Effects of omega-3 and -6 polyunsaturated fatty acids on ovine follicular cell steroidogenesis, embryo development and molecular markers of fatty acid metabolism

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Abstract

We previously reported increased follicular fluid progesterone (P₄) concentrations in ewes fed an n-3 compared to an n-6 polyunsaturated fatty acid (PUFA)-enriched diet, but detected no differential effect of n-3 and n-6 PUFA-enriched high-density lipoproteins (HDL) on granulosa cell (GC) steroidogenesis *in vitro*. Moreover, net n-6 PUFA-enriched HDL reduced early embryo development, but in the absence of a net uptake of FA. Consequently, we hypothesised that a) effects of n-3 PUFA on ovarian steroidogenesis are mediated by theca rather than GCs and b) during embryo culture lipids are acquired solely from the albumin fraction of serum, so that albumin-delivered n-3 and n-6 PUFA exert a greater differential effect on embryo development than either low-density lipoprotein (LDL)- or HDL-delivered PUFA. Data confirmed that n-3 PUFA increases P₄ production solely in theca cells and that this is associated with an increase in *STAR* transcript expression. Furthermore, LDL- and HDL-delivered n-3 PUFA are equally efficacious in this regard during the first 96 h of culture, but thereafter only HDL-delivered n-3 PUFA induces this effect in partially luteinised theca cells. We also demonstrate that albumin is the sole serum fraction that leads to a net uptake of FA during embryo culture. PUFA-enriched serum and albumin increased the yield of morphologically poorer quality blastocysts with increased transcript expression for the antioxidant enzyme *SOD1*. Important differential effects of n-3 and n-6 PUFA on ovarian steroidogenesis acting solely on theca cells are identified, but differential effects of PUFA on embryo development are less apparent.

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Introduction

Polyunsaturated fatty acids (PUFAs) are known to exert a broad range of effects on reproductive tissues (Wathes *et al.* 2007) although the benefits to fertility from feeding PUFA-enriched diets, particularly to lactating ruminants, have been inconsistent (Santos *et al.* 2008). In part, this may be due to modifications that occur to PUFA in the rumen of cattle and sheep. These modifications include a reduction in the number of double bonds and isomer reorientation leading to an increase in the production of saturated and monounsaturated FA (MUFA), as well as polyunsaturated *trans*-FA most notably vaccenic acid (C18:1 *trans*-11; Jenkins 1994). Long-chain PUFAs are, however, less affected by these processes and we (e.g. Fouladi-Nashta *et al.* 2009) and others (e.g. Childs *et al.* 2008) have successfully altered the PUFA composition of various tissues and biological fluids in lactating and non-lactating ruminants by feeding a variety of PUFA-rich plant and animal oils. We have also observed that saturated FAs are preferentially accumulated by the

follicle-enclosed oocyte, suggesting selective uptake mechanisms or *de novo* synthesis that favours this class of FA (Adamiak *et al.* 2006). As a consequence of this, and possibly because we used vegetable sources of PUFA, we have been unable to alter the FA composition of granulosa cells (GCs) and oocytes in lactating dairy cows (Fouladi-Nashta *et al.* 2009). In contrast, a combination of vegetable and fish oils in diets of non-lactating sheep significantly altered the PUFA composition of both GCs and oocytes (Wonnacott *et al.* 2010).

The feeding of n-3 relative to n-6 PUFA-enriched diets in that latter study also increased follicular fluid progesterone (P₄) concentrations, but we were unable to reproduce this effect in GCs cultured in the presence of n-3 and n-6 PUFA-enriched high-density lipoproteins (HDL) fractionated from the sera of these ewes. This led to the hypothesis that the differential effects of n-3 and n-6 PUFA on ovarian steroidogenesis may be mediated by theca rather than GCs. There was also no net uptake of FA by sheep embryos cultured in the presence of PUFA-enriched HDL in that study, although PUFA

supplementation altered the expression of transcripts for scavenger receptor class B member 1 (*SCARB1*), low-density lipoprotein receptor (*LDLR*) and stearoyl-CoA desaturase (*SCD*), indicating that embryos were responsive to the presence of PUFA-enriched HDL in culture. Indeed, n-6 PUFA-enriched HDL reduced the proportion of zygotes that developed to the blastocyst stage, although the mechanisms that underly this effect remain elusive.

The purpose of the current series of experiments, therefore, was to extend these observations to firstly investigate the differential effects of n-3 and n-6 PUFA-enriched HDL and LDL on granulosa- and theca-cell development and steroidogenesis during culture. Sheep zygotes were also cultured to the blastocyst stage in the presence of n-3 and n-6 PUFA-enriched LDL, albumin or serum. The purpose was to test the hypothesis that serum-derived lipids (Thompson *et al.* 1995) are delivered only by the albumin fraction of serum, so that we may expect albumin-delivered n-3 and n-6 PUFA to exert a greater differential effect on pre-implantation embryo development than either the LDL- or the HDL-delivered n-3 and n-6 PUFA tested by Wonnacott *et al.* (2010). Given the differences in PUFA content of GCs and embryos reported by Wonnacott *et al.* (2010), and the differences in PUFA composition anticipated in the current series of experiments, a further object of this study is to determine transcript expression for a number of enzymes involved in fatty acid elongation (i.e. *ELOVL2* and *ELOVL5*) and desaturation (i.e. *SCD*, *FADS1* and *FADS2*), together with enzymes associated with follicular cell steroidogenesis (*STAR*, *CYP11A1*, *CYP17A1* and *HSD3B1*) and cellular stress (*SOD1*). *ELOVL2* and *ELOVL5* are involved in the conversion of the essential FA precursors linoleic (C18:2n-6) and α -linolenic (C18:3n-3) acids to arachidonic acid (C20:4n-6) and docosahexaenoic acid (DHA; C22:6n-3) (Guillou *et al.* 2010), and the majority of *Elovl5*^{-/-} female mice are infertile (Moon *et al.* 2009). The $\Delta 6$ -desaturase *FADS2* is the sole enzyme that catalyses the initial, rate-limiting desaturation of linoleic (C18:2n-6) and α -linolenic (C18:3n-3) acids for the production of longer chain PUFA. Biological fluids and tissues (including ovaries and testes) of *Fads2*^{-/-} mice are devoid of PUFA and, while male and female *Fads2*^{-/-} mice are viable and have a normal lifespan, they too are sterile (Stoffel *et al.* 2008). As the feeding of fish oils to rats inhibits *de novo* lipogenesis and expression of all fatty acid desaturases and specific (i.e. *ELOVL5* but not *ELOVL2*) elongases in primary hepatocytes (Wang *et al.* 2005), we sought to determine what differential effect n-3 and n-6 PUFA-enriched lipoproteins would have on transcript expression for these enzymes in cultured ovarian follicular cells and pre-implantation embryos.

Results

A detailed breakdown of the FA composition of serum, LDL, HDL and albumin fractions used for cell and embryo culture in this study is provided in Table 1, and the composition of basal media can be found online (Supplementary Table 5, see section on supplementary data given at the end of this article). Maternal dietary treatment was effective in significantly altering the relative proportions of n-3 and n-6 PUFA in serum and each of the three serum fractions (Table 1; Supplementary Tables 1–4, see section on supplementary data given at the end of this article). Of further note, in Supplementary Table 5, the trace amounts of FA were detected in basal media, the inclusion of FA-free (FAF) BSA in synthetic oviductal fluid medium (SOFM) did not increase these further, and the measured total amount of FA in DMEM with added n-6 PUFA-enriched HDL (73.0 µg/ml) matched the calculated concentration (72.3 µg/ml) based on the composition of serum at an intended 5% v/v incorporation rate (Table 1).

Table 1 Fatty acid (FA) composition of (a) serum, (b) low-density lipoprotein (LDL), (c) high-density lipoprotein (HDL) and (d) albumin fractionated from the serum that was derived from ewes offered diets differing in n-3 and n-6 polyunsaturated FA (PUFA; Wonnacott *et al.* 2010). Analyses were based on two replicate batches.

Dietary treatment	g/100 g total FA		S.E.D.	P
	n-3	n-6		
(a) Serum TFA (µg/ml)	1439	1445		
Saturated	36.51	36.50	0.189	–
Unsaturated	59.08	59.13	0.718	–
MUFA	17.29	18.72	0.618	–
PUFA	41.79	40.41	0.544	–
n-6 series	23.51	36.62	0.432	<0.001
n-3 series	18.28	3.79	0.133	<0.001
Ratio of n-6:n-3	1.29	9.66	0.234	<0.001
(b) LDL TFA (µg/ml)	1519	1012		
Saturated	36.8	41.5	0.192	0.002
Unsaturated	58.8	53.9	0.624	0.015
MUFA	19.5	20.7	0.598	–
PUFA	39.3	33.1	0.149	<0.001
n-6 series	24.7	30.5	0.124	<0.001
n-3 series	14.6	2.63	0.045	<0.001
Ratio of n-6:n-3	1.68	11.6	0.020	<0.001
(c) HDL TFA (µg/ml)	3321	3171		
Saturated	33.9	30.2	0.612	0.026
Unsaturated	61.6	64.9	0.521	0.024
MUFA	18.2	20.1	0.796	–
PUFA	43.5	44.8	0.276	0.039
n-6 series	27.7	41.5	0.267	<0.001
n-3 series	15.7	3.30	0.085	<0.001
Ratio of n-6:n-3	1.76	12.5	0.034	<0.001
(d) Albumin TFA (µg/ml)	88.6	89.6		
Saturated	38.9	44.0	0.261	0.003
Unsaturated	57.1	51.4	0.258	0.002
MUFA	20.8	20.8	0.576	–
PUFA	36.3	30.6	0.754	0.017
n-6 series	16.7	25.7	0.298	<0.001
n-3 series	19.6	4.91	0.460	<0.001
Ratio of n-6:n-3	0.85	5.24	0.073	<0.001

Granulosa cell culture

The total FA content (TFA, $\mu\text{g}/10^5$ cells) of GCs following 144 h culture did not differ between lipoprotein fractions or between dietary sources of PUFA (Table 2). The percentage MUFA, however, was lower ($P=0.04$) whereas the percentage PUFA was higher ($P=0.004$) in GCs cultured in the presence of HDL than LDL; n-6-enriched LDL and HDL increased ($P=0.014$) n-6 series PUFA in GCs relative to n-3 series PUFA. Conversely, n-3-enriched LDL and HDL increased ($P=0.04$) n-3 series PUFA in GCs relative to n-6 series PUFA. The most abundant FA in GCs was oleic acid (C18:1n-9), comprising on average 30.9 ± 0.35 g/100 g of TFA (Supplementary Table 6, see section on supplementary data given at the end of this article). Its content was greater ($P<0.05$) in LDL and n-6 than HDL and n-3-cultured cells. The second most abundant FA was palmitic acid (C16:0), comprising 23.2 ± 0.18 g/100 g TFA, and stearic acid (C18:0) was the third (11.5 ± 0.66 g/110 g TFA). Of the seven n-6 PUFA identified, three were increased in cells cultured with n-6, regardless of lipoprotein fraction: linoleic (C18:2n-6) ($P<0.001$), γ -linolenic (C18:3n-6) ($P=0.02$) and eicosadienoic (C20:2n-6) ($P=0.02$). Similarly, of the five n-3 PUFA identified, four were increased in cells cultured with n-3 PUFA: α -linolenic acid (C18:3n-3) ($P=0.002$), eicosapentaenoic acid (C20:5n-3) ($P<0.001$), docosapentaenoic acid (C22:5n-3) ($P=0.003$) and DHA (C22:6n-3) ($P=0.02$).

There was no differential effect of lipoprotein fraction or dietary source of PUFA on cell proliferation or steroid production by GCs (Table 3). Cell number increased ($P<0.001$) between 48 and 96 h of culture, but decreased between 96 and 144 h. Cell-number-adjusted

oestradiol (E_2) concentrations in spent media decreased ($P<0.001$) with time during culture while P_4 concentrations increased ($P<0.001$).

Transcripts for *CYP17A* and *ELOVL2* were not expressed in GCs. All other transcripts were expressed. The following transcripts, however, were not affected by lipoprotein fraction or dietary PUFA source: *SCD*, *FADS1*, *FADS2*, *HSD3B1*, *SCARB1*, *LDLR*, *ELOVL5* and *CYP11A1*. The latter four transcripts were also not affected by duration of culture and so data are not presented. Transcript expression for *SCD* increased ($P<0.001$) by 7.5-fold between 48 and 144 h of culture. Transcript expression for *FADS1* and *FADS2* generally increased ($P<0.001$) during culture, although transcripts for the latter gene were barely detectable. In contrast, transcript expression for *HSD3B1* decreased ($P<0.001$) between 48 and 96 h of culture before increasing again by 144 h. *STAR* expression was greater ($P=0.018$) in GCs cultured in the presence of LDL than HDL and increased ($P<0.001$) with time during culture by more than fourfold.

Theca cell culture

The TFA content (TFA, $\mu\text{g}/10^5$ cells) of theca cells following 144 h culture did not differ between lipoprotein fractions or between dietary sources of PUFA, although it was lower for cells cultured with n-3 PUFA LDL than those with n-6 PUFA LDL ($P=0.04$; Table 2). Similarly, the percentage MUFA was greatest ($P=0.004$) for cells cultured with n-6 PUFA LDL and least for cells cultured with n-3 PUFA LDL. In keeping with GCs, n-6-enriched LDL and HDL increased ($P<0.001$) n-6 series PUFA relative to n-3 series PUFA, while n-3-enriched

Table 2 Effect of culture treatment (lipoprotein fraction and dietary source of polyunsaturated fatty acid (PUFA)) on total FA (TFA) and percentage (g/100 g TFA) of saturated FA, unsaturated FA, monounsaturated FA (MUFA) and PUFA in (a) granulosa and (b) theca cells following culture for 144 h.

Lipoprotein (L)	LDL		HDL		S.E.D.	Significance		
	n-3	n-6	n-3	n-6		L	D	L×D
Dietary source (D)								
(a) Granulosa cells								
<i>n</i>	6	6	6	6				
TFA ($\mu\text{g}/10^5$ cells)	5.55	6.50	6.11	6.04	0.70	–	–	–
Saturated	40.2	38.0	39.1	38.8	0.71	–	–	–
Unsaturated	54.0	56.2	55.2	55.6	0.76	–	–	–
MUFA	36.1	38.0	35.6	35.9	0.55	0.04	–	–
PUFA	17.9	18.2	19.6	19.6	0.41	0.004	–	–
n-6 series	10.4	12.3	10.8	13.4	0.73	–	0.014	–
n-3 series	7.51	5.95	8.82	6.22	0.86	–	0.04	–
Ratio of n-6:n-3	1.44	2.24	1.25	2.23	0.30	–	0.02	–
(b) Theca cells								
<i>n</i>	6	6	6	6				
TFA ($\mu\text{g}/10^5$ cells)	3.62	4.76	4.33	4.14	0.42	–	–	0.04
Saturated	38.6	35.3	35.9	36.9	1.12	–	–	–
Unsaturated	55.6	58.8	58.3	57.3	1.06	–	–	–
MUFA	32.6	37.2	34.7	35.5	0.77	–	<0.001	0.004
PUFA	22.9	21.7	23.6	21.8	0.89	–	–	–
n-6 series	11.7	15.8	12.2	16.1	0.44	–	<0.001	–
n-3 series	11.3	5.80	11.4	5.71	0.50	–	<0.001	–
Ratio of n-6:n-3	1.03	2.74	1.07	2.84	0.08	–	<0.001	–

Table 3 Effect of culture treatment (lipoprotein fraction and dietary source of polyunsaturated fatty acid (PUFA)) and culture duration on granulosa cell proliferation, oestradiol (E₂) and progesterone (P₄) production (both adjusted for cell number) and transcript expression.

Lipoprotein (L) ^a	LDL		HDL		S.E.D.	Time of culture (h) (T)			S.E.D.	Significance		
	n-3	n-6	n-3	n-6		48	96	144		L	D	T
Dietary source (D) ^a												
Cell proliferation and steroidogenesis												
<i>n</i>	36	36	36	36		48	48	48				
Cell number (×10 ⁵)	2.93	3.00	3.18	3.02	0.152	2.81	3.41	2.86	0.132	–	–	<0.001
E ₂ (pg/10 ⁵ cells)	1289	1276	1259	1186	77.1	2337	847	566	66.8	–	–	<0.001
P ₄ (pg/10 ⁵ cells)	345	281	256	232		49	442	959				
(log ₁₀)	(2.538)	(2.450)	(2.408)	(2.366)	(0.0577)	(1.694)	(2.645)	(2.982)	(0.0499)	0.012	–	<0.001
Transcript expression relative to <i>ACTB</i>												
<i>n</i>	9	9	9	9		12	12	12				
SCD	0.0753	0.0704	0.0734	0.0925	0.0080	0.0197	0.0666	0.1475	0.0070	–	–	<0.001
<i>FADS1</i>	0.0036	0.0030	0.0034	0.0038	0.0008	0.0024	0.0024	0.0056	0.0007	–	–	<0.002
<i>FADS2</i>	0.0007	0.0008	0.0008	0.0007	0.0001	0.0005	0.0010	0.0007	0.0001	–	–	<0.001
<i>HSD3B1</i>	0.0731	0.0677	0.0667	0.0782	0.0090	0.0796	0.0405	0.0941	0.0080	–	–	<0.001
<i>STAR</i>	0.0049	0.0044	0.0033	0.0033	0.0007	0.0015	0.0033	0.0069	0.0006	0.018	–	<0.001

^aData averaged over the three time points (i.e. 48, 96 and 144 h culture).

LDL and HDL increased ($P<0.001$) n-3 series PUFA relative to n-6 series PUFA, in theca cells. As for GCs, the most abundant FA was oleic acid (C18:1n-9c), comprising on average 31 ± 0.35 g/100 g of TFA (Supplementary Table 7, see section on supplementary data given at the end of this article). Its content was greatest ($P<0.001$) in cells cultured with n-6 PUFA. The second most abundant FA was palmitic acid (C16:0), comprising 21.2 ± 0.52 g/100 g TFA, and stearic acid (C18:0) was the third (12.35 ± 0.26 g/110 g TFA). Of the seven n-6 FA identified, four were increased in cells cultured with n-6 PUFA: linoleic (C18:2n-6) ($P<0.001$), eicosadienoic (C20:2n-6) ($P=0.002$), dihomo- γ -linolenic acid (C20:3n-6) ($P=0.009$) and arachidonic acid ($P<0.001$). Similarly, all five identified n-3 FA were increased ($P<0.001$) in cells cultured with n-3 PUFA. Lipoproteins did not have an effect on FA uptake in these cells.

Similar to GCs, there was no overall (i.e. across all time points) differential effect of lipoprotein fraction or dietary source of PUFA on theca cell proliferation (Table 4). Cell number increased ($P<0.001$) with time during culture, and there was evidence ($P=0.012$) of a lipoprotein fraction × culture time interaction. Cell number (×10⁵) increased with time (48, 96 and 144 h) in the presence of both HDL (1.79, 2.26 and 2.85; S.E.D. = 0.128) and LDL (1.61, 2.47 and 3.22; S.E.D. = 0.128) but the fold increase in number was greater for cells cultured in the presence of the latter lipoprotein fraction. Cell-number-adjusted E₂ concentrations in spent media were extremely low at all time points during culture and were neither affected by lipoprotein fraction nor dietary source of PUFA. In contrast, cell-number-adjusted P₄ concentrations in spent media increased ($P<0.001$) with time during culture and were greater ($P<0.001$) across all time points for cells cultured in the presence of n-3 PUFA than n-6 PUFA irrespective of lipoprotein source. However, when data for 144 h were analysed separately, there was evidence ($P=0.037$) of a lipoprotein

fraction × dietary PUFA source interaction, which indicated that P₄ production was greatest for cells cultured in the presence of n-3 HDL than for the other treatment combinations (geometric means for n-3 HDL, n-6 HDL, n-3 LDL and n-6 LDL of 1387, 592, 653 and 705 ng/10⁵ cells respectively).

As for GCs, transcripts for *ELOVL2* were not expressed in theca cells. Although transcripts for *LDLR* and *FADS2* were expressed in theca cells, they were not affected by culture treatment or duration of culture and so these data are not presented. The expression of all remaining transcripts was altered ($P<0.001$) during culture (Table 4). For most of these transcripts, expression increased with time during culture. The most dramatic increases in expression were for the two steroidogenic enzymes *STAR* (sixfold increase) and *HSD3B1* (eightfold increase). In contrast, *CYP17A1* transcript expression decreased with time during culture so that, by 144 h culture, levels were around 8% of those at 48 h. There was an interaction ($P=0.008$) between lipoprotein fraction and PUFA source with respect to *SCARB1* expression. Transcript expression was greater for n-3 LDL and n-6 HDL than for n-6 LDL and n-3 HDL. Transcript expression for *ELOVL5* was also greater ($P=0.018$) for n-3 LDL than for the other treatment combinations. The only clear-cut effect of dietary PUFA source, however, was for *STAR* expression; n-3 PUFA increased ($P=0.006$) *STAR* transcript expression relative to n-6 PUFA irrespective of lipoprotein fraction.

Embryo culture

Experiments were conducted with sheep embryos, which involved culturing two- to four-cell zygotes to the blastocyst stage in SOFM with one of three PUFA-enriched supplements added: a) serum, b) LDL or c) albumin (Table 1). Both LDL and albumin were fractionated from the serum used in this study, which in

Table 4 Effect of culture treatment (lipoprotein fraction and dietary source of polyunsaturated fatty acid (PUFA)) and culture duration on theca cell proliferation, oestradione (E_2) and progesterone (P_4) production (both adjusted for cell number) and transcript expression.

Lipoprotein (L) ^a	LDL			HDL			Time of culture (h) (T)				Significance			
	n-3	n-6	n-3	n-6	n-3	n-6	48	96	144	s.e.d.	L	D	T	L×D
Dietary source (D) ^a														
Cell proliferation and steroidogenesis														
n	36	36	36	36	36	36	48	48	48					
E_2 (pg/10 ⁵ cells)	69	70	69	66	66	66	74	62	69	6.5	-	-	-	-
P_4 (pg/10 ⁵ cells)	149	100	218	105	105	105	14	231	783	(0.0729)	-	-	-	-
(log ₁₀)	(2.172)	(2.000)	(2.339)	(2.020)	(2.020)	(2.020)	(1.139)	(2.364)	(2.894)					
Transcript expression relative to ACTB														
n	9	9	9	9	9	9	12	12	12					
SCARB1	0.2334	0.2017	0.1917	0.2208	0.1917	0.1917	0.1226	0.2347	0.2785	0.0129	-	-	<0.001	0.008
SCD	0.0142	0.0177	0.0115	0.0132	0.0115	0.0115	0.0085	0.0102	0.0191	0.0032	-	-	<0.001	-
FADS1	0.0029	0.0028	0.0029	0.0033	0.0029	0.0029	0.0017	0.0031	0.0041	0.0003	-	-	<0.001	-
ELOVL5	0.1069	0.0776	0.0836	0.0898	0.0836	0.0836	0.0741	0.0790	0.1153	0.0085	-	-	<0.001	0.018
CYP11A1	0.0633	0.0671	0.0642	0.0694	0.0642	0.0642	0.0423	0.0612	0.0945	0.0053	-	-	<0.001	-
CYP17A	0.0052	0.0049	0.0039	0.0045	0.0039	0.0039	0.0118	0.0012	0.0009	0.0010	-	-	<0.001	-
HSD3B1	0.0539	0.0612	0.0562	0.0655	0.0562	0.0562	0.0127	0.0599	0.105	0.0112	-	-	<0.001	-
STAR	0.018	0.010	0.018	0.012	0.018	0.018	0.0042	0.0123	0.0271	0.0027	-	0.006	<0.001	-

^aData averaged over the three time points (i.e. 48, 96 and 144 h culture).

turn was derived from the ewes that were offered n-3 or n-6 PUFA-enriched diets, as described earlier.

An orthogonal contrast analysis indicated that the inclusion of PUFA-enriched serum in SOFM increased ($P=0.036$) the FA content (ng/blastocyst) of Day-7 blastocysts relative to the embryos cultured with FAF-BSA (Table 5). The inclusion of serum also increased the percentage and absolute mass (ng/blastocyst) of n-3 PUFA but not of n-6 PUFA. Furthermore, relative to n-6 PUFA-enriched serum, n-3 PUFA-enriched serum increased the percentage n-3 PUFA and reduced the ratio of n-6:n-3 PUFA in blastocysts; n-3 PUFA-enriched serum was also effective in increasing ($P=0.004$) the percentage and absolute mass of eicosapentaenoic acid (C22:5n-3) and DHA (C22:6n-3) in Day-7 blastocysts (Supplementary Table 8, see section on supplementary data given at the end of this article).

Similar to serum, the inclusion of PUFA-enriched albumin in SOFM increased ($P=0.02$) the FA content of Day-7 blastocysts relative to embryos cultured with LDL and FAF-BSA (Table 6). Relative to n-6 PUFA-enriched albumin, the inclusion of n-3 PUFA-enriched albumin increased ($P=0.042$) the percentage n-3 PUFA and reduced ($P=0.047$) the ratio of n-6:n-3 PUFA in blastocysts. As for serum, notable PUFA increases in blastocysts by n-3 PUFA-enriched albumin were eicosapentaenoic acid (C22:5n-3) and DHA (C22:6n-3) (Supplementary Table 9, see section on supplementary data given at the end of this article).

There were marked differences between the embryo culture experiments described above in the relative percentages of the different FA classes, which could not be ascribed to the nature and composition of the supplements used. Blastocysts from the serum experiment had the greatest percentage saturated FA ($56.6 \pm 2.1\%$) consisting mostly stearic acid (C18:0, $30.5 \pm 1.3\%$) and palmitic acid (C16:0, $22.8 \pm 0.8\%$). The next most abundant FA was oleic acid (C18:1n-9, $29.1 \pm 1.5\%$) followed by linoleic acid (C18:2n-6, $4.03 \pm 0.06\%$). In contrast, embryos cultured in the presence of either LDL or albumin had the greatest percentage MUFA ($48.7 \pm 0.6\%$), where once again oleic acid (C18:1n-9, $46.0 \pm 0.5\%$) was the most abundant FA. Stearic acid (C18:0, $14.9 \pm 0.3\%$) was the next most abundant FA, followed by palmitic acid (C16:0, $13.6 \pm 0.2\%$) and then linoleic acid (C18:2n-6, $7.00 \pm 0.17\%$).

In a separate series of experiments, the inclusion of PUFA-enriched serum and albumin increased the proportion of blastocysts present on Days 6 ($P<0.001$) and 7 ($P<0.05$) of culture relative to the FAF-BSA control (Fig. 1A and C). In contrast, the inclusion of PUFA-enriched LDL had no effect on embryo development (Fig. 1B). There was no differential effect of dietary source of PUFA on the percentage of zygotes that developed to the blastocyst stage. Similarly, there was no effect of PUFA source nor indeed supplement type on the proportion of blastocysts classified (according to IETS

Table 5 Total fatty acids (TFA) and percentages (g/100 g TFA) of saturated FA, unsaturated FA, monounsaturated FA (MUFA) and polyunsaturated FA (PUFA) of Day-7 sheep blastocysts cultured^a *in vitro*.

Culture treatment		Serum			Orthogonal contrasts	
PUFA source	FAF-BSA	n-3	n-6	S.E.D.	BSA versus serum	n-3 versus n-6
<i>n</i>	4	4	4			
TFA (ng/blastocyst)	150.6	157.8	166.6	4.97	0.036	–
Saturated	55.5	56.2	58.0	1.01	–	–
Unsaturated	40.7	40.2	38.3	0.94	–	–
MUFA	32.6	31.8	30.8	0.60	0.05	–
PUFA	8.1	8.3	7.5	0.41	–	–
n-6 series	7.2	6.1	6.0	0.28	0.004	–
n-3 series	0.9	2.2	1.5	0.16	<0.001	0.004
Ratio of n-6:n-3	7.7	2.7	4.2	0.49	<0.001	0.002

^aCulture incorporated fatty-acid-free BSA (FAF-BSA) or serum obtained from ewes offered diets enriched with either n-3 or n-6 PUFA (Wonnacott *et al.* 2010).

(1998)) as being early, mid, expanding, hatching or hatched (data not presented). However, the inclusion of either PUFA-enriched serum or albumin, but not LDL, increased ($P<0.001$) the proportion of lower grade (IETS 1998) Day-7 blastocysts (Fig. 2). Once again, there was no differential effect of PUFA source.

The inclusion of all three PUFA-enriched supplements reduced transcript expression in Day-7 blastocysts for *SCARB1* and *LDLR* relative to the FAF-BSA control (Fig. 3A–F). This effect was generally greater for n-3 than n-6 PUFA-enriched supplements. Relative to FAF-BSA control, the inclusion of serum or albumin, but not LDL, increased ($P\leq 0.05$) transcript expression for *SOD1* (Fig. 3G–I). Differences ($P<0.001$) in *SCD* expression between treatments were evident for the serum supplement experiment but not for the other two experiments, where *SCD* transcript expression for FAF-BSA control blastocysts was reduced in line with that for embryos cultured in the presence of PUFA-enriched LDL and albumin (Fig. 3J–L). Transcripts for *FADS1* were not detected in blastocysts, but transcripts for *FADS2* were present and their expression was increased in embryos cultured in the presence of n-6 PUFA-enriched serum, relative to the FAF-BSA control, but not by the other

supplements (Fig. 3M–O). Transcripts for *ELOVL5* were more abundantly expressed in the 'serum experiment' (i.e. both in supplemented and FAF-BSA control blastocysts; Fig. 3P) than in experiments in which the other two supplements were added (Fig. 3Q and R). The inclusion of PUFA-enriched LDL reduced ($P=0.003$) transcript expression for *ELOVL5* relative to FAF-BSA control blastocysts. Neither PUFA-enriched serum nor albumin altered *ELOVL5* expression in blastocysts relative to FAF-BSA controls.

Discussion

Several important and novel findings emerge from these experiments, which add to our previous observations (Wonnacott *et al.* 2010). It was reported in that study that the increase in follicular fluid P_4 concentrations in ewes fed n-3 PUFA compared to n-6 PUFA-enriched diets could not be recapitulated in GCs cultured in the presence of PUFA-enriched HDL. This study confirms this observation and now reveals, for the first time, that the n-3 PUFA-associated increase in P_4 levels within the ovarian follicle arises solely in theca cells, and is mediated in part by an n-3 PUFA-associated increase

Table 6 Total fatty acids (TFA) and percentages (g/100 g TFA) of saturated, unsaturated, monounsaturated (MUFA) and polyunsaturated (PUFA) fatty acids of Day-7 sheep blastocysts cultured^a *in vitro*. Analysis was based on three replicate groups of 10–35 embryos.

Serum fraction		LDL		Albumin (Alb)		S.E.D.	Orthogonal contrasts			
PUFA source	FAF-BSA	n-3	n-6	n-3	n-6		FAF-BSA versus suppl	LDL versus Alb	LDL n-3 versus LDL n-6	Alb n-3 versus Alb n-6
<i>n</i>	3	3	3	3	3					
TFA (ng/blastocyst)	117.2	115.3	121.1	126.6	131.1	4.17	0.09	0.02	–	–
Saturated	33.0	34.2	33.2	31.0	33.4	1.34	–	–	–	–
Unsaturated	63.9	61.9	63.1	65.4	62.7	1.21	–	–	–	0.06
MUFA	48.9	47.5	47.9	50.2	48.9	1.52	–	–	–	–
PUFA	14.3	14.4	15.2	15.2	13.9	0.95	–	–	–	–
n-6 series	12.9	13.3	14.4	11.6	11.6	1.01	–	0.014	–	–
n-3 series	1.4	1.1	0.7	3.6	2.3	0.48	–	0.001	–	0.042
Ratio of n-6:n-3	9.8	12.2	19.8	3.3	6.8	1.32	–	<0.001	0.011	0.047

^aCulture incorporated fatty-acid-free BSA (FAF-BSA) or a supplement (suppl) of low-density lipoprotein or albumin fractionated from serum of ewes offered diets enriched with either n-3 or n-6 PUFA (Wonnacott *et al.* 2010).

Figure 1 Embryo development following 6 (open bars) or 7 (filled bars) days of culture in synthetic oviductal fluid media supplemented with either fatty-acid-free BSA (FAF-BSA) or one of the three PUFA-enriched supplements (A) serum, (B) low-density lipoproteins (LDL) or (C) albumin; the latter two supplements were fractionated from the serum used in (A). This serum was derived from ewes offered either n-3 or n-6 polyunsaturated fatty acid (PUFA)-enriched diets. Levels of significance represent orthogonal contrasts between each supplement and FAF-BSA. There was no differential effect of n-3 and n-6 PUFA on embryo development.

in *STAR* transcript expression. Furthermore, delivery of n-3 PUFA from either LDL or HDL, in a manner that supports both theca cell proliferation and steroidogenesis, appears equally efficacious although, by 144 h culture, only n-3 PUFA-enriched HDL in this study was associated with increased P_4 production in partially luteinised theca cells. Although the mechanisms underlying this effect are not clear they are consistent with the observations of Bao *et al.* (1995), who also found that HDL, but not LDL, increased P_4 production in bovine theca cells between 96 and 144 h culture. Furthermore, this effect may be induced by the underlying protein molecule as Chandras *et al.* (2004) found that lipid-free apolipoprotein-A1 increased P_4 production in human granulosa-lutein cells following 3 days of culture.

The study of Wonnacott *et al.* (2010) also revealed that there is no net uptake of FA from HDL by sheep embryos, so that FA composition was similar to embryos cultured in the presence of FAF-BSA. This study extends this observation to demonstrate that there is also no net increase in FA content of sheep embryos when cultured in the presence of LDL, but that culture in the presence of either PUFA-enriched serum or albumin leads to a net increase in FA content of Day-7 blastocysts. Furthermore, in terms of absolute mass (ng/blastocyst), culture of sheep embryos in the presence of n-3 PUFA-enriched serum or albumin, but not n-3 PUFA-enriched LDL, increased the intra-cellular content of n-3 series PUFA. A greater proportion of embryos cultured in the presence of either serum or albumin developed to the blastocyst stage, but more of these were classified as having moderate to poor morphological grades associated with increased transcript expression for the antioxidant enzyme *SOD1*.

Finally, this study reports for the first time the expression of transcripts for specific enzymes involved in fatty acid elongation and desaturation in the ruminant ovarian follicle and in the mammalian pre-implantation embryo. Transcripts for *SCD*, *FADS1* and *FADS2*, along with transcripts for *ELOVL5*, were expressed in the three cell types studied. However, although expressed in sheep liver (data not presented), transcripts for *ELOVL2* were not detected in either granulosa or theca cells, or in Day-7 blastocysts. This latter observation is somewhat at odds with an earlier study revealing mRNA expression for *ELOVL2* in the mouse testis (Tvrdik *et al.* 2000), suggesting either species- and/or a gender-specific tissue distribution for this enzyme.

Granulosa and theca cell culture

Lipoprotein fractions in this study were added to TCM199 and DMEM/Hams-F12 so that FA supply was equivalent to that provided by serum incorporated at 5% v/v. Measured concentrations of FA in supplemented media matched predictions (Supplementary Table 5) but, understandably, were considerably lower than those

found in body fluids including follicular fluid to which granulosa and theca cells are exposed (Sinclair *et al.* 2008). Under such circumstances, cholesterol provision for steroidogenesis will be limiting so that the provision of lipoprotein fractions in this study and that of Wonnacott *et al.* (2010), albeit at low levels, enhanced

Figure 2 Morphological grade (IETS 1998; Grade 1 is good, Grade 4 is poor) of Day-7 blastocysts cultured in synthetic oviductal fluid media supplemented with either fatty-acid-free BSA (FAF-BSA) or one of the three PUFA-enriched supplements described in Fig. 1. The inclusion of either serum (A) or albumin (C) increased ($P < 0.001$) the proportion of embryos with poor grades. In contrast LDL (B) had no effect on embryo grade.

P_4 and E_2 production by cultured follicular cells. This is perhaps further exemplified in this study where the provision of LDL, which contains proportionally more cholesterol and cholesteryl esters than HDL (Christie 1981), increased P_4 production and *STAR* expression relative to HDL in GCs. Consistent with the previous observations of Wonnacott *et al.* (2010), there was no differential effect of n-3 and n-6 PUFA on P_4 production by cultured GCs, which is in stark contrast to that observed for theca cells in this study, where both n-3 PUFA-enriched LDL and HDL increased P_4 production and *STAR* transcript expression relative to n-6 PUFA-enriched lipoproteins. These observations support the increase in P_4 concentrations in follicular fluid reported for ewes offered an n-3 PUFA-enriched diet (Wonnacott *et al.* 2010). The mechanisms that explain this effect are complex, not fully understood and are beyond the immediate scope of this study to explore in any great depth. A putative stimulatory effect on steroidogenesis by n-3 PUFA, however, could arise as a consequence of their known inhibitory effects on prostaglandin-endoperoxide synthase 2 (PTGS2) activity (Ringbom *et al.* 2001), as inhibition of PTGS2 activity with indomethacin in MA-10 mouse Leydig cells enhanced *STAR* transcript expression (Wang *et al.* 2003). Alternatively, or in addition, this increase in *STAR* expression and P_4 production by theca cells may have arisen as a consequence of reduced (to around 50%) levels of arachidonic acid (C20:4n-6) in n-3 PUFA-supplemented cells. Certain metabolites of arachidonic acid are known to inhibit *STAR* expression (Wang *et al.* 2006).

The 7.5-fold increase in *SCD* transcript expression in GCs is consistent with that reported previously (Wonnacott *et al.* 2010) for this cell type, and probably represents a feature of cells cultured with relatively high (i.e. 10 ng/ml) concentrations of insulin, which is known to increase *SCD* expression in sheep tissues (Daniel *et al.* 2004). Both LH and insulin have been reported to increase *STAR* expression in cultured porcine theca cells (Zhang *et al.* 2000), and transcript expressions of *ELOVL5* and *FADS1* are also known to be responsive to insulin (Brenner *et al.* 2003). In theca cells, the increase in *CYP11A*, *HSD3B1* and *STAR* over time was consistent with the temporal increase in P_4 production. The temporal decrease in *CYP17A* expression in theca cells, on the other hand, was consistent with the onset of luteinisation in this cell type (Demeter-Arlotto *et al.* 1993).

Embryo culture

In contrast to LDL, which had no effect, serum and albumin supplementation of SOFM enhanced blastocyst yields and PUFA-enriched serum and albumin increased the FA content of Day-7 sheep blastocysts. However, this was associated with poorer morphological grades and increased *SOD1* transcript expression, indicative of enhanced superoxide toxicity (Lequarré *et al.* 2001).

Lipid accumulation by ovine embryos cultured in the presence of serum has been reported previously and this was also associated with markers of impaired embryo quality (Thompson *et al.* 1995, Reis *et al.* 2003a, 2005). Lipid peroxidation was also enhanced in Day-3 sheep embryos cultured with FAF-BSA, to which DHA

(C22:6n-3) was complexed, leading to arrested development at the 8–16-cell stage (Reis *et al.* 2003b).

Considered together, evidence from this study and that of Wonnacott *et al.* (2010) demonstrate that pre-implantation embryos acquire FA, particularly n-3 PUFA, from the albumin fraction of serum and not from the other major lipoprotein fractions. However, in the absence of a net uptake of FA, HDL, presumably acting via SCARB1, reduced the transcript expression for *LDLR* and *SCD* and, in the case of n-6 PUFA-enriched HDL, reduced the proportion of zygotes that developed to the blastocyst stage (Wonnacott *et al.* 2010). Despite PUFA compositional differences in this study, however, there was evidence of only a partial effect of PUFA source on transcript expression (e.g. for *SCARB1* and *LDLR*) and no differential effect on embryo development or quality. The increase in *FADS2* expression in embryos cultured with n-6 PUFA-enriched serum compared to control and n-3 PUFA-exposed embryos is enigmatic as neither LDL nor albumin-derived PUFAs had any differential effect on expression of transcripts for this gene. It may merely reflect some obscure mechanism associated with the relatively low percentage of linoleic (C18:2n-6) and α -linolenic (C18:3n-3) acids in embryos from this experiment.

A notable feature of Day-7 blastocysts in this study was their high FA content (110–170 ng/blastocyst), which was greater than that reported by Wonnacott *et al.* (2010) (60–80 ng/blastocyst) and Reis *et al.* (2002) (68–124 ng/blastocyst). From Table 4, it is apparent that the differences between the current three culture experiments are inherent to the FA composition of oocytes from which these embryos were derived; they are clearly not a feature of the culture conditions *per se*. Basic SOFM and SOFM+FAF-BSA had only trace amounts of FA (i.e. 0.0025 that of serum), which are probably contaminants in some of the organic supplements (e.g. amino acids) added. Oocytes used in the current series of experiments were from abattoir-derived ovaries for which we have no information on genotype, age or dietary background of donors. This non-descript background also probably accounts for the reduced blastocyst yields observed in the LDL and albumin experiment (which was conducted between January and March, 2010) compared with the serum experiment (conducted during November and December, 2009). Support for the contention that the differences in FA composition of embryos between experiments are biological rather than analytical in nature are gained, firstly, from the increase in transcript expression for *ELOVL5* in the serum experiment relative to either the LDL or albumin experiment, in which PUFA levels were almost twofold higher. Support is also provided by the negative relationship between transcript expression for *SCD* and the percent oleic (C18:1) and linoleic (C18:2n-6) acid of Day-7 blastocysts cultured in FAF-BSA across the three experiments (Supplementary Figure 1, see section on supplementary data given at the end of this article).

Figure 3 Transcript expression (relative to *GAPDH*) of Day-7 blastocysts cultured in synthetic oviductal fluid media supplemented with either fatty-acid-free BSA (BSA, open bars) or one of the three PUFA-enriched supplements (filled bars) (serum, left column; LDL, middle column; albumin, right column). Levels of significance are for orthogonal contrasts between (i) each supplement (Supp) and BSA controls and (ii) within n-3 versus n-6 PUFA-enriched supplements (PUFA); NS, not significant.

Diets rich in PUFA are known to reduce *SCD* transcript expression in mice and recently, using promoter–reporter gene constructs transfected into kidney- and liver-derived cell lines, Zulkifli *et al.* (2010) demonstrated that the addition of both oleic and linoleic acid to culture media reduced the activity of the *SCD* promoter from four different species (i.e. human, mouse, pig and sheep) in a dose-dependent manner.

Conclusions

PUFA act on both granulosa and theca cells within the ovarian follicle to promote steroidogenesis, but the differential effects of n-3 and n-6 PUFA on P_4 production are mediated solely within theca cells, and this may primarily arise as a consequence of increased *STAR* expression. Although n-3 PUFA from both LDL and HDL enhance P_4 production by theca cells, relative to n-6 PUFA, only HDL-derived n-3 PUFA enhance P_4 synthesis by luteinising theca cells. The FA composition of sheep embryos is highly variable, largely as a consequence of inherent differences in the FA composition of oocytes. Both serum and albumin promote blastocyst yields when added to culture, but they each impair embryo quality. Given that there was no net uptake of FA from LDL in this study or from HDL in the study of Wonnacott *et al.* (2010), and that very low density lipoprotein (VLDL) and chylomicrons account for just 0.2% (by weight) total lipoprotein lipid in serum (Christie 1981), it would appear that albumin is the primary if not sole fraction of serum responsible for FA uptake by embryos during culture, and is effective in the delivery of n-3 PUFA. This study highlights important differential effects of n-3 and n-6 PUFA on ovarian steroidogenesis, but differential effects on pre-implantation development are less apparent. Future attention will be directed towards assessing the importance of PUFA assimilated during oocyte growth and maturation, relative to PUFA acquired during pre-implantation development, on embryo viability and pregnancy establishment. Indeed, emerging evidence indicates that PUFA supplementation during oocyte maturation *in vitro* can influence early embryo development (Marei *et al.* 2010).

Materials and Methods

All reagents were obtained from Sigma–Aldrich unless otherwise stated.

Fractionation of lipoproteins from serum

Very low-density ($d < 1.006$ g/ml), low-density ($d = 1.006$ – 1.06 g/ml) and high-density ($d = 1.06$ – 1.24 g/ml) lipoprotein fractions were separated from pooled sera, obtained from ewes offered n-3 and n-6 PUFA-enriched diets, by sequential preparative ultracentrifugation in a Beckman XL-70 Preparative Centrifuge (Beckman Coulter UK Ltd, Buckinghamshire, UK) as

described previously (Wonnacott *et al.* 2010). The remaining fraction was free of lipoproteins and contained mainly albumin. Following dialysis to remove KBr, fractionated lipoproteins and albumin were aliquoted and frozen at -20°C until analysis by gas chromatography or added to culture media of follicular cells or embryos.

Culture of primary granulosa and theca cells

Granulosa and theca cell experiments were conducted separately, each as a $2 \times 2 \times 3$ factorial (i.e. LDL versus HDL; n-3 versus n-6 PUFA; and three sample times (48, 96 and 144 h)) arrangement in 6×24 -well plates (Nunc, Thermo Fisher Scientific, Loughborough, UK). Cells within plate and within treatment combination were harvested from separate wells at 48, 96 and 144 h for FA and transcript analyses, and spent culture media was analysed for steroid hormones.

Large (>5 mm) amber-coloured clear follicles were dissected from abattoir-derived sheep ovaries, and GCs were scraped from inner follicle wall using a sterile inoculation loop and oocytes were removed. Theca cells were removed by pinching the inner wall with curved forceps and peeling away from the outer shell. GCs were resuspended in a culture medium (90% v/v TCM199 and 10% v/v double distilled water) supplemented with 1 mg/ml BSA, 100 ng/ml L-glutamine, 10 ng/ml insulin, 0.5 $\mu\text{g/ml}$ transferrin, 0.5 ng/ml sodium selenite, 50 IU/ml penicillin, 50 $\mu\text{g/ml}$ streptomycin, 1 ng/ml FSH and 100 ng/ml testosterone, at an initial seeding density of 3.5×10^5 viable cells (determined by Trypan blue exclusion)/500 μl . Theca cells underwent protease digestion in DMEM/Hams F12 media supplemented with 50 IU/ml penicillin, 50 $\mu\text{g/ml}$ streptomycin, 5 mg/ml collagenase I, 1 mg/ml hyaluronidase and 25 g/ml (188 U/ml) DNase1. Cells were incubated at 37°C for 30 min, washed and then resuspended in a culture medium (DMEM/Hams F12) supplemented with 1 mg/ml BSA, 100 ng/ml L-glutamine, 10 ng/ml insulin, 0.5 $\mu\text{g/ml}$ transferrin, 0.5 ng/ml sodium selenite, 50 IU/ml penicillin, 50 $\mu\text{g/ml}$ streptomycin, 1 ng/ml LH, at an initial seeding density of 2.0×10^5 viable cells/500 μl . Plates used for theca cultures were pre-coated with fibronectin (500 μl of 6 $\mu\text{g/ml}$ fibronectin/well at 39°C for 4 h, removed then air dried for 2 h). Basal media for both cell types was supplemented with lipoproteins at 57 $\mu\text{l/ml}$ media (LDL fraction) and 22 $\mu\text{l/ml}$ media (HDL fraction). These inclusion rates were calculated to provide FA at level equivalent to that of 5% v/v serum (Table 1). Both media were kept at pH 7.4 and 270–280 mOsm and cells were incubated at 38.8°C in a humidified atmosphere of 5% CO_2 for up to 144 h. Eighty percent of the media was collected at 48 h intervals, and replaced with fresh media. All cells were snap-frozen in liquid nitrogen prior to storage at -80°C .

Embryo culture

The culture of sheep zygotes was based on in-house protocols described previously (Powell *et al.* 2006, Wonnacott *et al.* 2010), and only a brief summary is provided here. Grades 1 and 2 cumulus-oocyte complexes (COCs) were matured in groups of 50 in 500 μl bicarbonated-buffered TCM199 with supplements. For fertilisation, 1×10^6 motile spermatozoa from a

single ram was added/ml of media containing matured oocytes (up to 60/well) and incubated for 22 h at 38.8 °C in a humidified atmosphere of 5% CO₂ in air. Over the course of three experiments, zygotes (four to eight cells) were selected and cultured in groups of 30–50 in 400 µl SOFM (Supplementary Table 5) to which various supplements were added. Experiment 1: i) 0.1% w/v FAF-BSA (control), ii) n-3 PUFA-enriched serum (5% v/v) or iii) n-6 PUFA-enriched serum (5% v/v). Experiment 2: i) 0.1% w/v fFAF-BSA (control), ii) 11 µl n-3 PUFA-enriched LDL/ml media or iii) 11 µl n-6 PUFA-enriched LDL/ml media. Experiment 3: i) 0.1% w/v FAF-BSA (control), ii) 160 µl n-3 PUFA-enriched albumin/ml media or iii) 160 µl n-6 PUFA-enriched albumin/ml media. The inclusion rates for LDL and albumin were calculated to provide FA at level equivalent to that of 1% v/v serum (Table 1). The serum experiment was replicated 8 times, whereas the LDL and albumin experiments were replicated 10 and 11 times respectively. Embryo quality (i.e. stage of development and

grade) was assessed on Days 6 and 7 of culture according to the International Embryo Transfer Society (IETS) classification system (IETS 1998). Day-7 embryos within well were washed in 1 × PBS and 0.1% polyvinyl alcohol (PVA), snap-frozen in minimal volume and stored at –80 °C until either FA or transcript analysis.

Fatty acid analysis

Fatty acid analyses of culture media, serum and lipoprotein fractions (i.e. LDL, HDL and albumin), GCs, theca cells and blastocysts were based on established in-house protocols (Fouladi-Nashta *et al.* 2009, Wonnacott *et al.* 2010). Twenty-six quantitatively significant and/or biologically important fatty acids were identified for subsequent statistical analysis. Blastocyst FA were extracted from pools of 10–35 embryos, together with 50 µl of 100 µg/ml internal standard (pentadecanoic acid; C15:0), using a 2:1 mixture of

Table 7 Alphabetical list of primer and probe sequences used for real-time quantitative PCR analysis of transcript expression in follicular cells and embryos.

Name	Name	Sequence 5'–3'	NCBI accession numbers
ACTB	F	TGTGCGTGACATCAAGGAGAA	AF129289 <i>Ovis aries</i>
	R	CGCAGTGGCCATCTCCTG	
	Probe	FAM-CTGCTACGTGGCCCTGGACTTCGA-TAMRA	
CYP11A1	F	TCGGCAATTTGGAGTCAGTTT	D50057 <i>Ovis aries</i>
	R	GGTAGGATCCCTCGAACTTAAAGAG	
	Probe	FAM-ATCATTACCCTGAAGGCGTGGCC-TAMRA	
CYP17A	F	TCATCTCGCCATCGTTAAGCT	AF251388 <i>Ovis aries</i>
	R	CGGGCTAGCATCTCACCTACA	
	Probe	FAM-TTGCCCTTTGGAGCCGGACCC-TAMRA	
ELOVL2	F	TTCCTTGTGGGCAAAGTTTCTT	BC114786 <i>Bos taurus</i>
	R	GCCCCGTAGTAGGAGTACATGAGGAT	
	Probe	FAM-ACGCTACCACCACGCCTCCATGTTT-TAMRA	
ELOVL5	F	TCACGGTCTCGCATGTGTATC	BC105391 <i>Bos taurus</i>
	R	GACCCAGTTCATCACGAACCA	
	Probe	FAM-CCACGCCAGCATGCTCAACATCTG-TAMRA	
FADS1	F	AGCTGCCCCCTGAGGATATTC	EU272818 <i>Ovis aries</i>
	R	CGACCACTCGAAGATGTCGTT	
	Probe	FAM-CTGCTGCCAACTCCATGGCTTTCC-TAMRA	
FADS2	F	TGGCCTGCATGCTCATCA	BC133520 <i>Bos taurus</i>
	R	TGGAAGATGTTGACGTGGAGAT	
	Probe	FAM-AGGTCCCTGCTGGCCACCC-TAMRA	
GAPDH	F	TCCGTTGTGGATCTGACCTG	AF030943 <i>Ovis aries</i>
	R	TGCTTCACCACCTTCTTGATCTC	
	Probe	FAM-CGCCTGGAGAAACCTGCCAAGTATGA-TAMRA	
HSD3B1	F	CCAGGCTAGTGTGCCAGTCTTT	NM_001135932 <i>Ovis aries</i>
	R	CTCCCTGTAGGCGTTGGGCC	
	Probe	FAM-TCCACACCAGCACCATAGAGGTGGCT-TAMRA	
LDLR	F	GAGCGTGGGTGCCCTATACA	K01830 <i>Bos taurus</i>
	R	TTCCGAAGGCCAGGAGG	
	Probe	FAM-CGTCTCCCCATCGACTGCTC-TAMRA	
SCARB1	F	TCCAAGGCCAGAAGCCAC	AF019384 <i>Bos taurus</i>
	R	GCTCTTGTCCTGAACCTCC	
	Probe	FAM-TGCAGGAGCACGGCCCTTATGTGA-TAMRA	
SCD	F	CGAACCTACAAAGCTCGGCT	NM_001009254 <i>Ovis aries</i>
	R	TGGAACGCCATGGTGTG	
	Probe	FAM-CCCCTACGGGTCTTCCTGATCATCG-TAMRA	
SOD1	F	GCCGTCTGCGTGTGAA	XM 584414 <i>Bos taurus</i>
	R	ACTGTATTTCCCTTTGCCTCGA	
	Probe	FAM-CCCGGTGCAAGGCACCATCC-TAMRA	
STAR	F	GCGACGTTAAAGCTGTGTCT	AF290202 <i>Ovis aries</i>
	R	TGCTGCCGACGCC	
	Probe	FAM-AGCTCTATAGACACGTGCCGAGCATG-TAMRA	

chloroform:methanol and methylated using methanolic HCl using the same protocol as described for GCs (Fouladi-Nashta *et al.* 2009). A dedicated Varian CP-Sil 88-fused silica capillary column (100 m×0.25 mm ID, 0.2 µm film) was used to analyse blastocysts (Wonnacott *et al.* 2010) as the quantities of fatty acid methyl esters were so low.

Steroid analysis

Culture media E₂ (diluted 1:10 for GCs) were analysed in duplicate by an in-house ELISA; 96-well plates (Immuno-Maxisorp; Nuncbrand Fisher Scientific) were coated with E₂ 3 antibody (Abcam, Cambridge, UK) at a dilution of 1:64 000 in coating buffer (50 mM sodium bicarbonate, 50 mM sodium carbonate, pH 9.6), sealed and incubated overnight at 4 °C. Plates were washed four times in wash buffer (PBS, 0.05% Tween20, pH 7.4) and 100 µl blocking solution (PBS, 3% BSA) added to each well and incubated at room temperature for 2 h. Following washing, 100 µl of either sample, standard or QC, was added to each well together with 100 µl HRP-conjugate and incubated for 2 h at room temperature. Plates were washed again and 50 µl tetramethylbenzidine were added to each well and incubated for 12 min at room temperature; 50 µl stop solution (0.5 M H₂SO₄) were added to each well and plates were read at 450 nm. The limit of detection was 39 pg/ml, and the working range extended to 10 ng/ml. Intra- and inter-assay coefficient of variation (CV) values were 9.8 and 4.2% respectively. P₄ ELISA was carried out on spent media as previously described (Wonnacott *et al.* 2010). Samples were diluted up to 1:400 (for theca cells) and up to 1:100 (for GCs). Intra- and inter-assay CV values were 7.9 and 8.4% respectively.

Transcript analysis

Total RNA was extracted from cultured ovine granulosa and theca cells using RNeasy Mini kit, followed by RNase-free DNase (Promega) to remove genomic DNA contamination, and reverse transcribed using Omniscript RT kit (Qiagen Ltd; Kanakkaparambil *et al.* 2009, Wonnacott *et al.* 2010). RNA was extracted from pools of 10 to 35 ovine blastocysts using Dynabeads mRNA DIRECT kit (Invitrogen Ltd; Kwong *et al.* 2006). RT of mRNA from blastocysts was performed using QuantiTect RT kit (Qiagen Ltd), which eliminates genomic DNA and efficiently reverse transcribes small quantities of RNA in a two-step reaction. To ensure no genomic DNA contamination in samples, –RT (equivalent to 0.7 blastocyst) was used in quantitative RT-PCR (qRT-PCR) with *ACTB* qRT-PCR. qRT-PCR was performed using Roche LightCycler 480 (Roche Diagnostics Ltd) with gene-specific primers and TaqMan probes (Table 7). For blastocysts, cDNA equivalent to 0.7 embryo was added to each PCR. The probes and primers for qRT-PCR were designed using Primer Express. All TaqMan probes were labelled with 6'-carboxyfluorescein (FAM) and 6'-carboxy-*N,N,N',N'*-tetramethylrhodamine (TAMRA) at 5' and 3' end respectively (Eurofins MWG Operon, Raynes Park, London, UK). For each target gene, sequence homology was matched to the ovine sequence but where this was not

possible the bovine sequence was used. The size of PCR product was confirmed by gel electrophoresis.

Statistical analysis

All statistical analyses were conducted using Genstat release 11.1 (Genstat 2008). FA and steroid concentrations in media and cells were analysed by using ANOVA. P₄ data were transformed (log₁₀) prior to analysis to satisfy the condition of 'additivity', i.e. to correct for the differences in means and variance across the three time points. Data are presented as geometric means with log₁₀ means and S.E.D. presented in italics. Where an FA was not detectable in a single treatment (i.e. an FA percentage <0.03 g/100 g TFA), then the FA percentage in the other treatment was analysed by using a two-tailed *t*-test to determine whether it was significantly greater than the detection limit. In the case of cultured granulosa and theca cells, data were analysed by ANOVA as a three-way factorial randomised block design as described earlier.

The proportions of zygotes that developed into blastocysts were analysed by analysis of deviance using generalised linear models assuming binomial errors and with logit link functions. Data are presented as predicted back-transformed means ± S.E.M. Transcript and FA data for blastocysts were analysed by ANOVA. For FA analysis of embryos in the serum experiment, and in the analysis of transcript expression for each of the three supplements, the variations associated with two degrees of freedom related to the three treatment groups were, in each case, partitioned into two orthogonal linear contrasts (i.e. FAF-BSA versus supplement (i.e. serum, LDL or albumin) and n-3 supplement versus n-6 supplement). For the embryo culture experiment that assessed the effects of n-3 and n-6 PUFA-enriched LDL and albumin on the fatty acid composition of embryos, the variations associated with four degrees of freedom related to the five treatment groups were partitioned into the following orthogonal contrasts (FAF-BSA versus supplement, LDL versus albumin, n-3 PUFA LDL versus n-6 PUFA LDL and n-3 albumin versus n-6 albumin).

Finally, the effects of culture treatment and morphological assessments of embryo stage on embryo grade were assessed by ordinal regression analysis assuming multinomial errors with logit link functions. Data are presented as the proportion of embryos within each of the four embryo-grade categories.

Supplementary data

This is linked to the online version of the paper at <http://dx.doi.org/10.1530/REP-10-0337>.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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