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Fatty acid composition of lipids in immature cattle, pig and sheep oocytes with intact zona pellucida

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Cattle, pig and sheep oocytes isolated from healthy cumulus–oocyte complexes were pooled, within species, to provide samples of immature denuded oocytes with intact zona pellucida ($n = 1000$ per sample) for determination of fatty acid mass and composition in total lipid, constituent phospholipid and triglyceride. Acyl-containing lipid extracts, transmethylated in the presence of a reference penta-decaenoic acid (15:0), yielded fatty acid methyl esters which were analysed by gas chromatography. Mean (± SEM) fatty acid content in samples of pig oocytes (161 ± 18 µg per 1000 oocytes) was greater than that in cattle (63 ± 6 µg; $P < 0.01$) and sheep oocytes (89 ± 7 µg; $P < 0.05$). Of 24 fatty acids detected, palmitic (16:0; 25–35%, w/w), stearic (18:0; 14–16%) and oleic (18:1n-9; 22–26%) acids were most prominent in all three species. Saturated fatty acids (mean = 45–55%, w/w) were more abundant than mono- (27–34%) or polyunsaturates (11–21%). Fatty acids of the n-6 series, notably linoleic (18:2n-6; 5–8%, w/w) and arachidonic acid (20:4n-6; 1–3%), were the most abundant polyunsaturates. Phospholipid consistently accounted for a quarter of all fatty acids in the three species, but ruminant oocytes had a lower complement of polyunsaturates (14–19%, w/w) in this fraction than pig oocytes (34%, w/w) which, for example, had a three- to fourfold greater linoleic acid content. An estimated 74 ng of fatty acid was sequestered in the triglyceride fraction of individual pig oocytes compared with 23–25 ng in ruminant oocytes ($P < 0.01$). It is concluded that the greater fatty acid content of pig oocytes is primarily due to more abundant triglyceride reserves. Furthermore, this species-specific difference, and that in respect of polyunsaturated fatty acid reserves, may underlie the contrasting chilling, culture and cryopreservation sensitivities of embryos derived from pig and ruminant (cattle, sheep) oocytes.

Introduction

Oocytes of all mammals contain an endogenous lipid reserve. This feature reflects their shared ancestral origin, the yolk-rich amniote egg. However, the lipid is species-specific in terms of its apparent abundance and utilization. Despite the significant role of the lipid reserve in cell structure and function, very few studies have provided detailed descriptions of its nature and composition in mammalian oocytes. Notable studies include those for oocytes or early embryos of cats and dogs (Guraya, 1965), humans (Matorras *et al*., 1998), mice (Loewenstein and Cohen, 1964), rabbits (Khandoker *et al*., 1996; Khandoker and Tsujii, 1998) and sea hares (Yamaguchi *et al*., 1992). Among farm animal species, only oocytes of cows and pigs have been studied in detail (Homa *et al*., 1986; Khandoker *et al*., 1997) and there is one report on the triglyceride content of newly fertilized cattle embryos (Ferguson and Leese, 1999). With the exception of one study, which included pig oocytes as small as 75 µm in diameter (Homa *et al*., 1986), the fatty acid profiles of separate lipid classes, notably phospholipid and triglyceride, have not been reported.

Most studies on lipids in the oocytes or embryos of farm animal species concern their accumulation in culture or highlight their damaging influence during cryopreservation rather than their composition or contribution to cell structure and metabolism. This is in marked contrast to the understanding of the role of lipids in spermatozoa (Nissen and Kreysel, 1983; Roldan and Harrison, 1993; Cerolini *et al*., 1997; Kelso *et al*., 1997) and is surprising in view of the importance of phospholipid, triglyceride and other lipid fractions in mammalian ovum development. Phospholipid and cholesterol, for example, are prerequisites for the formation of membranes that are a crucial requirement during the rapid cell divisions after fertilization, while triglycerides represent compact reserves of stored energy.

It is not sensible to assume that *in vitro* culture procedures for oocytes and embryos of farm animal and other species

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can be optimized when basic information concerning lipid biochemistry is lacking; however, this has been the approach in recent decades. In addition to other aspects of ovum culture (for example, see Bavister, 1995; Thompson, 1996), knowledge of the lipid and fatty acid composition of cattle, pig and sheep oocytes and early embryos is essential to the development of optimal procedures for their safe developmental programming and culture *in vitro*. If the role of lipids in membrane receptor biology (Litman and Mitchell, 1996), signal transduction (Downes and Currie, 1998) and growth regulation (Sellmayer *et al*., 1996) is not understood, these processes may be disrupted *in vitro* and thereby compromise both short- and long-term embryonic and fetal function. Information concerning lipid reserves and their uptake and utilization by oocytes and embryos is also crucial in the improvement of cryopreservation practices, which can be hampered by the excessive accumulation of lipid in embryos cultured in the presence of serum (Shamsuddin and Rodriguez-Martinez, 1994).

The aim of the present study was to measure the fatty acid content in lipid of immature cattle, pig and sheep oocytes with intact zona pellucida, and to determine the fatty acid composition of total lipid and of the phospholipid and triglyceride fractions. Preliminary findings have been published (McEvoy *et al*., 1997; Coull *et al*., 1998).

Materials and Methods

Oocyte collection and storage

Ovaries were collected from cattle, pigs and sheep immediately after animals were killed at local abattoirs and were stored at 37°C in Dulbecco's PBS (Imperial Laboratories, Andover) during transport to the laboratory. Only ovaries from a single species were collected on any one day. All cattle ovaries came from pubertal heifers aged between 14 and 30 months; pig ovaries were from prepubertal gilts, and sheep ovaries were collected from adult ewes early in the breeding season. The ovaries of all three species were from a cross-section of commercially reared animals and were not chosen to represent any particular breed or production system.

The oocyte harvesting and processing procedures were identical for all three species. On arrival at the laboratory, ovaries were rinsed in PBS at 37°C and cumulus–oocyte complexes (COC) with good cumulus investment were retrieved by surface slicing in a glass Petri dish within 2 h after the animals were killed. Sufficient ovaries to yield at least 200 oocytes per session were processed in this manner and the COC were transferred in approximately 1.0 ml PBS to a glass vial (2-SV, Chromacol Ltd, Welwyn Garden City; 2.0 ml capacity), which was then capped using a seal with a PTFE-coated inner surface (8-TSTI, Chromacol Ltd) and subjected to vigorous vortexing for up to 10 min to remove adherent cumulus cells from the oocytes. After vortexing, the fluid was placed in a clean pre-warmed glass Petri dish and oocytes with intact zona pellucida that had been fully divested of all adherent cells and had uniform cytoplasm and an outer diameter ≥ 125 µm (pig) or ≥ 140 µm (cattle, sheep) were re-selected. The different threshold diameters for oocyte selection (including the zona pellucida) were determined to reflect the contrasting diameters of antral stage pig and ruminant oocytes (Webb *et al*., 1999). Oocyte selection and handling at this and all other processing stages relied on the use of disposable 5 µl glass pipettes (Microdispenser Model 105G; Drummond Scientific Company, Broomall, PA), thus avoiding contact with non-PTFE plastics which might distort later analyses. Each aggregate sample of 1000 re-selected oocytes was distributed across a number (n = 3–5) of microsampling glass inserts (02-MTV, Chromacol Ltd; 200 µl capacity), in 50 µl PBS per insert, within vials identical to those used previously. Two such samples of sheep oocytes and four each of cattle and pig oocytes were prepared for the study; all vials contributed to the ten completed samples sealed as described previously and stored upright (–20°C) for later analysis.

Oocyte processing and lipid analysis

Samples allowed to thaw at room temperature were homogenized immediately in a suitable excess (30 ml per 1000 oocytes) of chloroform:methanol (2:1, v/v) and total lipids were extracted (Christie, 1982) and prepared for analysis of fatty acid content, on the basis of total lipid (cattle, n = 2; pigs, n = 2) or after separation into major lipid classes (n = 2 per species). However, one of the cattle oocyte samples separated into lipid classes was subsequently omitted from the estimation of total fatty acid content and overall fatty acid composition because the assay of its constituent cholesterol ester fraction was unreliable.

Total lipid extracts (1000 oocytes per sample) were subjected to TLC on silica gel G using a solvent system of hexane:diethyl ether:formic acid (80:20:1, v/v) to separate oocyte lipids into the four major lipid fractions: phospholipid, triglyceride, cholesterol ester and free fatty acid. Individual fractions were eluted from the silica with methanol (phospholipid) or diethyl ether (all other fractions). The acyl-containing lipids were subjected to transmethylation by refluxing for 30 min with methanol:toluene:sulphuric acid (20:10:1, v/v/v) in the presence of a pentadecaenoic acid (15:0) standard (Christie *et al*., 1970). The resultant fatty acid methyl esters were analysed, via a CP9010 autosampler (Chrompack Ltd, London), onto a capillary column (Carbowax, 30 m × 0.25 mm, film thickness 0.25 µm; Alltech Ltd, Carnforth) within a CP9001 gas chromatograph (Chrompack, Middleburg) to determine fatty acid composition; identities of peaks were verified by comparison with the retention times of standard fatty acid methyl esters (Sigma Chemical Co., Poole).

Analysis of the fatty acid composition of the total lipid of the cattle and pig oocyte samples was carried out using the same procedures, with the exception that the TLC separation step was omitted.

Expression of results and statistical analysis

An EZ Chrom data system (Scientific Software Inc., San Jose, CA), for integration of the peaks and subsequent data

handling, enabled calculation of fatty acid composition and distribution in lipid classes (phospholipid, triglyceride, cholesterol ester and free fatty acids). Values (mean ± SEM) were expressed as percentages (w/w) of the total fatty acid content of specific lipid classes or of total oocyte lipid, as appropriate. The data system also enabled calculation, by reference to the C15:0 standard, of the amount of fatty acid present in the total lipid of the oocytes. Estimates expressed on a per oocyte basis (ng) reflect actual mean values (μg) per sample of 1000 oocytes.

Mean (± SEM) fatty acid mass in the oocyte total lipid samples of the three species was compared by single-factor ANOVA and Tukey's pair-wise HSD test. The same tests were used to compare all three species in respect of their oocyte phospholipids and triglycerides.

Results

Fatty acid composition of lipid in oocytes of cattle, pigs and sheep

The mean (± SEM) fatty acid content of pig oocyte samples was 161 ± 18 μg, approximately 2.5-fold the amount in cattle oocytes (63 ± 6 μg, $P < 0.01$) and 1.8 times that in sheep oocytes (89 ± 7 μg, $P < 0.05$); the two ruminant species did not differ significantly.

A total of 24 different fatty acids were detected in acyl-containing mammalian oocyte lipid, but only nine of these consistently averaged ≥ 1% (w/w) of the total fatty acid content in all three species. Of these nine fatty acids, only three, which together accounted for approximately two-thirds of total fatty acid mass, exceeded 10% (w/w) in all species (Table 1). Palmitic acid (16:0) was the most abundant in cattle (32%, w/w) and pig oocytes (35%) and, in accounting for 25% of the fatty acid mass in sheep oocyte lipid, was second only to oleic acid (18:1n-9). The amounts of oleic acid in cattle, pig and sheep oocytes were 25%, 22% and 26% (w/w), respectively. In all three species, the third most abundant fatty acid was stearic acid (18:0), representing 14–16% of the total fatty acids in the oocytes studied. Linoleic acid (18:2n-6) was fourth most abundant on the basis of mass in all three species, but the mean values recorded for cattle, pig and sheep oocytes were between 5 and 8% of total fatty acids.

In oocyte lipid, saturated fatty acids (mean = 45–55%, w/w) were more abundant than monounsaturates (27–34%) which, in turn, were more abundant than polyunsaturated fatty acids (11–21%). Most polyunsaturated fatty acids were of the n-6 series, but only linoleic (see above) and arachidonic

Table 1. Fatty acid composition of total lipid extracted from zona-intact oocytes of cattle, pigs and sheep

		Mean (± SEM) distribution of fatty acids (%, w/w)[a]		
Name	Formula	Cattle (n = 3)[b]	Pigs (n = 4)	Sheep (n = 2)
Lauric	12:0	0.23 ± 0.15	0.13 ± 0.075	nd
Myristic	14:0	2.48 ± 1.02	2.28 ± 1.35	0.39 ± 0.032
Palmitic	16:0	32.0 ± 1.64	34.9 ± 2.92	24.7 ± 0.74
Palmitoleic	16:1n-7	2.24 ± 0.45	1.25 ± 0.55	4.38 ± 0.20
Heptadecanoic	17:0	0.76 ± 0.14	0.64 ± 0.154	0.41 ± 0.407
Stearic	18:0	14.2 ± 2.47	14.4 ± 1.06	16.2 ± 0.30
Oleic	18:1n-9	25.1 ± 1.75	21.7 ± 1.00	26.2 ± 0.23
Vaccenic	18:1n-7	3.71 ± 0.12	3.18 ± 0.15	3.64 ± 0.32
Linoleic	18:2n-6	5.17 ± 0.12	7.40 ± 1.52	6.98 ± 0.10
γ-Linolenic	18:3n-6	0.75 ± 0.16	0.26 ± 0.088	1.01 ± 0.06
α-Linolenic	18:3n-3	0.49 ± 0.09	0.67 ± 0.254	2.01 ± 0.41
Stearidonic	18:4n-3	nd	0.75 ± 0.437	1.68 ± 0.30
Arachidic	20:0	1.35 ± 0.70	1.10 ± 0.38	3.11 ± 0.45
Eicosenoic	20:1n-9	0.27 ± 0.13	0.32 ± 0.014	0.19 ± 0.187
Eicosadienoic	20:2n-6	0.54 ± 0.20	0.51 ± 0.096	0.91 ± 0.476
Eicosatrienoic	20:3n-6	0.27 ± 0.15	0.57 ± 0.063	0.52 ± 0.070
Arachidonic	20:4n-6	1.13 ± 0.57	3.17 ± 0.46	1.50 ± 0.60
Eicosapentaenoic	20:5n-3	1.15 ± 1.15	1.52 ± 0.89	3.03 ± 1.09
Behenic	22:0	1.23 ± 0.63	0.38 ± 0.225	nd
Erucic	22:1n-9	0.20 ± 0.10	0.10 ± 0.060	nd
Docosatetraenoic	22:4n-6	0.27 ± 0.15	1.20 ± 0.14	nd
Docosapentaenoic	22:5n-3	0.88 ± 0.28	1.07 ± 0.44	1.41 ± 0.33
Docosahexaenoic	22:6n-3	0.50 ± 0.25	1.14 ± 0.47	1.74 ± 0.06
Lignoceric	24:0	2.30 ± 1.21	0.60 ± 0.383	nd

Each sample (n = 2–4) represents 1000 oocytes.
[a]Percentage (w/w) of the total fatty acids in oocyte lipid.
[b]One of the four original cattle oocyte samples was excluded because of uncertainty about the validity of the assay result involving the cholesterol ester component of total lipid.
nd: not detected.

acid (20:4n-6) exceeded 1% (w/w) of total fatty acids in all three species. Of the n-3 polyunsaturated fatty acids, only eicosapentaenoic acid (20:5n-3) exceeded this same threshold in all three species, although docosapentaenoic (22:5n-3) and docosahexaenoic acid (22:6n-3) did in pig and sheep oocyte lipids. Other n-3 fatty acids detected were α-linolenic (18:3n-3) and stearidonic (18:4n-3) acids, but these exceeded 1% (w/w) of total fatty acids only in sheep oocytes.

Phospholipid

Phospholipid consistently accounted for one-quarter (25–29% w/w) of the oocyte fatty acid mass in all three species, equivalent to 18, 41 and 25 ng per individual cattle, pig and sheep oocyte, respectively.

The fatty acid profiles detected in the oocyte phospholipid fractions of each ruminant (cattle, sheep) species were similar (Table 2), and 16:0 (25 versus 22%, w/w), 18:1n-9 (23 versus 20%) and 18:0 (16 versus 16%) were most abundant. Overall, almost half of the fatty acid component of phospholipid in cattle and sheep oocytes was saturated (49 versus 47%, w/w, respectively); polyunsaturates accounted for only 14 and 19%, respectively. In contrast, pig oocyte phospholipids were characterized by a three- to fourfold enrichment with 18:2n-6 (23%, w/w) relative to ruminant oocytes (cattle, 5.3%; sheep, 6.7%), increasing the polyunsaturate component to 34% (w/w). The relative contribution of 18:0 (16%) and 18:1n-9 (24%) was equal to that for cattle oocytes, but that of 16:0 was lower (16%). However, in absolute terms (ng per oocyte), the phospholipid reserves of these three major fatty acids were highest in pig oocytes. On the same basis, the advantage in terms of polyunsaturated fatty acid reserves was even more marked; for example, the estimated mass of 18:2n-6 in phospholipid was 9.41 ng for pig oocytes, compared with 0.95 ng and 1.65 ng for cattle and sheep oocytes, respectively.

Triglyceride

In absolute terms, mean estimates for the fatty acid mass in triglycerides of individual cattle and sheep oocytes were similar (23 versus 25 ng per oocyte, respectively) but that of individual pig oocytes was threefold higher (74 ng; $P < 0.01$). These values represent approximately 36, 46 and 28% (w/w) of total fatty acids in cattle, pig and sheep oocyte lipid, respectively.

The monounsaturate 18:1n-9 accounted for almost 40% (w/w) of all fatty acids in triglycerides from cattle (8.9 ng) and sheep oocytes (9.5 ng; Table 3) but, although this fatty acid was more abundant in absolute terms in pig oocytes (14.6 ng), 16:0 was the predominant fatty acid in the monogastric species, because of its twofold higher concentration (41% versus 20%, w/w). In pig oocytes, the saturated fatty acid component of triglycerides was greater than in ruminant oocytes (55% versus 40%, w/w), a reversal of the trend for phospholipid (36 versus 48%, w/w). Another difference was that the long-chain polyunsaturated fatty acid docosahexaenoic (22:6n-3), not detected in phospholipid from pig and sheep oocytes, was present in the triglyceride fraction of both species. As in the oocyte phospholipid fraction, the content of stearic acid (18:0) in triglyceride was consistent in all three species (12–13%, w/w).

Table 2. Most abundant fatty acids in phospholipid from zona-intact oocytes of cattle, pigs and sheep

	Cattle	Pigs	Sheep
Fatty acid mass in phospholipid (μg per sample)	17.95 ± 0.75[a]	40.9 ± 5.3[b]	24.6 ± 1.7[ab]
Fatty acid abundance (%, w/w)[c]			
16:0	25.2 ± 0.87	16.2 ± 0.59	22.4 ± 2.62
16:1n-7	6.2 ± 0.98	3.1 ± 0.04	6.3 ± 0.46
18:0	16.0 ± 1.31	15.8 ± 1.30	15.7 ± 1.66
18:1n-9	23.3 ± 0.67	24.0 ± 0.24	19.9 ± 1.41
18:1n-7	8.1 ± 0.26	3.5 ± 0.37	7.7 ± 0.46
18:2n-6	5.3 ± 0.47	23.0 ± 2.10	6.7 ± 1.31
g18:3n-6	3.4 ± 0.63	1.28[d]	3.7 ± 0.75
18:3n-3	1.43[d]	3.7 ± 1.46	3.17[d]
20:0	7.6 ± 0.30	3.4 ± 0.34	8.6 ± 0.15
20:4n-6	1.65[d]	4.1 ± 2.05	5.53[d]
Fatty acid sub-classes (%, w/w)[c]			
Saturated	49 ± 1.9	36 ± 1.1	47 ± 5.2
Monounsaturated	38 ± 1.9	31 ± 0.6	34 ± 0.5
Polyunsaturated	14 ± 3.8	34 ± 1.6	19 ± 4.8

Values are means (± SEM) of duplicate samples, each comprising 1000 oocytes.
[ab]Phospholipid fatty acid mass values (μg) with different superscripts are significantly different ($P < 0.05$).
[c]Percentage (w/w) of the total fatty acids in oocyte phospholipid.
[d]Value for one sample; not detected in other sample.

Table 3. Most abundant fatty acids in triglycerides from zona-intact oocytes of cattle, pigs and sheep

	Cattle	Pigs	Sheep
Fatty acid mass in triglyceride (μg per sample)	22.95 ± 0.55[a]	74.25 ± 4.45[b]	24.95 ± 3.55[a]
Fatty acid abundance (%, w/w)[c]			
16:0	27.7 ± 1.00	40.9 ± 1.33	26.4 ± 0.14
16:1n-7	2.7 ± 0.62	1.09 ± 0.04	2.1 ± 0.26
18:0	13.2 ± 0.05	12.6 ± 0.47	12.2 ± 0.28
18:1n-9	38.8 ± 0.36	19.7 ± 1.19	37.9 ± 1.13
18:1n-7	4.6 ± 0.36	5.2 ± 0.11	3.9 ± 0.06
18:2n-6	9.4 ± 0.19	5.5 ± 0.47	9.3 ± 0.48
18:3n-3	1.94[d]	nd	2.6 ± 0.40
20:4n-6	nd	5.4 ± 0.12	1.3 ± 0.01
22:4n-6	nd	2.7 ± 0.16	nd
22:6n-3	3.82[d]	2.1 ± 0.25	2.1 ± 0.41
Fatty acid sub-classes (%, w/w)[c]			
Saturated	41 ± 1.0	55 ± 1.0	39 ± 0.3
Monounsaturated	46 ± 0.6	27 ± 1.1	45 ± 0.6
Polyunsaturated	13 ± 0.3	19 ± 0.1	16 ± 0.4

Values are means (± SEM) of duplicate samples, each comprising 1000 oocytes.
[ab]Triglyceride fatty acid mass values (μg) with different superscripts are significantly different ($P < 0.01$).
[c]Percentage (w/w) of the total fatty acids in oocyte triglyceride.
[d]Value for one sample; not detected in other sample.
nd: not detected.

Discussion

The results of this study provide the first detailed quantitative account of the fatty acid content and composition of sheep oocytes and augment other reports of cattle and pig oocyte lipid content (Homa *et al*., 1986; Khandoker *et al*., 1997). Moreover, analysis of the fatty acid composition of both phospholipids and triglycerides in immature oocytes of all three species provides a sound basis for improved understanding of their shared and separate developmental roles and relevance.

In contrast to a study of zona-intact pig oocytes as small as 75 μm in diameter (Homa *et al*., 1986), the diameter of the oocytes used in the present study, including the zona pellucida, was ≥ 125 μm (pig) or ≥ 140 μm (cattle, sheep) and consequently represented a more mature sample. For example, Fair *et al*. (1995) reported that cattle oocytes become more competent with increasing diameter, and Hyttel *et al*. (1997) reported that cattle oocytes lack full developmental capability until they reach a diameter of 110 μm, excluding the zona pellucida, which is approximately equal to the 140 μm zona-intact selection threshold applied to cattle and sheep oocytes in the present study. The exclusion of smaller oocytes in the present study also took into account the studies of Fair *et al*. (reviewed by Hyttel *et al*., 1997), indicating that bovine oocyte lipid content increases with increasing size.

The most notable species-related contrast detected in the present study was the approximately twofold greater complement of fatty acid, reflecting the acyl-containing lipid mass, found in pig oocytes compared with ruminant oocytes. This was not reported in the study of Khandoker *et al*. (1997) on oocytes and reproductive fluids (follicular, oviductal and uterine) from cattle and pigs, which concluded that samples from each species had similar lipid contents. This may be the case for the fluids studied by Khandoker *et al*. (1997), but the results of the present study indicate that this is not the same for oocytes. The greater lipid content of pig oocytes has also been observed, but not quantified, in studies on the effects of centrifugation (Cran, 1987) and on sensitivity to chilling or cryopreservation (Nagashima *et al*., 1994; Dobrinsky, 1996).

Despite gross differences in total fatty acid reserves, the percentage concentrations of particular fatty acids was similar for all three species studied. The identification of palmitate (16:0), stearate (18:0) and oleate (18:1n-9) as the most abundant fatty acids in all samples is consistent with a preliminary study on cattle oocytes (Zeron *et al*., 1999), a study of immature pig oocytes (Homa *et al*., 1986) and findings for other mammalian species (human: Matorras *et al*., 1998; rabbit: Khandoker *et al*., 1996). As in rabbit oocytes (Khandoker *et al*., 1996), palmitate and oleate together accounted for more than half of the total fatty acid complement in the three species studied. The same fatty acids are also prominent in oocytes of frogs (*Xenopus laevis*; Mes-Hartree and Armstrong, 1976) and toads (*Bufo arenarum* Hensel; Alonso *et al*., 1986; Caldironi and Alonso, 1996), and these may function as fuel reserves for energy provision via β-oxidation, as has been postulated for amphibian oocytes (Alonso *et al*., 1986; Caldironi and Alonso, 1996).

Palmitoleic and linolenic acids were not detected in rabbit oocyte lipids (Khandoker *et al*., 1996), but were present at low concentrations in oocytes in the present study, whereas Khandoker *et al*. (1997) detected them in pig but not in cattle oocytes. In the present study, in all three species, palmitoleate was consistently two- to threefold more abundant in the scarcer phospholipid fraction (3–6%, w/w) than in triglyceride, whereas γ-linolenic acid exceeded 1% (w/w) only in phospholipid. Arachidonic acid, an important

precursor of short-lived eicosanoids including prostaglandins, thromboxanes and leukotrienes, was detected in oocytes of all three species at approximately the same concentration reported by Khandoker *et al*. (1997) for pig (4.9%, w/w) but not cattle oocytes (15.1%, w/w), although Zeron *et al*. (1999) detected very little arachidonic acid in cattle oocytes, which supports the results of the present study. Arachidonic acid can be synthesized from linoleic acid, and oocytes may benefit by storing the more versatile precursor preferentially, which is at lesser risk of free radical damage.

Lipids may act as a reservoir of latent energy (Betteridge and Fléchon, 1988; Thompson, 1996), although metabolic studies indicate that ATP requirements of embryos are met by utilization of carbohydrates (Thompson *et al*., 1996). Nevertheless, oxygen consumption by one-cell mouse ova (3.84 nl per day) indicates that there is scope for utilization of endogenous fatty acids (515 pl oxygen used per pmol palmitate) without precluding alternative endogenous or exogenous fuel sources (Leese, 1991). Such a contribution from endogenous fatty acids may not be detected in embryo metabolism studies, and Bavister (1995) noted that there is very little information on fatty acids as substrates for embryos; the notable exception is the study of Kane (1979) who investigated exogenous but not endogenous fatty acid effects on rabbit embryos *in vitro*.

One function attributed to fatty acid utilization is the generation of water for blastocoele fluid by mitochondrial β-oxidation (Wiley, 1987); in mice, this is preceded by cortical localization of randomly dispersed cytoplasmic droplets and mitochondria to apposed cell surfaces (Wiley, 1984). Cell–cell compaction, which precedes normal blastulation, depends on the lipid composition of embryo plasma membranes (Pratt, 1978) and, as shown in sheep (Walker *et al*., 1998), can be compromised in conditions that alter embryo lipid content artificially.

Just over a quarter of fatty acid mass in the oocytes of all three species studied was in phospholipid, consistent with the observation that the phospholipid content of pig oocytes was about threefold less than that of neutral lipid (Homa *et al*., 1986) and also reflecting the estimate for phospholipid in *Xenopus laevis* embryos (28% of total; Mes-Hartree and Armstrong, 1976). In the present study, pig oocyte phospholipid was found to contain a higher proportion of polyunsaturated fatty acids than ruminant oocyte phospholipid. This difference, which reflects an estimated tenfold greater abundance of linoleic acid (9.41 versus 0.95 ng) in pig than in cattle oocyte phospholipid, could have marked effects on the nature of membranes subsequently derived from phospholipid reserves (Stryer, 1988) and may contribute to contrasting responses of embryos from these farm animal species to chilling or cryopreservation (Pollard and Leibo, 1994).

Phospholipid, together with cholesterol, is an important component of biological membranes, which must be synthesized as the fertilized mammalian ovum repeatedly undergoes cell division. Pratt and George (1989) estimated that the membrane surface area of a two-cell and a four-cell mouse ovum was approximately 33% and 74% greater, respectively, than that of the original membrane surrounding the one-cell ovum. Thus, the additional membrane required for each cell division must be provided by reorganization of the existing membranes together with phospholipid turnover or *de novo* synthesis. Pratt and George (1989) proposed that *de novo* synthesis was concentrated at a girdle-like region of intercellular apposition of daughter cells. In addition to cell surface membrane requirements, studies on mouse embryos have shown that phospholipid is increasingly utilized for intracellular membrane-bound organelles as cell division proceeds (Pratt, 1980). Phospholipid also may facilitate protein attachment to cell membranes in the mammalian ovum; glycosyl-phosphoinositides fulfil such a role in other types of cell (Kane and Fahy, 1993).

Second messengers reflect other phospholipid roles in cell function and, thereby, mammalian ovum development. Hydrolysis of membrane-bound phosphatidylinositol 4,5-bisphosphate (PIP_2) yields two second messengers, inositol 1,4,5-trisphosphate (IP_3) and diacylglycerol. Diacylglycerol contains both stearic and arachidonic acids. IP_3 and its derivative (IP_4) increase Ca^{2+} concentrations, while diacylglycerol stimulates protein kinase C (Cran and Moor, 1990; Nishizuka, 1992; Roldan and Harrison, 1993). Thus, phosphatidylinositol, which represents approximately 6% of pig oocyte phospholipid (Homa *et al*., 1986), probably influences the calcium-centred cascade of events at fertilization indirectly (Cran and Moor, 1990; Ducibella, 1998). Phosphatidylserine, which accounts for almost 10% of phospholipid in pig oocytes (Homa *et al*., 1986), also influences second messenger functions (Berridge, 1987) and plays a vital role in purine synthesis (Kane, 1989).

The present study indicates that triglyceride is most abundant in pig oocytes, which contain an estimated 50 ng greater fatty acid mass than ruminant oocyte triglycerides. Consequently, this form of lipid may impede conventional embryo cryopreservation protocols when applied to pig zygotes, morulae and unexpanded blastocysts. Such problems are not encountered after blastocyst expansion when, coincident with significant increases in energy needs, blastocyst neutral lipid content is reduced (Nagashima *et al*., 1992). Physical removal of excess lipid from ova of cattle (Tominaga *et al*., 1998; Ushijima *et al*., 1999) and pigs (Nagashima *et al*., 1994) at earlier developmental stages also reduces sensitivity to chilling. Moreover, Ferguson and Leese (1999) reported that triglyceride concentrations in cattle embryos remain stable from the two-cell to the blastocyst stage *in vivo* (32.2 versus 33 ng per embryo), whereas *in vitro*, in embryos exposed to serum (10%, v/v) from the four-cell stage, the triglyceride reserves can double by the blastocyst stage. Ferguson and Leese (1999) estimated triglyceride concentrations by assaying glycerol content, but nevertheless their results are consistent with the results of the present study. Given that embryos from such culture conditions are poorly suited to conventional cryopreservation (Nagashima *et al*., 1992; Leibo and Loskutoff, 1993; Shamsuddin and Rodriguez-Martinez, 1994), triglyceride content may influence their sensitivity to chilling. This may also be true for pig ova *in vivo*, which have similar chilling injury kinetics (Pollard and Leibo, 1994). Shamsuddin and Rodriguez-Martinez (1994) suggested that numerous vesicles and lipid droplets in the cytoplasm of cattle blastocysts, which had

been co-cultured in the presence of serum, may account for their sensitivity. This view is supported by Thompson *et al*. (1995) who demonstrated that lipid increased in cultured sheep embryos exposed to serum, possibly reflecting mitochondrial degeneration (Dorland *et al*., 1994). Thompson *et al*. (1995) concluded that most additional lipid was probably stored as triglycerides in cytoplasmic droplets, as was observed for fatty acids sequestered by rabbit embryos *in vitro* (Waterman and Wall, 1988). It was also observed that the droplets were osmophilic, indicating the presence of a significant proportion of unsaturated lipid (Thompson *et al*., 1995); however, the proportion was not specified, thus it is not possible to determine whether it exceeded the amount in the natural triglyceride reserves of sheep oocytes in the present study.

The amounts of unsaturated fatty acids sequestered *in vivo* or accumulated *in vitro* may be more critical determinants of oocyte and embryo chilling sensitivity than gross lipid content. Antioxidant-mediated protection of *in vitro* produced bovine embryos achieved by injection of α-tocopherol conferred benefits equivalent to removal of excess lipid (Pangestu *et al*., 1996). This finding may be explained by a study of incubated turkey spermatozoa (Surai *et al*., 1998) in which α-tocopherol completely preserved phosphatidyl-serine in conditions that otherwise caused an almost 50% reduction in this vital lipid component; α-tocopherol also prevented depletion of several polyunsaturated fatty acids, including docosapentaenoic and docosahexaenoic from the n-3 series. Thus, protection of the fatty acid and lipid components of oocytes and embryos that render them susceptible to free radical or other oxidative injury may prevent the significant damage currently associated with chilling, cryopreservation and culture.

In conclusion, the present study quantified the fatty acid content of cattle, sheep and pig oocytes with intact zona pellucida and indicated that most of the additional lipid naturally sequestered in pig oocytes is triglyceride. In all three species, palmitic and oleic acid together accounted for more than 50% of fatty acid reserves, but combined polyunsaturate reserves ranged only from 10 to 20% of the total mass. Triglyceride was the most fatty acid-rich lipid fraction in oocytes from the three species but phospholipid was most consistent, accounting for one-quarter of total fatty acid mass. Pig oocyte phospholipid showed at least a threefold linoleic acid enrichment relative to ruminant oocytes, reflecting its more unsaturated nature. These findings indicate that cattle, pig and sheep oocytes reflect their shared ancestry, through a reliance on the same few abundant fatty acids, but also have distinctive features indicative of different strategies for ruminant and monogastric species. Information on fatty acid composition is relevant to oocyte and embryo competence, culture and cryopreservation and phospholipid in particular has a vital role in development during and after fertilization.

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