCs) were collected by aspirating follicles of 2–8 mm in diameter with an 18-gauge needle followed by three washes in Dulbecco's PBS (Sigma, St Louis, MO). COCs were selected and classified as A or B using the criteria of de Loos *et al.* (1989). COCs ranked as A, equivalent to category 1 of the criteria described by de Loos *et al.* (1989), had homogeneous ooplasm and compact multi-layered cumulus cells tightly adherent to the zona pellucida. COCs ranked as B, combining categories 2 and 3 of the criteria of de Loos *et al.* (1989), were of coarse appearance with a dark zone at the periphery of the oocytes, but also had multi-layered cumulus cells tightly adherent to the zona pellucida. COCs ranked as A or B were assigned for freezing and thawing, and *in vitro* maturation, or were prepared immediately for lipid extraction. COCs were denuded by vigorous pipetting in four-well dishes (NUNCLON; Nunc, Roskilde) with 500 µl of 1% (w/v) sodium citrate. The denuded immature oocytes were washed three times in 500 µl PBS with 0.3% BSA (essentially fatty acid free; Sigma) with repeated gentle pipetting. Oocytes were transferred to a Petri dish containing 3 ml PBS with 0.1% BSA for reselection, and were washed three times. Finally, oocytes were washed twice in PBS without BSA. Subsequently, 250–400 fully denuded oocytes with intact zonae pellucidae were centrifuged at 4000 ***g*** for 5 min and resuspended by vortexing in 500 µl PBS. Oocytes were transferred into a glass vial with 1.0 ml PBS for total lipid extraction.

Freezing and thawing

COCs were frozen–thawed using techniques reported by Asada *et al.* (2000). Dulbecco's PBS containing 10% (v/v) fetal calf serum (FCS), 1.8 mol ethylene glycol l^{-1} and 0.1 mol sucrose l^{-1} was used as the cryoprotective medium. Ranked COCs were transferred to a cryoprotective medium and were equilibrated for 10 min at room temperature (20–25°C). During the equilibration, groups of 20–25 COCs were loaded into 0.25 ml straws (Fujihira Industry, Co., Tokyo), powder-sealed and transferred into a pre-cooled methanol bath (0°C) of a programmable freezer (ET-1, Fujihira). The straws were cooled from 0°C to –6°C at 0.5°C per min. At –6°C, ice formation (seeding) was induced in the medium by touching the straw with frozen forceps. After 12 min, cooling was resumed at 0.3°C min^{-1} to –35°C. The straws were placed in liquid nitrogen vapour for 10 s and immersed in liquid nitrogen. Before lipid analysis, straws were thawed by exposure to the air for 10 s at 25°C and immersed immediately in water at 37°C for 20 s. The COCs were denuded and washed three times in PBS containing 0.3% BSA. Intact oocytes with no discoloration of the ooplasm, cell disruption or pyknosis or those that had a dark ooplasm were selected according to the criteria described by Al Hasani and Diedrich (1995). Selected frozen–thawed oocytes were washed twice in PBS containing 0.1% BSA and then washed in PBS without BSA. Oocytes were subsequently treated for lipid extraction in the same manner as fresh immature oocytes.

In vitro *maturation*

COCs were matured *in vitro* using procedures modified from those of Lee and Fukui (1995). Briefly, COCs were cultured for 24 h in four-well dishes containing tissue culture medium 199 (TCM-199 with Earle's salts and glutamine; ICN Biomedicals, Inc., Costa, CA) supplemented with either 10% heat-inactivated FCS or 0.1% (w/v) polyvinyl alcohol, 1 µg oestradiol ml^{-1} (Sigma) and 0.02 AU FSH ml^{-1} (Antrin, Denka Chemical Co., Ltd, Kawasaki) (35–40 COCs per well), at 39°C in 5% CO_2 and 95% humidified air.

After maturation, the COCs were denuded and washed as described for immature oocytes. The oocytes were examined using a microscope to determine the stage of maturation. Only those oocytes that had extruded the first polar body were selected and prepared for total lipid extraction.

Total lipid extraction

Total lipids of fresh and frozen–thawed immature bovine oocytes and those of *in vitro* matured oocytes were extracted using the procedure of Bligh and Dyer (1959), with a slight modification. The samples (n = 250–400), suspended in 1.0 ml PBS, were added immediately to 3.75 ml methanol:chloroform (1:2; v/v) containing 0.002% (w/v) butylated hydroxytoluene (to inhibit free radical-initiated oxidation reactions) and sonicated for 5 min. After sonication, chloroform (1.25 ml) and water (1.25 ml) were added to the mixture, which was centrifuged at 1500 ***g*** for 10 min. The lower chloroform layer was transferred to a new tube and 2 ml methanol:water (10:9; v/v) was added. The samples were mixed vigorously and centrifuged at

1500 ***g*** for 10 min. The lower chloroform layer was withdrawn and evaporated to dryness under nitrogen. The residues (total lipids) were dissolved into chloroform:methanol (2:1; v/v) and stored in a freezer (–70°C) until analysis.

As a control, total lipid of FCS was also extracted using the procedure of Bligh and Dyer (1959).

Lipid analysis

Fatty acid analysis. The total lipid extracts from oocytes and FCS were evaporated to dryness under a stream of nitrogen, and treated with 5% (v/v) HCl in methanol in sealed vials for 2 h at 100°C. After methanolysis, the fatty acid methyl esters were extracted using water-saturated hexane. The resultant fatty acid methyl esters were analysed by gas chromatography (G-3000, Hitachi; equipped with a hydrogen flame-ionization detector) and separated on a capillary column (0.25 mm i.d. × 50 m) of CP-Sil 88. The column temperature was programmed from 160°C to 220°C at 2°C min^{-1}. Injection and detection temperatures were maintained at 210°C. The amount of each fatty acid was quantified through automatic calculation of peak area by a D-2500 chromato-integrator (Hitachi) to determine fatty acid composition.

Lipid content assay. Triglyceride, total cholesterol, phospholipids (phosphocholine-containing phospholipid) and non-esterified fatty acids from the oocytes were determined in fresh immature bovine oocytes and in aliquots of maturation medium containing FCS and polyvinyl alcohol using the triglyceride E test, total cholesterol E test, phospholipid C test, and the non-esterified fatty acid C test (Waco Pure Chemical Co., Osaka), respectively. Phosphocholine-containing phospholipid and non-esterified fatty acid contents were determined in frozen–thawed immature bovine oocytes to investigate the change in membrane phospholipids. For assays using triglyceride and non-esterified fatty acid kits, 100 µl total lipid extracts from 100 oocytes was dried under a stream of nitrogen and reconstituted with 100 µl isopropanol, and for assays using the cholesterol and phosphocholine-containing phospholipid kits, 100 µl total lipid extracts from 200 oocytes was reconstituted with 100 µl isopropanol. A 20 µl aliquot was taken for assay of triglyceride, total cholesterol and phosphocholine-containing phospholipid (Youngs *et al.*, 1994), and a 50 µl aliquot was taken for assay of non-esterified fatty acid. All colorimetric assays were conducted in microplates and read at the appropriate wavelengths (600–700 nm for triglyceride, total cholesterol and phosphocholine-containing phospholipid and 546–660 nm for non-esterified fatty acid) on a microtitre plate spectrophotometer (ELSIA Reader, International Reagent's Corp, Tokyo).

Statistical analysis

All comparisons were made between fresh and frozen–thawed immature oocytes. Data were analysed by one-way ANOVA and the Student's *t* test. The same tests were used to compare immature and *in vitro* matured oocytes cultured in medium supplemented with FCS or polyvinyl alcohol.

Results

Fatty acid composition

The fatty acid composition in fresh and frozen–thawed immature bovine oocytes and in oocytes matured *in vitro* in medium containing FCS or polyvinyl alcohol is shown (Table 1). At least 22 fatty acids were detected, but only eight of these averaged > 2% (w/w) of the total fatty acid content, and these were compared among experimental treatment groups. Palmitic acid (16:0) was the most abundant fatty acid in immature oocytes (A: 35%; B: 36%) and in *in vitro* matured oocytes cultured in medium supplemented with FCS (A: 36%; B: 35%) or polyvinyl alcohol (A: 33%; B: 36%). Palmitic acid content was not affected significantly by oocyte quality, freezing and thawing or by the degree of maturity of oocytes. However, the second most abundant fatty acid differed significantly: oleic (18:1) and stearic (18:0) acids occupied this position in A and B ranked oocytes, respectively (fresh and frozen–thawed immature and *in vitro* matured oocytes). In addition, there were significant differences ($P < 0.05$) between A and B ranked fresh immature oocytes in the proportions of linoleic (18:2; A: 6.5 ± 0.3; B: 4.6 ±0.3) and arachidonic (20:4; A: 4.4 ± 0.3; B: 2.3 ± 0.4) acids. In contrast, regardless of ranking, *in vitro* matured oocytes showed significant decreases ($P < 0.05$) in the proportions of linoleic and arachidonic acids. Similarly, significant decreases in the proportion of arachidonic acid were observed in both A and B ranked frozen–thawed oocytes (A: 2.4 ± 0.5, B: 1.7 ± 0.2) compared with fresh immature oocytes. There was no significant difference in the fatty acid composition between *in vitro* matured oocytes cultured in the medium supplemented with FCS or polyvinyl alcohol. However, there was a tendency towards higher concentrations of oleic and stearic acids in *in vitro* matured oocytes cultured in medium containing FCS.

Linoleic acid was the most abundant fatty acid (37.6 ± 0.4%) and palmitic and stearic acids were the second and third most abundant.

Lipid content

Triglyceride was the most abundant lipid class in both immature and *in vitro* matured oocytes in medium containing FCS and polyvinyl alcohol (Table 3). The triglyceride content in immature oocytes (57.6 pmol per oocyte) decreased after 24 h of maturation (FCS: 36.6 pmol per oocyte; polyvinyl alcohol: 27.7 pmol per oocyte). Thus, the decrease in triglyceride varied depending on the presence of FCS in the culture medium. The total cholesterol content (16.3 pmol per oocyte) was not altered after maturation in the presence of FCS (15.1 pmol per

Table 1. Fatty acid composition of fresh and frozen–thawed immature bovine oocytes and of bovine oocytes matured *in vitro* in a medium supplemented with fetal calf serum or polyvinyl alcohol

		Immature oocytes				*In vitro* matured oocytes			
		Fresh		Frozen–thawed		Fetal calf serum (10%)		Polyvinyl alcohol (0.1%)	
Fatty acids*	Structure	Rank A	Rank B	Rank A	Rank B	Rank A	Rank B	Rank A	Rank B
Myristic	C14:0	5.9 ± 0.2	6.7 ± 1.0	7.3 ± 1.1	10.8 ± 0.9	8.1 ± 0.4	8.1 ± 0.6	3.0 ± 0.2	4.4 ± 0.9
Palmitic	C16:0	35.3 ± 0.1	35.6 ± 0.3	34.7 ± 0.2	35.2 ± 0.6	35.7 ± 0.9	34.9 ± 0.4	33.0 ± 0.9	35.9 ± 0.8
Palmitoleic	C16:1	5.7 ± 0.2	7.0 ± 0.7	5.1 ± 0.3	4.5 ± 0.4	5.7 ± 0.3	6.0 ± 0.3	4.4 ± 0.1	6.5 ± 1.5
Stearic	C18:0	12.1 ± 0.1^{A}	18.8 ± 0.4^{B}	10.7 ± 0.3^{A}	19.3 ± 1.3^{B}	16.3 ± 1.8	18.0 ± 0.8^{B}	12.6 ± 1.0^{A}	17.2 ± 1.0^{B}
Oleic	C18:1^{9}	18.8 ± 0.1^{C}	14.5 ± 0.7^{D}	19.3 ± 0.2^{C}	13.7 ± 0.6^{D}	18.8 ± 2.3^{C}	16.7 ± 1.0	16.0 ± 0.1	15.3 ± 1.4
Cis-Vaccenic	C18:1^{11}	4.0 ± 0.1	2.6 ± 0.4	3.1 ± 0.1	2.4 ± 0.1	3.1 ± 0.4	2.3 ± 0.2	2.7 ± 0.1	2.8 ± 0.8
Linoleic	C18:2	6.5 ± 0.3^{a}	4.6 ± 0.7^{b}	6.3 ± 0.2^{a}	3.2 ± 0.2^{b}	3.9 ± 0.2^{b}	3.3 ± 0.3^{b}	3.8 ± 0.1^{b}	3.5 ± 0.5^{b}
γ-Linolenic	C18:3γ	0.4 ± 0.1	0.5 ± 0.0	0.4 ± 0.2	0.8 ± 0.2	0.2 ± 0.2	0.3 ± 0.1	0.5 ± 0.3	0.5 ± 0.4
α-Linolenic	C18:3α	0.5 ± 0.1	0.3 ± 0.1	0.4 ± 0.2	0.3 ± 0.0	0.2 ± 0.1	0.2 ± 0.1	0.4 ± 0.2	0.2 ± 0.1
Parinaric	C18:4	0.2 ± 0.1	0.6 ± 0.2	0.7 ± 0.1	0.1± 0.1	0.2 ± 0.2	0.3 ± 0.1	0.4 ± 0.3	0.3 ±0.1
Arachidic	C20:0	0.7 ± 0.2	1.0 ± 0.2	0.9 ± 0.4	0.9 ± 0.0	0.8 ± 0.1	0.9 ± 0.1	0.7 ± 0.3	0.7 ± 0.3
Gadoleic	C20:1	0.9 ± 0.1	1.4 ± 0.2	0.9 ± 0.5	0.7 ± 0.1	0.5 ± 0.3	0.8 ± 0.1	1.2 ± 0.5	1.1 ± 0.1
Eicosadienoic	C20:2	1.7 ± 0.2	1.2 ± 0.2	1.6 ± 0.3	0.7 ± 0.0	1.8 ± 0.6	1.2 ± 0.3	0.7 ± 0.3	1.1 ± 0.2
Dimono-γ-linolenic	C20:3(n-6)	0.5 ± 0.1	0.7 ± 0.1	1.5 ± 0.4	0.5 ± 0.0	0.3 ± 0.3	0.5 ± 0.1	0.6 ± 0.1	0.5 ± 0.1
Arachidonic	C20:4	4.4 ± 0.2^{c}	2.3 ± 0.3^{d}	2.4 ± 0.2^{d}	1.7 ± 0.1^{e}	2.6 ± 0.5^{d}	2.1 ± 0.4^{d}	2.2 ± 0.2^{d}	2.2 ± 0.6^{d}
Eicosapentaenoic	C20:5	0.2 ± 0.2	ND	ND	ND	0.2 ± 0.1	0.1 ± 0.1	0.1 ± 0.0	0.1 ± 0.1
Behenic	C22:0	0.1 ± 0.1	0.8 ± 0.1	0.8 ± 0.1	1.2 ± 0.2	0.4 ± 0.3	0.7 ± 0.2	0.6 ± 0.2	0.6 ± 0.2
Aadremic	C22:4	ND	0.3 ± 0.1	0.7 ± 0.2	0.2 ± 0.1	0.1 ± 0.1	0.1 ± 0.0	0.1 ± 0.1	0.2 ± 0.1
Docosapentaenoic	C22:5	0.7 ± 0.1	ND	ND	ND	1.5 ± 1.1	0.5 ± 0.5	ND	ND
Docosahexaenoic	C22:6	0.3 ± 0.1	0.5 ± 0.1	0.1 ± 0.2	0.1 ± 0.1	0.2 ± 0.2	0.3 ± 0.2	0.1 ± 0.1	0.1 ± 0.1
Lignoceric	C24:0	0.6 ± 0.2	0.5 ± 0.5	2.6 ± 0.4	1.6 ± 0.2	2.3 ± 0.0	2.3 ± 0.2	1.3 ± 0.2	1.9 ± 0.1
Nervonic	C24:1	0.4 ± 0.1	ND	0.5 ± 0.2	0.1 ± 0.0	0.1 ± 0.2	0.3 ± 0.1	1.4 ± 1.1	0.2 ± 0.1
Degree of unsaturation		0.6 ± 0.01	0.5 ± 0.02	0.5 ± 0.	0.4 ± 0.1	0.5 ± 0.2	0.5 ± 0.03	0.5 ± 0	0.5 ± 0

Values are means ± SEM of samples ($n = 7$), each comprising 250–400 oocytes (sample of A ranked frozen–thawed oocytes: $n = 3$).
*Percentage (w/w) of total fatty acids.
Degree of unsaturation: ($\Sigma m_i n_i/100$), where m_i is the percentage and n_i is the number of C–C double bonds of fatty acid *i*.
ND: not determined.
Within rows, values with different letters are significantly different ($P < 0.05$): A–B; C–D; a–b; c–e.

Table 2. Fatty acid composition of fetal calf serum used in the *in vitro* maturation medium

Fatty acids	Structure	Percentage of total fatty acid
Palmitic	C16:0	14.5 ± 1.2
Palmitoleic	C16:1	6.3 ± 1.2
Stearic	C18:0	14.1 ± 2.6
Oleic	$C18:1^9$	7.4 ± 0.7
Linoleic	C18:2	37.6 ± 0.4
γ-Linolenic	C18:3γ	9.4 ± 0.2
Parinaric	C18:4	0.7 ± 0.2
Arachidic	C20:0	1.9 ± 0.2
Dimono-γ-linolenic	C20:3(n-6)	2.6 ± 0.2
Arachidonic	C20:4	2.1 ± 0.1
Eicosapentaenoic	C20:5	1.6 ± 0.4

Values are means ± SEM of three replicates and are expressed as a percentage of the total fatty acid composition.

oocyte). In contrast, oocytes matured in medium containing polyvinyl alcohol had significantly ($P < 0.01$) lower total cholesterol content (9.2 pmol per oocyte). There were no differences in phospholipid and non-esterified fatty acid concentrations between immature and *in vitro* matured oocytes.

There was a significant ($P < 0.05$) decrease in both phosphocholine-containing phospholipid (15.8 pmol per oocyte and 10.6 pmol per oocyte) and non-esterified fatty acid (11 pmol per oocyte and 4.1 pmol per oocyte) concentrations between fresh and frozen–thawed immature bovine oocytes.

Discussion

The results of this study provide the first detailed data on the changes in lipid and fatty acid composition in fresh and frozen–thawed immature, and *in vitro* matured, bovine oocytes cultured in media containing FCS or polyvinyl alcohol. In addition, differences in fatty acid composition were observed between morphologically distinct groups of bovine oocytes.

In mammals, storage lipids tend to be predominantly saturated and monosaturated fatty acids (Gurr and Harwood, 1991). Jeffcoat (1979) reported that the high concentrations of palmitic and oleic acids were found in oocyte neutral lipids, which were also required as substrates for fatty acid elongation and desaturation. In the present study, the analysis of fatty acid composition in all A ranked oocytes showed that palmitic (16:0), oleic (18:1) and stearic (18:0) acids were the first, second and third most abundant fatty acids, respectively. This finding was similar to those of studies in bovine (Zeron *et al.*, 1999a; McEvoy *et al.*, 2000) and pig oocytes (Homa *et al.*, 1986; McEvoy *et al.*, 1997). In contrast, Sata *et al.* (1999) showed that myristic acid (14:0) was the most abundant fatty acid in bovine embryos. Sata *et al.* (1999) indicated that the differences in fatty acid composition could be attributed to species differences and the different ages or breeds of cow used in the experiment. The present study demonstrates a difference in fatty acid composition of A and B ranked oocytes and indicates that the appearance of the ooplasm may reflect its lipid and fatty acid content.

Lower concentrations of oleic, linoleic and arachidonic acids were observed in B ranked oocytes compared with those of A ranked oocytes. However, the lower concentrations of linoleic and arachidonic acids observed in B ranked oocytes were similar to those concentrations in *in vitro* matured oocytes regardless of rank. It is possible to relate these findings to those of other studies (Kruip *et al.*, 1983; de Loos *et al.*, 1989). de Loos *et al.* (1989) observed that bovine oocytes in categories 3 and 4 had larger lipid droplets than those in oocytes in categories 1 and 2, and that oocytes in categories 3 and 4 had maturation-like organelle arrangements. However, there was no significant difference in the ability of oocytes in categories 1, 2 and 3 to undergo maturation *in vitro*, whereas oocytes in category 4 had a significantly diminished ability to mature *in vitro* (de Loos *et al.*, 1989). There was also no significant difference in the *in vitro* maturation rates between A and B ranked oocytes in the present study (data not shown). Thus, it is possible to conclude that the different fatty acid composition in each ranked oocyte did not directly influence maturation, although fatty acid composition may be important for

Table 3. Lipid contents (pmol per oocyte) of fresh and frozen–thawed immature bovine oocytes and of bovine oocytes matured *in vitro* in a medium supplemented with fetal calf serum (FCS) or polyvinyl alcohol

	Immature oocytes		*In vitro* matured oocytes	
Lipid class	Fresh	Frozen–thawed	FCS (10%)	Polyvinyl alcohol (0.1%)
Triglyceride	57.6 ± 1.2^a	ND	36.6 ± 1.2^b	27.7 ± 1.0^c
Total cholesterol	16.3 ± 1.3^d	ND	15.1 ± 1.1^d	9.2 ± 0.5^e
Phospholipid	15.6 ± 1.0^f	10.5 ± 0.9^g	13.4 ± 1.1^f	12.1 ± 0.7^f
Non-esterified fatty acid	11.0 ± 1.2^h	5.5 ± 0.3^i	13.5 ± 1.4^h	10.3 ± 1.5^h

Values are means ± SEM of five replicate samples of 100–200 oocytes in 100–200 µl.
ND: not determined.
Within rows, values with different letters are significantly different: $P < 0.01$: a–c, d–e; $P < 0.05$: f–g, h–i.

oocyte competence and for the differences in fertilization and developmental potential.

Homa and Brown (1992) reported that a critical relative concentration of linoleic acid in follicular fluid may be important in regulating meiotic arrest of bovine oocytes at the germinal vesicle stage. In addition, Murakami *et al.* (1986) reported that linoleic acid stimulates protein kinase C, which plays a significant role in cell growth and differentiation (Nishizuka, 1988). In the present study, significantly lower ($P < 0.05$) amounts of linoleic and arachidonic acids were observed in the *in vitro* matured oocytes than in the immature oocytes. This difference is probably a result of the decrease in these acids during *in vitro* maturation as linoleic acid cannot be synthesized in animal cells. However, there was no significant difference in the concentrations of linoleic and arachidonic acids between oocytes matured in the media supplemented with FCS and those matured in media supplemented with polyvinyl alcohol. This finding indicates that the fatty acids in FCS did not affect the change in fatty acid composition in oocytes during *in vitro* maturation, although the FCS had a high concentration of linoleic acid.

Several studies have reported a positive effect of linoleic acid on cryopreservation of bovine embryos (Hochi *et al.*, 1999a; Tominaga *et al.*, 2000). It has been suggested that the concentration of polyunsaturated fatty acid in immature bovine oocytes may affect chilling sensitivity (Zeron *et al.*, 1999b; McEvoy *et al.*, 2000). In the present study, there was no significant difference in the survival rate between A ranked (76.8%) and B ranked (67.2%) frozen–thawed immature oocytes. However, arachidonic acid content and the concentration of unsaturated fatty acids were decreased in both A and B ranked frozen–thawed immature oocytes. Our unpublished data (J. Y. Kim, M. Kinoshita, M. Ohnishi and Y. Fukui) in another study observed that degenerated frozen–thawed oocytes, which usually are not selected, showed significantly lower concentrations of arachidonic acid (0.8 ± 0.2; w/w). These lower concentrations may be due to the oxidative damage of oocyte membrane lipids during and after freezing and thawing. Arachidonic acid can be synthesized from linoleic acid, and oocytes may benefit by storing the more versatile precursor preferentially, which is at lower risk of free radical damage (McEvoy *et al.*, 2000). Hochi *et al.* (1999b) found that a high linoleic acid concentration had a detrimental effect on cryopreservation of bovine embryos. Preliminary studies and the results of the present study indicate that the positive effects of linoleic acid may be dependent on the fatty acid components in an oocyte.

The contents of both phosphocholine-containing phospholipid and non-esterified fatty acid in the frozen–thawed immature oocytes were lower than those in the fresh immature oocytes. This difference may reflect a loss of phospholipid in oocyte membranes. The loss of phosphocholine-containing phospholipid may increase the cholesterol:phospholipid ratio, leading to a decrease in membrane fluidity and reduced ability to tolerate low temperature (Anash and Buckland, 1994). In the present study, because the loss of phosphocholine-containing phospholipid occurred without an increase in non-esterified fatty acid, it can be postulated that phosphocholine-containing phospholipid was metabolized to phosphatidic acids rather than deacylated to the water-soluble form. Fuku *et al.* (1995) speculated that the possible change of phosphocholine-containing phospholipid to phosphatidic acids may disrupt the oocyte membrane. It has been suggested that changes in lipid content of bovine oocytes may be associated with changes in the sensitivity of intracellular organelles to low temperature (Diez *et al.*, 1996; Otoi *et al.*, 1997). Therefore, the reduction in arachidonic acid content and the loss of phospholipid in frozen–thawed oocytes, as observed in the present study, may be the main reasons for the low survival and development rates after freezing and thawing procedures in other studies (Lim *et al.*, 1992; Parks and Ruffing, 1992; Van Blerkum and Davis, 1994). However, it has not been established whether the membrane damage that occurred as a result of the freezing and thawing procedure resulted from the decrease of phosphocholine-containing phospholipid content *per se* or from the conversion of phosphocholine-containing phospholipid to phosphatidic acids. Further studies on oxidative damage of membranes in frozen–thawed oocytes are required to confirm these observations.

The fatty acid composition of cells grown in lipid-free media differs from that of cells grown in media containing serum (Geyer *et al.*, 1961). Cells grown in a serum-containing medium have a fatty acid profile that reflects the composition of the serum (Geyer *et al.*, 1961; Boyle and Ludwig, 1962). Spector (1972) demonstrated that cells readily take up fatty acids, phospholipids and triglycerides from culture medium containing serum. In the present study, there was no significant difference in fatty acid composition between *in vitro* matured oocytes in the media containing FCS and those in the media containing polyvinyl alcohol. However, *in vitro* matured oocytes in the media supplemented with FCS tended to have higher concentrations of stearic (18:0) and oleic (18:1) acids regardless of ranking. The triglyceride and the total cholesterol contents in *in vitro* matured oocytes cultured in the medium containing FCS were significantly ($P < 0.05$) higher than those of *in vitro* matured oocytes cultured in the medium containing polyvinyl alcohol. These findings indicate that lipids and fatty acids may be incorporated into the oocyte cytoplasm during *in vitro* maturation.

Several studies have reported that triglyceride is the major lipid component of bovine and pig oocytes (Homa *et al.*, 1986; McEvoy *et al.*, 1997, 2000). Similarly, triglyceride was also found to be the most abundant lipid in immature and *in vitro* matured bovine oocytes in the present study. However, in the present study, the triglyceride content in *in vitro* matured oocytes was significantly ($P < 0.01$) lower than that of immature oocytes. This finding is in agreement with the study of Ferguson and Leese (1999), who reported

that the triglyceride content of bovine oocytes before *in vitro* maturation was 59 ± 1.37 ng and decreased to 46 ± 0.85 ng after *in vitro* maturation. These findings and preliminary studies indicate that triglyceride can be used as an energy source for *in vitro* maturation of oocytes. However, as the triglyceride content in *in vitro* matured oocytes cultured in the media containing FCS was higher than that of *in vitro* matured oocytes cultured in the media containing polyvinyl alcohol, there is still a great need to understand the mechanisms involved in lipid nutrition and mechanisms for transfer of lipids from supplemented medium into oocytes. Further research on lipid biochemistry in bovine oocytes, including lipid peroxidation and endogenous lipid metabolism in the membrane, is required to determine the factors that influence *in vitro* maturation and the sensitivity to cryopreservation. By altering the effects of these factors before or during cryopreservation, improved results may be obtained after freezing and thawing of oocytes.

In conclusion, the present study indicates that the fatty acid composition of bovine oocytes is relevant to oocyte competence. Thus, oocyte selection for *in vitro* maturation and the cryopreservation tolerance of immature bovine oocytes may eventually be evaluated predictably by analysis of fatty acid composition. In addition, the protection of fatty acid and lipid components may play a vital role in the prevention of the damage currently related to cryopreservation and in the improvement of *in vitro* maturation systems for bovine oocytes.

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