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Dietary omega-3 and -6 polyunsaturated fatty acids affect the composition and development of sheep granulosa cells, oocytes and embryos

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Abstract

The evidence that omega-3 (n-3) and -6 (n-6) polyunsaturated fatty acids (PUFAs) have differential effects on ovarian function, oocytes and embryo quality is inconsistent. We report on the effects of n-3 versus n-6 PUFA-enriched diets fed to 36 ewes over a 6-week period, prior to ovarian stimulation and follicular aspiration, on ovarian steroidogenic parameters and embryo quality. Follicle number and size were unaltered by diet, but follicular-fluid progesterone concentrations were greater in n-3 PUFA-fed ewes than in n-6 PUFA-fed ewes. The percentage of saturated FAs (mostly stearic acid) was greater in oocytes than in either granulosa cells or plasma, indicating selective uptake and/or *de novo* synthesis of saturated FAs at the expense of PUFAs by oocytes. High-density lipoproteins (HDLs) fractionated from sera of these ewes increased granulosa cell proliferation and steroidogenesis relative to the FA-free BSA control during culture, but there was no differential effect of n-3 and n-6 PUFAs on either oestradiol or progesterone production. HDL was ineffective in delivering FAs to embryos during culture, although n-6 PUFA HDL reduced embryo development. All blastocysts, irrespective of the treatment, contained high levels of unsaturated FAs, in particular linoleic acid. Transcripts for HDL and low-density lipoprotein (LDL) receptors (*SCARB1* and *LDLR*) and stearoyl-CoA desaturase (*SCD*) are reported in sheep embryos. HDL reduced the expression of transcripts for *LDLR* and *SCD* relative to the BSA control. The data support a differential effect of n-3 and n-6 PUFAs on ovarian steroidogenesis and pre-implantation development, the latter in the absence of a net uptake of FAs.

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Introduction

Dietary polyunsaturated fatty acids (PUFAs) are known to mediate a broad range of actions in reproductive tissues including effects on membrane fluidity, intracellular cell-signalling cascades and susceptibility to oxidative injury (Wathes *et al*. 2007). Central to the current thesis are the reported differential effects of omega-3 (n-3) and -6 (n-6) PUFAs on fertility, ovarian function and oocyte quality, where the literature to date has been inconsistent (Santos *et al*. 2008). By way of example, in the rat, studies by the same group have reported n-3 PUFA to both enhance and reduce egg recovery following ovarian stimulation (Trujillo & Broughton 1995, Broughton *et al*. 2008). Ovulation rate and embryo yield following ovarian stimulation were not altered in cattle fed an n-3 PUFA-enriched diet (Childs *et al*. 2008). Similarly, embryo development and expression of a number of developmentally related transcripts were unaltered in that study. In contrast, the culture of mouse embryos in the presence of both n-3 and n-6 PUFAs increased lipid peroxidation, intracellular reactive oxygen species (ROS) and decreased embryo development, which are the effects attenuated by the addition of antioxidants (Nonogaki *et al*. 1994). More recently, ROS production has been increased and intracellular Ca^{2+} homeostasis was perturbed in mature oocytes from mice offered n-3 PUFA-enriched diets, resulting in a reduced proportion of cleaved zygotes (Wakefield *et al*. 2008).

Previous studies at our laboratory with lactating and non-lactating cattle have shown that cells within the ovarian follicle preferentially accumulate saturated FAs at the expense of PUFAs, thereby nullifying the dietary-induced differences in PUFAs measured in plasma (Adamiak *et al*. 2006, Fouladi-Nashta *et al*. 2009). As a consequence of this, and in the absence of the differences in circulating metabolic hormone concentrations, contrasting n-3 and n-6 PUFA diets had no effect on post-fertilisation developmental potential of oocytes (Fouladi-Nashta *et al*. 2009). Dietary FAs in that study, however, were of vegetable origin and contained

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relatively low amounts of long-chain bio-active PUFAs such as eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA). Furthermore, these lactating dairy cows were in negative energy balance for much of the time, and this metabolic state may have increased the availability of saturated non-esterified fatty acids (NEFA) to the ovarian follicle (Leroy *et al*. 2004), thereby lessening the effects of dietary PUFAs.

The current study, therefore, sought to establish the contrasting effects that dietary n-3 and n-6 PUFAs have on the development, function and FA composition of granulosa cells and oocytes where the principal source of n-3 PUFA was salmon oil, which is rich in both EPA and DHA. To avoid the potentially confounding metabolic effects of lactation, non-lactating ewes were selected for the study. The choice of sheep over cattle was made largely on the basis of convenience and cost, but the genotype of ewe, and the protocols for oestrous synchronisation, ovarian stimulation and oocyte/granulosa cell recovery were consistent with those previously used at our laboratory (Sinclair *et al*. 2007, Kanakkaparambil *et al*. 2009), so permitting cross-study comparisons.

This animal study was extended by considering the effects of n-3 and n-6 PUFA-rich high-density lipoproteins (HDLs) on cultured granulosa cells and embryos. HDLs were fractionated from sera of ewes fed the aforementioned n-3 and n-6 PUFA diets. Transcripts for the following genes involved in lipid uptake, metabolism, steroidogenesis and cell signalling were selected for analysis in granulosa cells and/or blastocysts by quantitative real-time PCR (qRT-PCR) as, collectively, they have not been studied in the context of differential effects of n-3 and n-6 PUFAs within the ovary and the early embryo. Scavenger receptor class B member 1 (*SCARB1*) binds to HDL and mediates selective uptake of cholesterol esters from HDL as well as HDL-dependent cholesterol efflux from cells (Fidge 1999). In contrast, cholesterol uptake via low-density lipoprotein receptor (*LDLR*) involves internalisation of both LDL and its receptor. *STAR* then regulates cholesterol transfer within the mitochondria, and this is the rate-limiting step in ovarian steroidogenesis (Stocco & Clark 1996). Prostaglandin-endoperoxide synthase 2 (*PTGS2*) is responsible for the production of inflammatory eicosanoids from n-6 PUFA, notably arachidonic acid (Wathes *et al*. 2007), and can influence steroidogeneic capacity of cells via effects on *STAR* expression (Wang *et al*. 2003). Peroxisome proliferator-activated receptor-γ (*PPARG*) is a member of a family of nuclear receptors that are activated by binding of PUFAs, and it is highly expressed in granulosa cells where it is involved in regulating *PTGS2* and endothelial nitric oxide synthase (*NOS3*) expression (Froment *et al*. 2006). Nitric oxide regulates oxidative metabolism in mouse embryos (Manser *et al*. 2004) and is required to support the pre-implantation development in bovine embryos (Tesfaye *et al*. 2006). Furthermore, it is believed that SCARB1-mediated actions of HDL involve *NOS3* activation and signalling (Mineo & Shaul 2003). The final two transcripts selected for the analysis are involved in FA metabolism and antioxidant defence. Stearoyl-CoA desaturase (delta-9-desaturase) (*SCD*) is a microsomal, rate-limiting enzyme involved in the desaturation of stearic acid to oleic acid and is expressed in the rat ovary (Moreau *et al*. 2006), and copper–zinc superoxide dismutase (*SOD1*) is expressed at all the stages of pre-implantation bovine embryo development where it serves to protect against superoxide anion toxicity (Lequarré *et al*. 2001).

Results

During the 6-week experimental period, ewes gained (mean ± S.E.M.) 117 ± 6.5 g/day. Live-weight gain, however, was not affected by the dietary treatment.

Lipid analysis of plasma

The HDL-cholesterol component of plasma was more abundant than the LDL-cholesterol component (0.84 vs 0.22 mmol/l); however, it was not affected by the experimental diet. In contrast, plasma LDL-cholesterol concentrations were lower ($P<0.001$) for n-3 ewes than for n-6 ewes (0.17 vs 0.26 mmol/l; S.E.D. = 0.025). Plasma cholesterol and triglyceride concentrations averaged 1.62 and 0.24 mmol/l and were not affected by diet.

Follicle number, follicular-fluid composition and granulosa cell transcript expression

Follicle number and diameter were recorded for all 36 ewes. Individual responses, in terms of growing antral follicles, to gonadotrophin treatment were highly variable. However, neither the median number of follicles (37 (26 and 47 for 25th and 75th percentiles)) nor the follicle diameter (4.0 ± 0.15 vs 3.9 ± 0.14 mm for n-3 versus n-6 PUFA diets) was affected by treatment. The HDL-cholesterol fraction of follicular fluid, at 760 nmol/ml (Table 1), was comparable to that of plasma, but the LDL-cholesterol fraction, at around 36 nmol/ml, was sixfold lower than that of plasma. Both HDL-cholesterol and NEFA concentrations in the follicular fluid were greater ($P<0.05$) for n-6 ewes than for n-3 ewes (Table 1). Neither LDL-cholesterol nor total cholesterol concentrations in the follicular fluid were altered by diet. However, progesterone concentrations in the follicular fluid were greater ($P<0.001$) for ewes offered the n-3 diet than the n-6 diet. In contrast, neither oestradiol (E_2) concentrations nor the ratio of E_2 to progesterone was altered by diet. The expression of transcripts for *PPARG*, *PTGS2* and *STAR* in granulosa cells did not differ between dietary treatments (data not presented).

Table 1 Concentrations of steroids and lipids (lipoproteins, non-esterified fatty acid (NEFA) and cholesterol) in follicular fluid of ewes offered diets differing in n-3 or n-6 polyunsaturated fatty acids.

Experimental diet	n-3	n-6	S.E.D.	*P*
Lipids				
No. of animals	18	18		
HDL-cholesterol (nmol/ml)	696.0	818.0	45.7	0.011
LDL-cholesterol (nmol/ml)	40.0	34.4	5.13	–
NEFAs (nmol/ml)	249.0	301.0	20.1	0.018
Cholesterol (nmol/ml)	980.0	1054.0	57.2	–
Steroid hormones				
No. of animals	18	18		
Oestradiol (E_2; ng/ml)	30.2	23.4		
(*log_{10}*)	(*1.48*)	(*1.37*)	(*0.186*)	–
Progesterone (P_4; ng/ml)	52.5	22.4		
(*log_{10}*)	(*1.72*)	(*1.35*)	(*0.092*)	*<0.001*
E_2:P_4	0.56	1.05		
(*log_{10}*)	(*−0.25*)	(*0.02*)	(*0.156*)	–

Data are presented as geometric means with log_{10} means and S.E.D. presented in italics.

FA composition of plasma, granulosa cells and oocytes

All 36 ewes were blood sampled on three occasions (−20, −10 and 0 days (day of slaughter)) for plasma FA analysis. Total FA (μg/ml) was similar at all three time points (1411, 1317 and 1392; S.E.D. = 39.9). The percentage (g/100 g TFA) of the different classes of plasma FAs pooled across all 36 animals, averaged over the three time points, also agreed with the values for the reduced cohort of animals ($n=7$ ewes per diet; Table 2) on the day of slaughter. For these animals, the percentage of saturated FAs was greater, whereas the percentage of unsaturated FAs was lower ($P<0.05$) in oocytes than in either granulosa cells or plasma. Of particular interest was the fact that PUFA was greatest in plasma (34.6) with intermediary levels in granulosa cells (28.5) and lowest levels in oocytes (13.0; S.E.D. = 1.24). Furthermore, the ratio of n-6:n-3 PUFA also differed between compartments (6.78, 4.52 and 3.33 for plasma, oocytes and granulosa cells respectively (S.E.D. = 0.95)).

For the combined dietary treatments, stearic acid (C18:0), linoleic acid (C18:2n6c) and palmitic acid (C16:0) were the most abundant FAs in plasma respectively. While the former two were found in greater concentrations in plasma of n-6 diet-fed ewes, palmitic acid was greater in plasma of n-3 diet-fed ewes (Supplementary Table 1, see section on supplementary data given at the end of this article).

Palmitic acid (C16:0) was also the most abundant FA in granulosa cells, comprising 30.3 ± 1.24 g/100 g TFA (Supplementary Table 2, see section on supplementary data given at the end of this article). Oleic acid (C18:1n9c) was the second most abundant FA, and stearic acid (C18:0) was third; both FAs were greater in ewes offered n-6 diets than in those offered n-3 diets. Arachidonic acid (C20:4n6) was greater for animals offered the n-6 diet than for those offered the n-3 diet. All n-3 FAs (i.e. linoleic acid, α-linolenic acid, eicosatrienoic acid, EPA and DHA) were greater for animals offered n-3 diets than for those offered n-6 diets.

Table 2 Total fatty acids (TFAs) and percentages (g/100 g TFA) of saturated FAs, unsaturated FAs, monounsaturated fatty acids (MUFAs), polyunsaturated fatty acids (PUFAs), n-3 and n-6 series and ratio of n-6 to n-3 in plasma, granulosa cells and oocytes of ewes offered diets enriched with n-3 ($n=7$) or n-6 ($n=7$) PUFA.

Compartment	Dietary treatment	n-3	n-6	S.E.D.	*P*
Plasma	TFAs (μg/ml plasma)	1177.0	1519.0	152.6	0.045
	Saturated	30.9^{a}	42.1^{x}	1.11	<0.001
	Unsaturated	59.3^{a}	49.4^{x}	1.41	<0.001
	MUFAs	21.5^{a}	18.0^{x}	0.78	<0.001
	PUFAs	37.8^{a}	31.4^{x}	1.50	0.001
	n-6 PUFA	25.4^{a}	28.9^{x}	1.25	0.015
	n-3 PUFA	12.4^{a}	2.5^{x}	0.42	<0.001
	Ratio of n-6:n-3	2.1^{a}	11.5^{x}	0.59	<0.001
Granulosa cells	TFAs (μg/pellet)	1768	1925	847.0	–
	Saturated	39.1^{b}	39.7^{x}	2.29	–
	Unsaturated	49.7^{b}	52.1^{x}	1.64	–
	MUFAs	20.9^{a}	23.9^{y}	2.15	–
	PUFAs	28.9^{b}	28.2^{y}	1.13	–
	n-6 PUFA	8.5^{b}	24.1^{y}	1.00	<0.001
	n-3 PUFA	20.4^{b}	4.1^{y}	0.86	<0.001
	Ratio of n-6: n-3	0.4^{a}	6.2^{y}	0.52	<0.001
Oocytes	TFAs (ng/oocyte)	63.9	68.3	15.65	–
	Saturated	75.7^{c}	71.1^{y}	2.18	–
	Unsaturated	20.9^{c}	25.8^{y}	1.76	0.015
	MUFAs	7.8^{b}	12.9^{z}	1.09	<0.001
	PUFAs	13.0^{c}	12.9^{z}	2.07	–
	n-6 PUFA	5.2^{c}	10.9^{z}	1.95	0.014
	n-3 PUFA	7.8^{c}	2.1^{z}	0.90	<0.001
	Ratio of n-6:n-3	0.7^{a}	8.3^{y}	2.06	0.003

Within each fatty acid class (e.g. saturated), means within a column with different superscripts are significantly different between compartments at $P<0.05$.

The most abundant FA in oocytes was stearic acid (C18:0) comprising on average 36.5±0.97 g/100 g TFA (Supplementary Table 3, see section on supplementary data given at the end of this article). Palmitic acid (C16:0) was the second most abundant FA, and oleic acid (C18:1n9c) was third. In contrast to plasma and granulosa cells, neither of these two FAs in oocytes differed between treatments. However, in keeping with granulosa cells and plasma, the percentage of AA (C20:4n6) was greater, whereas the percentage of EPA (C20:5n3) was less in oocytes from n-6 animals than in those from n-3 animals.

Primary granulosa cell culture and transcript expression

The FA content of both HDL- and LDL-cholesterol fractions of sera (Table 3) closely reflected that of plasma (Table 2). In keeping with the HDL- and LDL-cholesterol concentrations of plasma, TFA concentration (μg/ml) of the HDL-cholesterol fraction was fourfold greater than that of the LDL-cholesterol fraction. The predominant PUFA in all the fractions was linoleic acid (C18:2n6c). However, given that the LDL-cholesterol concentration of follicular fluid was 20-fold less than that of HDL-cholesterol, only HDL was subsequently used to supplement the culture media at a level that closely matched that of the follicular fluid.

Compared to the BSA control, inclusion of HDL in granulosa cell culture media increased ($P<0.001$) granulosa cell number, E_2 and progesterone production, all of which were independent of dietary (i.e. n-3 versus n-6) source of HDL (Table 4). By 144 h, cell number was greater than the original plating density for both HDL treatments (data not presented). Inclusion of HDL during culture also led to a reduction ($P<0.001$) in the expression of transcripts for *SCARB1*, *LDL*, *SCD* and *PPARG* relative to the BSA control (Table 4). Again, dietary source of HDL had no effect on transcript expression. Granulosa cell number increased ($P<0.001$) between 48- and 96-h culture as did progesterone production. However, E_2 production by granulosa cells decreased ($P<0.001$) during this period. By 96 h of culture, the expression of transcripts for *SCARB1*, *SCD*, *PPARG* and *STAR* had increased ($P<0.001$). Levels of the former two transcripts, along with those for *LDLR*, declined between 96 and 144 h.

In vitro *embryo development and transcript expression*

A total of 2101 cumulus–oocyte complexes (COCs) were matured and inseminated over a 7-week period. From these, a total of 2030 presumptive zygotes were transferred to FA-free BSA, n-3 or n-6 HDL-supplemented synthetic oviductal fluid (SOF) media in four-well dishes. The proportion of cleaved inseminated oocytes was unaffected by treatment and averaged 0.77±0.012, 0.79±0.011 and 0.77±0.012 for BSA, n-3 PUFA HDL and n-6 PUFA HDL treatments respectively. Of those that were inseminated, the proportion of presumptive zygotes that developed to the blastocyst stage by day 6 of culture tended ($P=0.068$) to be less for n-6 PUFA HDL treatment than for the other two culture treatments (Table 5). By day 7, however, fewer ($P=0.006$) of these zygotes had developed to the blastocyst stage. Similarly, of those oocytes that cleaved following insemination, fewer ($P=0.016$) on the n-6 PUFA HDL treatment had developed to the blastocyst stage by day 7. By day 7 of culture, there was also an indication ($P=0.07$) that embryo grade was affected by culture treatment, with a lower proportion of grade 1 embryos and a higher proportion of grade 3 embryos in the n-6 PUFA HDL group (Table 5).

Transcript expression in day 7 blastocysts adjusted for *GAPDH* mRNA was found to be highly ($P<0.001$) correlated to transcript expression when adjusted for *ACTB* mRNA ($r=0.86$, *SCARB1*; $r=0.92$, *LDLR*; and $r=0.93$, *SCD*). Transcript expression is presented in relation to *GAPDH*, as this was the lesser abundant of the two housekeeping transcripts (Table 5). Consistent with the observation for granulosa cells (Table 4), transcripts for *LDLR* and *SCD* were lower ($P<0.001$) for embryos

Table 3 Fatty acid (FA) composition of high- (HDL) and low- (LDL) density lipoprotein fractions from pooled sera of all 36 ewes offered diets differing in n-3 and n-6 polyunsaturated fatty acids.

Dietary treatment (D)	**n-3**		**n-6**			**Significance** (*P*)	
Lipoprotein fraction (F)	HDL	LDL	HDL	LDL	S.E.D.	D	F
Total FA (μg/ml)	1903[a]	431[b]	2110[a]	483[b]	105	–	<0.001
FA (g/100 g)							
Saturated	28.5[a]	29.6[a]	30.3[a]	41.2[b]	0.71	<0.001	<0.001
Unsaturated	58.6[a,b]	55.0[a]	60.0[b]	47.8[c]	1.72	–	0.003
Monounsaturated	23.0	26.5	21.5	21.9	1.36	0.034	–
Polyunsaturated	35.6[a]	28.5[b]	38.5[c]	25.9[d]	0.71	–	<0.001
n-6 series	22.3[a]	17.6[b]	37.0[c]	25.4[d]	0.33	<0.001	<0.001
n-3 series	13.3[a]	10.9[b]	1.5[c]	0.5[c]	0.52	<0.001	0.010
Ratio of n-6:n-3	1.7[a]	1.6[a]	25.0[a]	59.1[b]	16.14	0.024	–
Unidentified	13.0	15.4	9.8	11.4	2.23	–	–

Means within a row with different superscripts are different ($P<0.05$).

Table 4 Effect of culture treatment* and duration on granulosa cell proliferation, oestradiol (E_2) and progesterone (P_4) production (both adjusted for cell number), and transcript expression in granulosa cells.

	Treatment			**Time of culture** (h)			**Significance** (*P*)		
	BSA	n-3	n-6	48	96	144	S.E.D.	Treatment	Time
n	30	30	30	30	30	30			
Cell proliferation and steroidogenesis									
Cell number ($\times 10^5$)	3.02^a	3.35^b	3.36^b	3.11^x	3.31^y	3.31^y	0.041	<0.001	<0.001
E_2 (pg/10^5 cells)	2.11^a	2.57^b	2.47^b	4.32^x	0.86^y	1.97^z	0.101	<0.001	<0.001
P_4 (pg/10^5 cells)	259^a	308^b	312^b	161^x	373^y	343^z	6.2	<0.001	<0.001
Transcript expression relative to *ACTB*									
SCARB1	0.483^a	0.373^b	0.355^b	0.353^x	0.566^y	0.292^x	0.0324	<0.001	<0.001
LDLR	0.418^a	0.312^b	0.317^b	0.404^x	0.400^x	0.243^y	0.0297	<0.001	<0.001
SCD	0.250^a	0.185^b	0.177^b	0.083^x	0.370^y	0.159^z	0.0236	0.007	<0.001
PPARG	0.461^a	0.372^b	0.381^b	0.326^x	0.449^y	0.438^y	0.0526	0.003	<0.001
PTGS2	0.108	0.105	0.111	0.106	0.109	0.110	0.0095	–	–
STAR	11.03	10.24	9.03	3.84^x	13.96^y	12.5^y	1.236	–	<0.001

Means within a row for each main effect (i.e. treatment and time) with different superscripts are significantly different ($P<0.05$).
*Culture medium was supplemented with fatty acid (FA)-free BSA or HDL varying in n-3 or n-6 PUFA.

cultured in the presence of HDL relative to the BSA control, although in this case the reduction in expression was greater for the n-3 PUFA HDL treatment than the n-6 PUFA HDL treatment. In contrast to granulosa cells, *SCARB1* transcript expression, together with *NOS3* and *SOD1* transcript expression, was not altered by culture media supplement.

FA composition of cultured granulosa cells and blastocysts

The TFA content of granulosa cells at 144 h differed ($P<0.001$) between the three culture groups, but on the whole it was greater for the two HDL groups than for the BSA control (Table 6). The percentage of saturated FAs, unsaturated FAs, MUFAs and PUFAs did not vary greatly between the culture groups, but the treatments (i.e. n-3 versus n-6 HDL) were effective in altering the percentages of n-3 and n-6 PUFAs in granulosa cells relative to the BSA control. In contrast to freshly harvested granulosa cells from ewes offered n-3 and n-6 diets, the most abundant FA in cultured granulosa cells was oleic acid (C18:1n9c) at 31.3 ± 0.33 g/100 g TFA (Supplementary Table 4, see section on supplementary data given at the end of this article). Palmitic acid (C16:0) comprising on average 23.9 ± 0.29 g/100 g TFA was the second most abundant FA, and stearic acid (C18:0) at 5.9 ± 0.11 g/100 g TFA was third. Arachidonic acid (C20:4n6) was not detectable in granulosa cells cultured in the presence of n-3 HDL, but was abundant in cells

Table 5 Embryo development and transcript expression following 6 or 7 days of culture in synthetic oviductal fluid media supplemented either with fatty acid-free BSA or with high-density lipoproteins (HDLs) fractionated from sera of ewes offered either n-3 or n-6 polyunsaturated fatty acid-enriched diets.

	BSA	**n-3 HDL**	**n-6 HDL**	**Significance** (*P*)
Of inseminated				
Cleaved	0.769 ± 0.012	0.793 ± 0.011	0.768 ± 0.012	–
Day 6 blastocysts	0.261 ± 0.012	0.285 ± 0.012	0.244 ± 0.011	0.068
Day 7 blastocysts	$0.399^a \pm 0.016$	$0.413^a \pm 0.016$	$0.335^b \pm 0.015$	0.006
Of cleaved				
Day 6 blastocysts	0.339 ± 0.015	0.358 ± 0.015	0.318 ± 0.014	–
Day 7 blastocysts	$0.505^a \pm 0.019$	$0.520^a \pm 0.019$	$0.438^b \pm 0.018$	0.016
Morphological grade*				
1	0.244	0.301	0.199	0.07
2	0.312	0.325	0.292	
3	0.306	0.267	0.337	
4	0.064	0.051	0.078	
Transcript expression relative to *GAPDH*				
SCARB1	0.652 ± 0.072	0.682 ± 0.072	0.581 ± 0.071	–
LDLR	$1.616^a \pm 0.073$	$0.801^b \pm 0.073$	$1.124^c \pm 0.073$	<0.001
SCD	$0.308^a \pm 0.011$	$0.179^b \pm 0.010$	$0.223^c \pm 0.012$	<0.001
NOS3	1.059 ± 0.112	1.058 ± 0.112	1.057 ± 0.125	–
SOD1	0.307 ± 0.015	0.310 ± 0.015	0.290 ± 0.016	–

*Proportion of day 7 sheep blastocysts within each of the four IETS quality grades (grade 1, excellent; grade 4, degenerating).

Table 6 Total fatty acids (TFAs) and percentages (g/100 g TFA) of saturated FAs, unsaturated FAs, monounsaturated fatty acids (MUFAs) and polyunsaturated fatty acids (PUFAs) in granulosa cells and blastocysts cultured* *in vitro*.

Culture treatment	BSA	n-3	n-6	S.E.D.	*P*
Granulosa cells					
TFAs (μg/10^5 cells)	3.13[a]	3.80[b]	5.05[c]	0.141	<0.001
Saturated	34.3[a]	32.7[b]	33.7[c]	0.40	0.003
Unsaturated	58.8	57.5	57.4	0.76	–
MUFAs	38.2[a]	35.3[b]	35.1[b]	0.69	<0.001
PUFAs	20.5	22.1	22.3	0.78	–
n-6 series	12.8[a]	7.9[b]	18.4[c]	0.47	<0.001
n-3 series	7.8[a]	14.3[b]	3.9[c]	0.60	<0.001
Ratio of n-6:n-3	1.69[a]	0.56[b]	4.73[c]	0.152	<0.001
Blastocysts					
TFAs (ng/blastocyst)	59.6	55.0	70.7	7.43	–
Saturated	28.1	28.7	28.7	0.51	–
Unsaturated	65.7	65.0	65.0	0.94	–
MUFAs	24.7	24.2	23.8	0.37	–
PUFAs	41.1	40.8	41.2	0.58	–
n-6 series	34.7	34.1	35.0	0.61	–
n-3 series	6.5	6.6	6.2	0.23	–
Ratio of n-6:n-3	5.4	5.2	5.7	0.20	–

*Culture was incorporated with FA-free BSA or high-density lipoproteins (HDLs) obtained from serum of ewes offered diets enriched with either n-3 or n-6 PUFA. Means within a row with different superscripts are significantly different ($P<0.05$).

cultured with either BSA or n-6 HDL (5.2±0.17 and 7.8±0.17 g/100 g TFA respectively). In contrast, both α-linolenic acid (C18:3n3) and eicosatrienoic acid (C20:3n-3) were relatively abundant in cells cultured with n-3 HDL (2.1±0.24 and 2.0±0.24 g/100 g TFA respectively), but were undetectable in cells cultured with n-6 HDL.

The TFA content of blastocysts was not altered by culture treatment, nor was the percentage of any of the major classes of FAs (Table 6). However, the percentage of unsaturated FAs at 65.2±0.67 g/100 g TFA, consisting predominantly of PUFAs, was greater than that of granulosa cells and oocytes (Tables 2 and 6). The most abundant FA in blastocysts was linoleic acid (C18:2n6c) at 34.4±0.46 g/100 g TFA (Supplementary Table 5, see section on supplementary data given at the end of this article). Oleic acid (C18:1n9c) at 24.0 ±0.26 g/100 g TFA was second, and palmitic acid at 21.7±0.30 g/100 g TFA was third. Only trace quantities of long-chain PUFAs were detectable, and these varied little between the treatments.

Discussion

A significant finding from the current study was the effectiveness with which our dietary formulations, one of which included fish oil, altered the FA composition of both granulosa cells and oocytes leading to contrasting profiles of cellular n-3 and n-6 PUFAs. This is in stark contrast to our previous observations, utilising only vegetable sources of PUFAs although, in agreement with earlier findings, the percentage of saturated FAs was significantly greater in oocytes than in either plasma or granulosa cells (Adamiak *et al*. 2006, Fouladi-Nashta *et al*. 2009). The latter observation confirms the presence of selective uptake mechanisms and/or *de novo* synthesis within the ovarian follicle and oocyte that favour saturated FAs. Our contrasting n-3 and n-6 PUFA diets led to differences in progesterone concentrations in the follicular fluid, although they did not alter the number of maturing antral follicles following FSH stimulation. HDL fractionated from the sera of ewes fed these diets increased the proliferation and steroidogenesis of cultured granulosa cells although, in contrast to the animal study, there was no differential effect of n-3 and n-6 PUFAs on progesterone production. This probably reflects the important actions of PUFAs on steroidogenic pathways within thecal cells as opposed to granulosa cells, and merits further investigation. Finally, this study is the first to report transcripts for *SCARB1*, *LDLR* and *SCD* in mammalian pre-implantation embryos, and transcripts for *NOS3* in sheep embryos. In contrast to granulosa cells, HDL was ineffective in delivering FAs to embryos during culture, although it did alter the expression of transcripts for *LDLR* and *SCD*, and n-6 HDL reduced embryo development.

Dietary effects on ovarian follicular steroidogenesis

Follicular-fluid progesterone concentrations were significantly greater in ewes offered the n-3 diet than in those offered the n-6 diet (Table 1). Inhibition of PTGS2 activity is known to facilitate cAMP-induced steroidogenesis in mouse Leydig tumour cells via increased STAR protein expression (Wang *et al*. 2003), and n-3 PUFAs are potent inhibitors of PTGS2 activity (Ringbom *et al*. 2001). However, there was little indication that such mechanisms were operational in granulosa cells from

the current study. Although there was a hint ($P=0.1$) that *PTGS2* transcript expression was reduced in granulosa cells from n-3 ewes, *STAR* transcript expression was unaltered by maternal diet. It is quite likely, therefore, that the activity of these two enzymes (i.e. PTGS2 and STAR), together with that of PPARG, is either not regulated at the transcript level or the actions of n-3 PUFA were mediated within thecal cells for which we have no data. Furthermore, and in contrast to the study of Kanakkaparambil *et al*. (2009) which reported an effect of dietary B-vitamins and methionine on follicle number using the same stimulatory protocol, there was no effect of dietary treatment on follicle number in the current study.

As observed elsewhere (González-Bulnes *et al*. 2004), ovarian responses to our stimulation protocol varied greatly between animals, which may be due in part to the timing of ovarian stimulation with respect to specific aspects of follicular-wave development (Veiga-Lopez *et al*. 2005, Mossa *et al*. 2007). This may be significant because PUFAs are known to influence follicle number during natural cycles in ruminants (Zeron *et al*. 2002, Wathes *et al*. 2007).

Dietary effects on granulosa cell and oocyte FA composition

It is noteworthy that the contrasting profiles of n-3 and n-6 PUFAs in oocytes between treatments were evident even though the percentage of unsaturated FAs was significantly less than that in either granulosa cells or plasma (Table 2). This may have been due to the principal source of n-3 PUFA (i.e. salmon oil) used in the current study, which is rich in long-chain PUFAs such as EPA and DHA, as dietary differences in n-3 and n-6 PUFAs in plasma were absent in granulosa cells in the study of Fouladi-Nashta *et al*. (2009), where the principal source on n-3 PUFA was linseed oil. Similarly, and in keeping with previous observations by our laboratory (Adamiak *et al*. 2006), the percentage of saturated FAs was greater, and hence the percentage of unsaturated FAs was less, in oocytes than in granulosa cells. Collectively, these observations point to the selective uptake and/or *de novo* synthesis of saturated FAs, as opposed to PUFAs, within the follicular compartment, but the extent to which dietary-induced differences in plasma PUFAs can be negated within the follicle is dependent on the dietary source of FAs. Long-chain PUFAs were barely detectable in oocytes from ewes offered a supplement of calcium soaps of fish oil FA at 4% dietary dry matter in the study of Zeron *et al*. (2002), but the analysis was restricted to the phospholipid fraction, which constitutes 28% of the total lipids in sheep oocytes (McEvoy *et al*. 2000), and so PUFA levels were below the detection limit of their assay. This was the principal reason why we elected to measure FAs in total lipids. It permitted a quantitative comparison of changes in FA composition between plasma, granulosa cells and the limited number of oocytes that could be recovered within animals (10–28). A potential weakness of our approach is that the proportion of phospholipids, trigylcerides and cholesterol esters may have differed between plasma and tissues (Christie 1981), which could have accounted for some of the differences observed in FA profiles between fluid and cellular compartments in the current study. However, of note is the fact that phospholipids account for 28–35% of LDL and HDL fractions in bovine plasma (i.e. comparable with that of sheep oocytes indicated above), and were found to incorporate most of the $\geq$C20 n-3 PUFAs from dietary fish oil (Offer *et al*. 2001). Also, McEvoy *et al*. (2000) found that triglycerides, phospholipids and cholesterol esters did not differ in their proportions of saturated FAs, MUFAs and PUFAs in sheep oocytes, so this lipid class is unlikely to account for a major component of the treatment differences in FA composition, although this remains to be fully established.

Effects of n-3 and n-6 HDLs during granulosa cell culture

In the current study, follicular-fluid HDL-cholesterol concentration was similar to that of serum, but it was 20-fold greater than LDL-cholesterol concentration in follicular fluid (Table 1). Granulosa cells are therefore exposed to relatively high levels of HDL-cholesterol and so, following on from the bovine study of O'Shaughnessy *et al*. (1990), the effects of HDLs fractionated from the sera of ewes fed n-3 or n-6 PUFA diets on cultured ovine granulosa cells were assessed. From the observed increase in TFA content, cell number and steroidogenesis, it is clear that granulosa cells can acquire FAs from HDLs in a manner that supports both cell growth and function. In contrast to *LDLR* mRNA expression, which did not alter, transcript levels for *SCARB1* in granulosa cells increased by 1.6-fold between 48 and 96 h of culture consistent with the 2.3-fold increase in progesterone production (Table 4). This observation agrees with the findings of Rajapaksha *et al*. (1997), who witnessed a fivefold increase in *SCARB1* transcript expression in bovine granulosa cells over a similar time frame in dishes pretreated with calf serum, and with cells cultured in the presence of dibutyryl cAMP to maximise functional luteinisation. By 144 h of culture, however, both *SCARB1* mRNA and progesterone production had decreased and E_2 production had increased, indicating that the serum-free culture conditions in the present study were not conducive for luteinisation, so granulosa cells largely retained their primary phenotype.

Dietary source of HDL (i.e. n-3 versus n-6) had no effect on cell number and steroidogenesis in the current study, but transcript expression for *SCARB1*, *LDLR*, *SCD* and *PPARG* was reduced in granulosa cells cultured with

HDL relative to those cultured with FA-free BSA (Table 4), consistent with an increased uptake of cholesterol and PUFAs (Nakamura & Nara 2002). The HDL-mediated reduction in *SCD* mRNA is interesting because this was associated with a small but significant ($P<0.001$) reduction in the percentage of oleic acid (C18:1n9c) in granulosa cells (Supplementary Table 4). The expression of *SCD* mRNA increased, however, by 4.5-fold between 48 and 96 h, which may account for why oleic acid, at around 31 g/100 g TFA, was the most abundant FA in the cultured granulosa cells following 144-h culture. This level of oleic acid was twofold greater than that observed in freshly harvested granulosa cells from the *in vivo* study (Supplementary Table 2) and may represent a feature of cells that are cultured with relatively high concentrations of insulin (i.e. 10 ng/ml), as insulin is known to increase *SCD* mRNA and oleic acid concentrations in sheep adipose tissue (Daniel *et al*. 2004). The nearly fourfold increase in *STAR* transcript expression between 48 and 96 h in the current study may also have been induced by insulin (Wood & Strauss 2002). In keeping with the observations from *in vivo* derived granulosa cells, the PUFA composition of HDL had no effect on *PTGS2*, *PPARG* and *STAR* transcript expression in cultured granulosa cells. Given that there were dietary-induced differences in follicular-fluid progesterone concentrations (Table 1), it can be inferred that n-3 and n-6 PUFAs do not differentially act on granulosa cells to influence steroidogenesis, but rather act on other cellular components of the ovarian follicle. This remains to be confirmed.

Effects of n-3 and n-6 HDLs during embryo culture

Neither the TFA content (ng/blastocyst) nor the profile of FAs within blastocysts was altered by culture treatment in the current study (Table 6). Indeed, the mean TFA content of blastocysts at 62 ng was similar to that of oocytes from the *in vivo* study (i.e. 66 ng; Table 4). Curiously, the latter value for oocytes is 0.73 of the value (90 ng/oocyte) reported for immature sheep oocytes by McEvoy *et al*. (2000), and is more akin to levels predicted for mature oocytes and two-cell zygotes with triglyceride reserves depleted by around 40% (Ferguson & Leese 1999, 2006). In keeping with the present study, these authors cultured zygotes in SOF with FA-free BSA, and noted no net change in triglyceride content from the zygote to the hatched blastocyst stage. Of significance in the current study is the fact that there was no increase in the FA content of embryos cultured from zygotes in the presence of HDL, as was observed in granulosa cells. This observation is consistent with the FA composition of *in vivo* derived blastocysts, which did not differ from *in vivo* derived oocytes, in sheep (Reis *et al*. 2002), but contrasts with that of the studies that incorporated serum into their culture media. In the presence of serum, the FA content of cultured embryos increases by between 1.3- and 1.7-fold (Ferguson & Leese 1999, Reis *et al*. 2003, 2005). This emphasises the physiological basis of incorporating HDL fractions into culture media in the current study. Furthermore, we have shown, for the first time, that sheep blastocysts express transcripts for *SCARB1* and are clearly responsive to the presence of HDL in culture media, as demonstrated by the decrease in transcript expression for both *LDLR* and *SCD* and, in the case of the n-6 HDL treatment, the reduction in the proportion of zygotes that developed to the blastocyst stage (Table 5). A putative signalling mechanism for these SCARB1-mediated actions of HDL involves *NOS3* activation, which is known to operate at least in vascular endothelial cells (Mineo & Shaul 2003). Furthermore, both transcriptional and post-transcriptional mechanisms operate to regulate *NOS3* expression by endothelial cells (Searles 2006). *NOS3* transcripts and protein have been detected in mouse and cattle embryos (Tranguch *et al*. 2003, Tesfaye *et al*. 2006) where nitric oxide serves to regulate oxidative metabolism and developmental rate (Manser *et al*. 2004). We now show that *NOS3* transcripts are also present in sheep blastocysts, but that expression is unaffected by culture treatment, so that the mechanisms of action of HDL and n-6 PUFA on embryo development remain to be determined.

It is notable, however, that neither FA content (ng/embryo) nor the composition (percentage of TFAs) of blastocysts differed between the three culture treatments, indicating that there was no net uptake of FAs during culture (Table 6). Also of note was the high percentage of unsaturated FAs, in particular linoleic acid (C18:2n6c), in blastocysts, which was strikingly greater than that in the *in vivo* derived oocytes. Working with the discarded human pre-implantation embryos, Haggarty *et al*. (2006) observed those that developed beyond the four-cell stage to have a higher relative percentage of unsaturated FAs, particularly linoleic acid, although, unlike sheep blastocysts in the current study, saturated FAs at around 62% TFA remained the largest component. Furthermore, these authors reported that, relative to ^{13}C palmitic acid, the uptake of added ^{13}C linoleic acid increased in embryos that developed beyond the eight-cell stage. Collectively, these observations point to an important, albeit unknown, role of linoleic acid following compaction in mammalian embryos. Furthermore, in the absence of a significant net uptake of linoleic acid, the increased relative percentage of this FA in blastocysts most likely arose due to the selective oxidation of saturated FAs, mainly stearic acid and to a lesser extent palmitic acid, which could have had a sparing effect on linoleic acid. This proposition, together with the partition of FAs between polar and neutral lipids in developing pre-implantation embryos, merits further study.

Conclusions

Diets contrasting in n-3 and n-6 PUFAs were effective in altering the FA composition of both granulosa cells and oocytes and led to differences in progesterone concentrations in ovarian follicular fluid, but had no effect on follicle number following ovarian stimulation. Similarly, n-3 and n-6 PUFA-enriched HDLs fractionated from sera of ewes fed these diets had no differential effect on granulosa cell proliferation and steroidogenesis during *in vitro* culture. Consequently, the differential effect of n-3 and n-6 PUFA-enriched diets on ovarian steroidogenesis must reside in cells other than granulosa cells within the follicle, but this requires further investigation. This study is the first to report transcripts for *SCARB1*, *LDLR* and *SCD* in mammalian embryos and *NOS3* in sheep embryos. One of the most significant findings from the current study was the negative effect of n-6 PUFA-enriched HDL on embryo development to the blastocyst stage, which occurred in the absence of a net uptake of FAs. The mechanisms that underlie this effect and associated cell signalling pathways remain to be determined.

Materials and Methods

All procedures were reviewed by the Animal Ethics Committee of the University of Nottingham and were conducted in accordance with the requirements of the UK Home Office Animals (Scientific Procedures) Act 1986. All reagents were purchased from Sigma–Aldrich, unless otherwise stated.

Animals and dietary treatments

Thirty-six mature Scottish Blackface ewes with an average body weight (±S.E.M.) of 42.5±0.66 kg were allocated randomly to one of the two dietary treatments. Ewes were offered a diet enriched either with n-3 PUFA (n=18) or with n-6 PUFA (n=18; Table 7). Ewes received 1 kg of these complete pelleted diets, calculated to fully meet their energy and protein requirements (ARC 1980), daily as two meals of 500 g at 0900 and 1600 h. Hay (dry matter 855 g/kg; metabolisable energy 6.5 MJ/kg DM; crude protein 66.2 g/kg DM; acid ether extract 15 g/kg DM) was offered at 100 g/d along with the afternoon meal to provide a source of long-form roughage. These diets were offered for a period of 6 weeks.

Full details of the oestrous synchronisation and ovarian stimulation procedures employing progestogen-impregnated intra-vaginal sponges and oFSH were documented previously (Kanakkaparambil *et al.* 2009). Intra-vaginal sponges were withdrawn, on average, 26 h (24–28 h) prior to slaughter, whereas the final dose of FSH was administered, on average, 17 h (15–19 h), prior to slaughter.

Blood was collected from each animal on two occasions during the experiment (20 and 10 days prior to slaughter), and on the day of slaughter, by jugular venipuncture into heparinised vacuum tubes. Plasma was stored at −20 °C prior to analysis. An additional bulk collection of serum was derived from blood collected from each ewe prior to ovarian stimulation.

Table 7 Experimental diets and chemical analyses.

Diet	n-3	n-6
(A) Ingredient (g/kg)		
Nutritionally improved straw	250	250
Oat feed	190	190
Barley	225	225
Molassed sugar beet pulp	100	100
Hipro soya bean meal	150	150
Linseed oil[a]	10	–
Sunflower oil[a]	–	45
Salmon oil[b]	35	–
Molasses	20	20
Minerals and vitamins[c]	20	20
(B) Chemical analysis[d]		
Dry matter (g/kg)	888.9	895.8
ME (MJ/kg DM)	10.7	10.2
Crude protein (g/kg DM)	134	129
NDF (g/kg DM)	371	383
Starch (g/kg DM)	172	161
AHEE (g/kg DM)	64.1	57.3

[a]Statfold Seed Oil Ltd, Tamworth, England. [b]Rossyew Ltd, Greenock, Scotland. [c]Minerals and vitamins: Ca 170, P 60, Na 70, Mg 100 g/kg; Co 150, Mn 6000, Zn 4000, I 300, Se 30 mg/kg; Vit A 500 000, Vit D_3 100 000, Vit E 1200 IU/kg. [d]DM, dry matter; ME, metabolisable energy; NDF, neutral detergent fibre; AHEE, acid-hydrolysed ether extract.

Following slaughter by captive bolt and exsanguination, ovaries were recovered, the diameter of individual antral follicles was measured using callipers and the follicle number was counted. Follicular fluid, oocytes and granulosa cells were aspirated and pooled from all visible (predominantly 4–7 mm) follicles within the animals. COCs were identified and oocytes were denuded by vortexing in 500 µl PBS containing 0.1% polyvinyl alcohol (PVA). Denuded oocytes (n=10–28) were snap frozen in minimal volume (2–3 µl) PBS/PVA and stored at −80 °C until analysis. The remaining aspirants were centrifuged at 500 ***g*** for 10 min, and follicular fluid was stored at −80 °C prior to analysis. Granulosa cells were washed in 10 ml 1×PBS, snap frozen in liquid N and stored at −80 °C in minimal volume PBS/PVA until analysis.

Fractionation of lipoproteins from serum

Low- (d=1.006–1.060 g/ml) and high- (d=1.06–1.24 g/ml) density lipoprotein fractions were separated from pooled sera obtained from ewes offered n-3 and n-6 PUFA diets by sequential preparative ultracentrifugation in a Beckman XL-70 Preparative Centrifuge (Beckman Coulter UK Ltd, Buckinghamshire, UK) according to the method of Havel *et al.* (1955). Following dialysis to remove KBr, fractionated lipoproteins were aliquoted and frozen at −20 °C until analysed by gas chromatography or added to the culture media of primary granulosa cells or zygotes.

Culture of primary granulosa cells

Ovine granulosa cells from small and medium (2–4 mm) antral follicles of abattoir-derived ovaries were cultured under serum-free conditions as described previously

(Kanakkaparambil *et al.* 2009). Granulosa cells were resuspended in a culture medium (90% v/v TCM199 and 10% v/v of double distilled water) supplemented with: i) 0.1% w/v FA-free BSA (control), ii) 7 μl/ml n-3 HDL or iii) 7 μl/ml n-6 HDL. These levels of HDL were calculated to deliver FAs at concentrations similar to that found in serum, and so were deemed physiologically relevant. Granulosa cells were incubated in 24-well plates, at an initial seeding density of 3.5×10^5 viable cells (determined by Trypan blue exclusion) in 500 μl media, for up to 144 h at 38.8 °C in a humidified atmosphere of 5% CO_2 in air with 80% of the media renewed every 48 h. Spent media were stored at −20 °C until E_2 and progesterone analysis. The number of viable cells at each of these three time points was determined by Trypan blue exclusion.

Embryo culture

The culture of sheep embryos was based on in-house protocols described previously (Powell *et al.* 2006), and only a brief summary is provided here. Grades 1 and 2 COCs (Goodhand *et al.* 1999) were matured in groups of 50 in 450 μl bicarbonate-buffered TCM199 with supplements. For fertilisation, 1×10^6 motile spermatozoa from a single ram were added per ml of media containing matured oocytes (up to 30 per well) and incubated for 22 h at 38.8 °C in a humidified atmosphere of 5% CO_2 in air. Putative zygotes were cultured in groups of 40–50 in 400 μl SOF medium to which were added one of the three supplements: i) 0.1% w/v FA-free BSA (control), ii) 7 μl/ml n-3 HDL and iii) 7 μl/ml n-6 HDL. The experiment was repeated over 7 weeks with two four-well dishes, containing each of the three supplements, per week. 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 the wells were washed in 1×PBS, snap frozen in minimal volume and stored at −80 °C until either FA or transcript analysis.

Lipid and FA analyses

Plasma and follicular fluid were analysed for HDL- and LDL-cholesterol, total cholesterol, triglycerides and NEFAs using an Imola Autoanalyser (RX imola; Randox Laboratories Ltd, Antrim, UK). The kits were supplied by Randox Laboratories Ltd (HDL, catalogue no. CH3811; LDL, catalogue no. CH3841; cholesterol, catalogue no. CH3810; triglycerides, catalogue no. TR3823; NEFA, catalogue no. FA115).

FA analyses of biological fluids (including HDL- and LDL-cholesterol fractions, the former subsequently used for cell and embryo culture), granulosa cells, oocytes and blastocysts were based on established in-house protocols (Sinclair *et al.* 2008, Fouladi-Nashta *et al.* 2009). Twenty quantitatively significant and/or biologically important FAs were identified for subsequent statistical analysis. Oocyte and blastocyst FAs were extracted, together with 100 μl of 100 ng/ml internal standard (pentadecanoic acid; C15:0), using a 2:1 mixture of chloroform:methanol and were methylated using methanolic HCl

Table 8 Primers and probes used for real-time quantitative PCR analysis of transcript expression in granulosa cells and embryos.

Transcript	Primers and probe (5′–3′)	NCBI accession no.
ACTB	For: TGTGCGTGACATCAAGGAGAA Rev: CGCAGTGGCCATCTCCTG Probe: CTGCTACGTGGCCCTGGACTTCGA	AF129289 *Ovis aries*
GAPDH	For: TCCGTTGTGGATCTGACCTG Rev: TGCTTCACCACCTTCTTGATCTC Probe: CGCCTGGAGAAACCTGCCAAGTATGA	AF030943 *Ovis aries*
LDLR	For: GAGCGTGGGTGCCCTATACA Rev: TTCCGAAGGCCAGGAGG Probe: CGTCCTCCCCATCGCACTGCTC	K01830 *Bos taurus*
SCARB1	For: TCCAAGGCCAGAAGCCAC Rev: GCTCTTGTGCCTGAACTCCC Probe: TGCAGGAGCACGGGCCTTATGTGTA	AF019384 *Bos taurus*
SCD	For: CGAACCTACAAAGCTCGGCT Rev: TGGAACGCCATGGTGTTG Probe: CCCCTACGGGTCTTCCTGATCATCG	NM_001009254 *Ovis aries*
STAR	For: GCGACGTTTAAGCTGTGTGCT Rev: TGCTGCCGCAGCCC Probe: AGCTCCTATAGACACGTGCGCAGCATG	AF290202 *Ovis aries*
PTGS2	For: GCACAAATCTGATGTTTGCAT Rev: AGCTGGTCCTCGTTCAATATCTG Probe: TGCCCAGCACTTCACCCATCAATTTTT	U68486 *Ovis aries*
PPARG	For: AGAGATCTCCAGCGACATCGA Rev: TCAGCGGGAAGGACTTTATGTAC Probe: CAGAGTCTGCTGACCTCCGGGCC	AY137204 *Ovis aries*
NOS3	For: CCTGCGATGGTATGCCCT Rev: AGAACTCCAGACCCCCGATT Probe: CCGGCCGTGTCCAACATGCTG	DQ015701 *Ovis aries*
SOD1	For: GCCGTCTGCGTGCTGAA Rev: ACTGTATTTCCCTTTGCCTCGA Probe: CCCGGTGCAAGGCACCATCC	XM_584414 *Bos taurus*

using the same protocol as described for granulosa cells. The only difference being that a dedicated 100 m Varian CP7489 capillary column was used to analyse oocytes and blastocysts as the quantities of FA methyl esters were so low.

Steroid analysis

Follicular fluid and culture media E_2 (diluted 1:50 and 1:20 respectively) were analysed without prior extraction using a [^{125}I] E_2 double-antibody RIA (Grant *et al.* 1989). The assay was modified and validated to enable the use of a rabbit anti-E_2 first antibody and donkey anti-rabbit IgG, and normal rabbit serum, which were obtained as gifts from the Scottish Antibody Production Unit, Carluke, Lanarkshire, Scotland. The sensitivity of the assay at ED_{80} was 44.0 pg/ml. Mean (for low, medium and high controls) intra- and inter-assay coefficients of variation (CV) were 5.7 and 8.3% respectively. Similarly, follicular fluid and culture media progesterone (both diluted 1:50) were analysed using an ELISA kit obtained from Ridgeway Science, Gloucestershire, UK. The sensitivity of the assay was 0.9 ng/ml. Mean (for low, medium and high controls) intra- and inter-assay CV were 7.4 and 9.4% respectively.

Transcript analysis

Total RNA was extracted from cultured ovine granulosa cells using RNeasy Mini Kit, followed by RNase-free DNase (Promega) to remove genomic DNA contamination, and was RT using Omniscript RT kit (Qiagen; Kanakkaparambil *et al.* 2009). RNA was extracted from pools of 10–29 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 that there was no genomic DNA contamination in samples, −RT (equivalent to 0.7 blastocyst) was used in 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 8). 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′-carboxyfluorescin (FAM) and 6′-carboxy-*N*,*N*,*N*′,*N*′-tetramethylrhodamine (TAMRA) on 5′ and 3′ ends 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 the PCR product was confirmed by gel electrophoresis.

Statistical analysis

All statistical analyses were conducted using Genstat release 11.1 (Genstat 2008). Regression coefficients of body weight against time were analysed using ANOVA where the term 'dietary treatment' was fitted to the model. Follicle number was analysed using a generalised linear model (GLM) assuming a Poisson error distribution and with a logit link function. Lipid, FA and steroid concentrations in biological fluids and cells were analysed using ANOVA. E_2 and progesterone data, which were not normally distributed, were transformed ($\log_{10}$) prior to analysis. Data are presented as geometric means with $\log_{10}$ means and S.E.D. presented in italics. For plasma FAs, 'animal' was included as a blocking term in the statistical model and the data were analysed using repeated-measures ANOVA. FA percentages were also formally compared between plasma (from the slaughter day samples), granulosa cells and oocytes using ANOVA. If a FA was not detectable in a single treatment (i.e. a FA percentage <0.03 g/100 g TFA), then the FA percentage in the other treatment was analysed using a two-tailed t-test to determine whether it was significantly greater than the detection limit.

In the case of cultured granulosa cells, data were analysed using ANOVA as a one-way randomised block with culture week (of which there were five) as the block, and there were two replicate plates of each treatment every week. The proportions of inseminated oocytes and zygotes that developed into blastocysts were analysed by analysis of deviance using GLMs assuming binomial errors and with logit link functions. Data are presented as predicted back-transformed means $\pm$ S.E.M. Finally, the effects of culture treatment and morphological assessments of embryo stage on embryo grade were assessed by ordinal regression analysis assuming multinomial errors and 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-09-0219.

Declaration of interest

The authors declare that there is no conflict of interest that would prejudice the impartiality of this scientific work.

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