REPRODUCTION
RESEARCH

Oocyte quality in lactating dairy cows fed on high levels of n-3 and n-6 fatty acids

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Abstract

Different fatty acid (FA) sources are known to influence reproductive hormones in cattle, yet there is little information on how dietary FAs affect oocyte quality. Effects of three dietary sources of FAs (supplying predominantly palmitic and oleic, linoleic (n-6) or linolenic (n-3) acids) on developmental potential of oocytes were studied in lactating dairy cows. A total of 12 Holstein cows received three diets containing rumen inert fat (RIF), soyabean or linseed as the main FA source for three periods of 25 days in a Latin-square design. Within each period, oocytes were collected in four ovum pick-up sessions at 3–4 day intervals. FA profiles in plasma and milk reflected profiles of dietary FA sources, but major FAs in granulosa cells were not affected. Dietary FA source did not affect plasma concentrations of leptin, insulin, IGF1, GH, or amino acids. RIF led to a higher proportion of cleaved embryos than soya or linseed, but blastocyst yield and embryo quality were not affected. It is concluded that the ovary buffers oocytes against the effects of fluctuations in plasma n-3 and n-6 FAs, resulting in only modest effects on their developmental potential.

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Introduction

In high-yielding dairy cows, nutrition can have significant effects on resumption of ovarian cycles *post partum* and on subsequent conception rate (Gong *et al*. 2002). Nutrition acts at various points of the hypothalamus–pituitary–ovarian axis to control ovarian function and the follicular environment (Armstrong *et al*. 2001, Garnsworthy *et al*. 2008*a*). More specifically, nutrition influences circulating concentrations of metabolites and hormones, and also influences growth factors in the follicular fluid, oviducts and uterus (Webb *et al*. 2007). Indeed, adequate levels of these factors at critical stages during follicle and oocyte development are essential for successful ovulation and conception.

Supplementary fat is often used to increase energy concentrations of diets for high-yielding dairy cows. Although moderate levels of added fat can improve energy status of the cow, thereby improving potential fertility, high levels of supplementary fat can reduce plasma insulin (Garnsworthy *et al*. 2008*b*) or dry matter intake (Choi *et al*. 2000), thereby reducing potential fertility. However, fatty acids (FAs) can also affect reproductive processes in ways that are not related to energy (Staples *et al*. 1998). FAs have important roles in maintenance of structure and function of cell membranes, and in cholesterol metabolism, steroidogenesis and synthesis of prostaglandins. Therefore, the positive effects of dietary fat on fertility in dairy cows observed in some studies could be due to effects of specific dietary FAs acting at various sites in the reproductive system rather than via improved energy status (Lucy *et al*. 1992). FAs may also have direct effects on transcription of genes that encode proteins essential for reproduction (Mattos *et al*. 2000).

Polyunsaturated fatty acids (PUFA) have important effects on reproductive processes including ovarian follicular growth, corpus luteum function, and progesterone production, ovulation, fertilization, maintenance of pregnancy and parturition (Abayasekara & Wathes 1999). For example, feeding diets high in linoleic acid (n-6) during the prepartum period delayed parturition in sheep (Baguma-Nibasheka *et al*. 1999) and increased the incidence of retained placenta in cattle (Barnouin & Chassagne 1991).

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In contrast, increased availability of α-linolenic acid (n-3) during the *post partum* period improved pregnancy rate in cattle (Kassa *et al*. 2002).

Dietary FAs alter the FA composition of cumulus cells, granulosa cells, and oocytes which may be relevant for oocyte quality (Kim *et al*. 2001). For example, feeding PUFA to sheep altered phospholipid FA composition in plasma and cumulus cells and altered oocyte membrane properties and quality (Zeron *et al*. 2002). In the same study, small changes in FA composition of oocytes were found, and higher numbers of grade 1 oocytes were collected from PUFA-supplemented ewes (Zeron *et al*. 2002). Kim *et al*. (2001) also found differences in FA composition between grade 1 and grade 2/3 oocytes, suggesting that the appearance of the ooplasm may reflect lipid and FA content. These differences did not directly influence oocyte maturation, but effects on oocyte competence cannot be discounted and may be responsible for differences in fertilization rate and developmental potential (Kim *et al*. 2001). Adamiak *et al*. (2006) compared the FA composition of plasma to that of granulosa cells and cumulus–oocyte complexes (COCs) in heifers supplemented with 0 or 6% calcium soaps of palm oil. Supplementation increased the FA content of COCs, but selective uptake of saturated FAs at the expense of PUFA within the follicular compartment led to higher concentrations of saturated FAs within COCs compared with concentrations in plasma.

We have recently reported beneficial effects of rumen inert fat (RIF) on developmental potential of oocytes in high-yielding dairy cows. RIF, supplied as calcium salts of palm acid oil which contain predominantly palmitic acid (C16:0) and oleic acid (C18:1), significantly increased blastocyst production *in vitro* (Fouladi-Nashta *et al*. 2007).

In summary, previous studies have shown varying effects of dietary FAs on reproductive performance, oocyte quality and FA composition of oocytes, granulosa cells, and follicular fluid. Although level of fat supplement has been shown to influence oocyte quality, there is little information on the effects of different FAs on developmental competence of oocytes from high-yielding dairy cows. Bilby *et al*. (2006) found no effect of supplementary n-3 or n-6 PUFA on oocyte quality, when compared with monounsaturated FAs in dairy cows. However, because two of their treatments involved fat supplements in the form of oils, it is possible that rumen biohydrogenation could have saturated FAs before they were absorbed into the bloodstream. The objective of the study reported here was to examine developmental potential of oocytes from high-yielding dairy cows fed on three dietary sources of FAs (RIF, soyabean, and linseed) that were partially protected against rumen biohydrogenation. To permit direct comparison of FA sources, each cow received each fat source in a 3×3 Latin-square design.

Results

There was no effect of FA source on (mean±S.E.M.) dry matter intake (18.7±0.25 kg/d), milk yield (32.6±0.59 kg/d), metabolizable energy balance (4.2±0.08 MJ/d), or milk concentrations of fat (38.8±1.90 g/kg), protein (32.9±0.42 g/kg), or lactose (47.1±0.33 g/kg).

FA composition in biological fluids and cells

Significant differences between treatments were observed in profiles of FAs in plasma and milk reflecting differences in the dietary sources of FAs (Tables 1 and 2). Whereas soya and linseed had no effect on unsaturated FA in plasma, both PUFA sources increased unsaturated FA in milk. Diets had predicted effects on proportions and ratios of n-6 and n-3 FA in both plasma and milk. RIF led to higher ($P<0.001$) concentrations of C16:0 and C18:1 in plasma and higher ($P<0.001$) concentrations of C16:0 in milk, compared with the two other dietary treatments. Soya led to higher ($P<0.001$) concentrations of linoleic acid (C18:2) in plasma, compared with linseed, and higher ($P<0.001$) concentrations of C18:2 in milk, compared with linseed and RIF. Linseed led to higher ($P<0.001$) concentrations of linolenic acid (C18:3) in plasma and milk, compared with soya and RIF.

Differences were also observed in plasma and milk among treatments for FAs that were not supplied directly in the diet. Conjugated linolenic acid concentrations in plasma and milk were higher when cows were offered linseed than when they were offered RIF or soya. Some trans FAs were significantly higher when cows were offered the linseed diet: 18:1t11 (vaccenic acid) and 18:1t12 were higher in both plasma and milk; 18:1t15 and 18:1t16 were higher in plasma; 18:1t4, 18:1t5 and 18:1t6/8 were higher in milk. Although dietary concentration of stearic acid (18:0) was highest in the RIF diet, 18:0 concentrations in both plasma and milk were lower when cows were fed this diet than when they were fed the other two diets.

In contrast to plasma and milk, the proportions of saturated and unsaturated FA in granulosa cells were unaltered by diet (Table 3). Furthermore, the proportions and ratio of n-6 and n-3 FA were unaltered by diet. Interestingly, proportions of individual FA shorter than C20 were not affected by diet, although some long-chain PUFA (notably C20:3, C20:4, C20:5, and C22:6) were altered ($P<0.005$).

Endocrinology and amino acids

No difference was observed in plasma concentrations of leptin, insulin, insulin-like growth factor 1 (IGF1), GH (Table 4) or amino acids (data not presented) among the three dietary treatments.

Table 1 Fatty acid composition of plasma.

Dietary treatment	RIF[a]	Soya	Linseed	S.E.D.	*P*
Fatty acids (g/100 g total fatty acids)					
Saturated	39.1*	39.0*	37.6†	0.408	0.002
Unsaturated	52.6*,†	51.9*	54.0†	0.747	0.029
Monounsaturated	16.9*	13.5†	17.8*	0.598	<0.001
Polyunsaturated	35.7*	38.4†	36.2*	0.805	0.007
n6 series	32.3*	35.7†	30.5‡	0.800	<0.001
n3 series	3.1*	2.8*	5.4†	0.379	<0.001
Ratio of n6:n3	10.81*	13.95†	5.72‡	1.189	<0.001
C14:0	0.63	0.81	0.68	0.102	0.216
C15:0	0.86	0.70	0.79	0.150	0.556
C16:0	14.76*	12.79†	11.91‡	0.323	<0.001
C17:0	0.86	0.86	0.88	0.012	0.222
C18:0	21.98*	23.84†	23.09†	0.356	<0.001
C23:0	0.008*	0.001*	0.062†	0.013	<0.001
C24:0	0.001	0.004	0.18	0.144	0.393
C16:1	3.45*	3.12*	4.00†	0.163	<0.001
C17:1	0.18	0.10	0.18	0.042	0.106
C18:1c9	10.21*	6.84†	8.45‡	0.465	<0.001
C18:1c11	0.58	0.53	0.56	0.029	0.197
C18:1c12	0.42*	0.55†	1.01‡	0.044	<0.001
C18:1c13	0.08	0.07	0.10	0.033	0.721
C18:1t9	0.10	0.12	0.13	0.044	0.806
C18:1t10	0.19	0.17	0.20	0.061	0.845
C18:1t11	0.68*	0.75*	1.34†	0.115	<0.001
C18:1t12	0.16*	0.29†	0.38‡	0.032	<0.001
C18:1t13/14	0.43	0.45	0.64	0.103	0.105
C18:1t15	0.05*	0.13†	0.18†	0.033	0.002
C18:1t16	0.33*	0.25†	0.38*	0.030	0.001
C20:1	0.06	0.03	0.06	0.024	0.477
C24:1	0.03	0.039	0.21	0.150	0.425
C18:2c9,c12 (n6)	29.17*	26.21†	24.47‡	0.708	<0.001
C18:2t9,c12	0.14	0.17	0.13	0.032	0.426
CLAc9,t11	0.12*	0.12*	0.17†	0.016	0.006
C18:3c6,c9,c12	0.22*	0.26*	0.17†	0.22	0.003
C18:3c9,c12,c15 (n3)	1.68*	2.05†	4.36‡	0.103	<0.001
C20:3 (n6)	3.41*	3.43*	2.74†	0.178	0.001
C20:4 (n6)	2.11	2.14	2.21	0.160	0.791
C22:2 (n6)	0.28*	0.35*	0.88†	0.153	0.001
C20:3 (n3)	0	0	0.07	0.054	0.329
C20:5 (n3)	0.75	0.51	0.82	0.152	0.128
C22:6 (n3)	0.34	0.64	0.18	0.243	0.186
Unidentified	8.33	9.13	8.41	0.750	0.506

Means within a row with different superscripts are significantly different ($P<0.05$). The *P* value in the table shows the overall significant difference across all treatments.
[a]RIF, rumen inert fat, Megalac, Volac International, Orwell, UK, calcium salts of palm fatty acids.

Follicle number and in vitro *embryo development*

A total of 1765 follicles were observed. Follicle number per cow did not differ between dietary treatments (mean ± S.E.M.; RIF: 11.9 ± 0.6, soya: 12.7 ± 0.7, linseed: 12.1 ± 0.8), and there was no difference between dietary treatments in average numbers of small (<4 mm), medium sized (4–10 mm) or large (>10 mm) follicles (Table 5). A total of 953 oocytes were collected and assessed morphologically. There was no difference between dietary treatments in the average number of oocytes collected per cow (mean ± S.E.M.; RIF: 6.1 ± 0.5, soya: 6.7 ± 0.6, linseed: 7.1 ± 0.7), nor in the distribution of oocytes in each grade (Table 5).

Cleavage rate was higher ($P=0.029$) for oocytes collected from cows offered the RIF diet than from cows offered the other two diets (Table 5). However, diet did not affect the proportions of blastocysts that developed from the number of inseminated oocytes or the number of cleaved oocytes (Table 5). There was no effect of dietary treatment on proportions of trophectoderm (TE) or inner cell mass (ICM) cells in blastocysts, or the ratio of ICM to TE (Table 5). Linseed tended to increase the proportion of apoptotic cells within TE ($P=0.08$), but there was no effect of diet on the proportion of apoptotic cells within ICM.

Discussion

The major finding of this study is that, despite altering proportions of major FAs in plasma and milk, contrasting sources of dietary FAs had little effect on FA composition of granulosa cells. Consequently, there was little effect of

Table 2 Fatty acid composition of milk.

Dietary treatment	RIF[a]	Soya	Linseed	S.E.D.	*P*
Fatty acids (g/100 g total fatty acids)					
Saturated	62.1*	59.7†	56.4‡	1.132	<0.001
Unsaturated	28.0*	30.1†	31.5†	0.850	0.002
Monounsaturated	24.7*	25.5†	27.2†	0.772	0.014
Polyunsaturated	3.3*	4.6†	4.3†	0.185	<0.001
n6 series	2.2*	3.3†	2.2*	0.157	<0.001
n3 series	0.6*	0.7†	1.1‡	0.046	<0.001
Ratio of n6:n3	4.07*	5.10†	1.99‡	0.256	<0.001
C4:0	2.95	2.95	3.05	0.096	0.494
C6:0	1.84	1.88	1.92	0.074	0.538
C8:0	1.03	1.07	1.07	0.059	0.728
C10:0	2.50	2.52	2.48	0.109	0.947
C12:0	3.18	3.14	3.05	0.106	0.468
C14:0	10.40	10.00	10.00	0.268	0.346
C16:0	30.90*	26.20†	23.90‡	0.901	<0.001
C18:0	9.30*	11.95†	10.93‡	0.445	<0.001
C16:1	1.79*	1.53†	1.48†	0.052	<0.001
C18:1c9	20.10	20.90	21.10	0.616	0.238
C18:1t4	0.03*	0.03*	0.04†	0.002	0.003
C18:1t5	0.03*	0.03*	0.04†	0.002	<0.001
C18:1t6/8	0.37*	0.37*	0.49†	0.037	0.003
C18:1t9	0.33	0.28	0.29	0.068	0.741
C18:1t10	0.47	0.53	0.80	0.223	0.309
C18:1t11	1.22*	1.30*	2.29†	0.224	<0.001
C18:1t12	0.43*	0.54†	0.71‡	0.042	<0.001
C18:2c9,c12 (n6)	2.16*	3.33†	2.18*	0.157	<0.001
CLAc9t11	0.61*	0.61*	1.00†	0.066	<0.001
C18:3c9,c12,c15 (n3)	0.55*	0.65*	1.12†	0.046	<0.001
Unidentified	9.89*	10.19*	12.14†	0.367	<0.001

Means within a row with different superscripts are significantly different ($P<0.05$). The *P* value in the table shows the overall significant difference across all treatments.
[a]RIF, rumen inert fat, Megalac, Volac International, Orwell, UK, calcium salts of palm fatty acids.

diet on follicle numbers and post-fertilization development of oocytes *in vitro*. In previous studies with lactating dairy cows, increased levels of dietary FAs (calcium salts of palm FA) improved oocyte quality in terms of the proportion of zygotes that developed to the blastocyst stage (Fouladi-Nashta *et al*. 2007). However, levels of dietary fats were similar for all treatments in the present study, indicating that the level of dietary fat is more important than type of dietary fat in determining oocyte developmental competence in high-yielding dairy cows. This conclusion is supported by the study of Bilby *et al*. (2006), which found no significant difference in blastocyst yield when comparing sunflower oil (high in oleic and n-6 FA), calcium salts of trans FAs, calcium salts of vegetable oil (high in n-6 FA) and linseed oil (high in n-3 FA). Similarly, Thangavelu *et al*. (2007) found no difference in the number of transferable embryos recovered from cows fed supplements of saturated FAs, whole linseed, or sunflower seed.

FA composition in biological fluids and cells

Milk and plasma samples showed no difference between the three dietary groups in concentrations of total fat, proteins, amino acids and metabolic hormones. Although differences between the dietary groups were observed in concentrations of FAs in both milk and plasma, these differences were not observed in granulosa cells. FA composition of plasma and milk reflected the FA composition of dietary fat sources, showing that short-term feeding of dairy cows changes the FA profile in some, but not all, tissues. Whether the FA profile of granulosa cells would change with longer-term feeding of contrasting diets remains to be seen. The feeding period of 25 days, with a minimum of 14 days from diet change to first ovum pick-up (OPU), was chosen to be comparable with previous studies (Adamiak *et al*. 2006, Fouladi-Nashta *et al*. 2007) which had demonstrated effects of FA supply on oocyte quality.

In addition to changes in the major FAs supplied by the dietary fat (palmitic, oleic, linoleic, and linolenic), increases in trans FAs and stearic acid were associated with feeding soya and linseed. These changes are expected from rumen biohydrogenation of PUFA (Lock & Garnsworthy 2002). Rumen biohydrogenation can alter considerably the profile of FAs absorbed from the digestive tract. A crucial feature of the current study was that plasma and milk FA profiles confirmed that dietary treatments were effective in altering FA supply to tissues. However, FA composition of granulosa cells was largely unaltered in the current study, so it is unlikely that FA supply to the oocyte was altered, although this was

Table 3 Fatty acid composition of granulosa cells.

Dietary treatment	RIF[a]	Soya	Linseed	S.E.D.	*P*
Fatty acids (μg/ml)	1245	1112	1161	146.9	0.664
Fatty acids (g/100 g total fatty acids)					
Saturated	37.5	37.3	37.1	0.968	0.928
Unsaturated	54.4	55.3	54.9	0.433	0.162
Monounsaturated	20.3	20.0	20.9	0.708	0.473
Polyunsaturated	34.1*	35.6†	34.1*	0.605	0.032
n6 series	19.7	20.8	19.7	0.618	0.128
n3 series	13.0	13.3	12.8	0.374	0.324
Ratio of n6:n3	1.57	1.61	1.58	0.074	0.835
C16:0	19.07	18.46	17.90	0.566	0.142
C17:0	0.75	0.89	0.78	0.134	0.548
C18:0	15.74	16.14	15.98	0.690	0.841
C20:0	0.03	0.01	0.03	0.012	0.350
C21:0	0.27*	0.24*	0.59†	0.092	0.002
C22:0	1.04	1.04	1.04	0.092	0.999
C24:0	0.66*	0.54*	0.82†	0.067	0.002
C16:1	1.69	1.72	2.00	0.204	0.269
C17:1	0.22	0.21	0.18	0.061	0.834
C18:1t9	0.74	0.76	0.70	0.373	0.989
C18:1c9	16.91	16.38	16.66	0.647	0.717
C20:1	0.44*	0.53*	0.71†	0.118	0.079
C22:1	0.21*	0.21	0.44†	0.104	0.059
C24:1	0.13*	0.18†	0.16†	0.020	0.055
C18:2t9,c12	0.07	0.41	0.17	0.157	0.104
C18:2c9,c12 (n6)	17.70	18.51	17.76	0.766	0.510
C18:3c6,c9,c12 (n3)	0.44	0.67	0.51	0.264	0.669
C20:2 (n6)	1.60	1.82	1.63	0.113	0.147
C20:3 (n6)	1.40*	1.27*	1.09†	0.070	0.001
C20:4 (n6)	0.07*	0.05*	0.13†	0.023	0.005
C22:2 (n6)	0.04	0.03	0.07	0.026	0.322
C18:3 (n3)	0.53	0.32	0.53	0.193	0.471
C20:3 (n3)	8.00	8.12	7.48	0.388	0.240
C20:5 (n3)	1.38*	1.61†	1.10‡	0.114	0.001
C22:6 (n3)	3.15*	2.93*	3.64†	0.136	<0.001
Unidentified	7.81	6.98	8.09	1.104	0.586

Means within a row with different superscripts are significantly different ($P<0.05$). The *P* value in the table shows the overall significant difference across all treatments.
[a]RIF, rumen inert fat, Megalac, Volac International, Orwell, UK, calcium salts of palm fatty acids.

not determined. In nonlactating cattle, Adamiak *et al.* (2006) reported selective uptake of saturated FAs at the expense of PUFA within the follicular compartment. This led to lower proportions of PUFA within COCs compared to plasma. In contrast, the proportions of saturated and unsaturated FAs were similar in plasma and granulosa cells in the current study (Tables 1 and 3). This may reflect differences between studies in physiological status (lactating versus nonlactating) and dietary treatments. However, the ratio of n-6 to n-3 was considerably lower in granulosa cells than in plasma, in agreement with Adamiak *et al.* (2006).

The mechanism by which granulosa cells regulate uptake of PUFA from plasma has not been elucidated. Offer *et al.* (2001) found that concentrations of PUFA were 10 times greater in the HDL and LDL fractions of plasma than in the VLDL fraction, and proposed that this explained the poor uptake of PUFA by the mammary gland which has little capacity to absorb FAs from the HDL and LDL fractions. However, the ability of granulosa cells to utilize HDL and LDL is established. For example, O'Shaughnessy *et al.* (1990) found that bovine granulosa cells are able to utilize HDL for steroidogenesis, but only after luteinization; Bao *et al.* (1995)

Table 4 Effects of dietary fat source on metabolic hormones.

	RIF[a]	Soya	Linseed	S.E.D.	*P*
Insulin (ng/ml)	0.38	0.32	0.37	0.034	0.200
IGF1 (ng/ml)	87.8	89.7	87.3	10.85	0.973
GH (ng/ml)	4.63	4.47	4.51	0.380	0.915
Leptin (ng/ml)	2.40	2.31	2.44	0.141	0.625

The *P* value in the table shows the overall significant difference across all treatments.
[a]RIF, rumen inert fat, Megalac, Volac International, Orwell, UK, calcium salts of palm fatty acids.

Table 5 Effects of dietary fat source on follicle numbers, oocyte grades, developmental potential of oocytes and embryo quality.

	Dietary treatment				
Means per cow	RIF[a]	Soya	Linseed	S.E.D.	*P*
Follicles observed					
Small (<4 mm)	4.28	4.51	3.66	0.857	0.587
Medium (4–10 mm)	6.78	7.28	7.70	0.775	0.490
Large (>10 mm)	0.60	0.56	0.50	0.152	0.785
Oocyte grades					
G1	13.7	13.7	15.3	4.25	0.982
G2	31.2	27.0	25.1	5.23	0.470
G3	32.9	34.5	36.0	5.45	0.885
G4	22.2	24.8	23.6	4.76	0.577
Embryo development					
Cleaved of IVF	0.70*	0.63†	0.59†	0.04	0.029
Blastocyst of cleaved	0.29	0.30	0.32	0.06	0.741
Blastocyst of IVF	0.20	0.19	0.19	0.04	0.992
Embryo quality					
Total cell number	115	118	123	22.0	0.944
TE of total	72.5	75.6	77.3	4.27	0.482
ICM of total	27.5	24.4	22.6	4.26	0.457
ICM:TE ratio	0.40	0.34	0.30	0.076	0.344
Apoptotic cells of TE	1.9	2.1	6.2	2.19	0.080
Apoptotic cells of ICM	9.9	11.6	7.7	4.99	0.720
Apoptotic cells of total	4.0	3.4	4.6	2.58	0.298

Means within a row with different superscripts are significantly different ($P<0.05$). The *P* value in the table shows the overall significant difference across all treatments.
[a]RIF, rumen inert fat, Megalac, Volac International, Orwell, UK, calcium salts of palm fatty acids.

found that progesterone synthesis by granulosa cells of dominant follicles was stimulated by HDL and that both HDL and LDL increased cell viability. Argov *et al*. (2004) studied control of lipoprotein uptake by developing bovine follicles and found increases in expression of lipoprotein receptor-related protein 8 (LRP8) and scavenger receptor class B type 1 receptor (SRB1) with increasing follicle size, which were related to preferential uptake of LDL and VLDL, so that these fractions contributed 30% of total lipoproteins in large antral follicles, but only 10% in small follicles. However, changes in the relative proportions of HDL, LDL, and VLDL do not explain differences in FA profiles of follicular fluid, since HDL (which has the greatest concentration of PUFA) remained the dominant lipoprotein fraction. Although Argov *et al*. (2004) discussed LRP8 and SRB1 primarily in relation to uptake of cholesterol for steroid synthesis, the FA profile of follicular fluid became increasingly saturated as follicles developed, which is consistent with active or passive selective uptake of saturated FA at the expense of PUFA.

Follicle development

Dietary fat has been shown to increase the number and size of follicles in lactating dairy cows, independent of energy supply (Staples *et al*. 1998, Garnsworthy *et al*. 2008*a*). Changes in size categories have been seen, with the number of medium-sized follicles increasing, and the number of small follicles decreasing (Lucy *et al*. 1992). Mattos *et al*. (2000) reported that feeding PUFA increased the number of large follicles and their diameter, and suggested that the increase in size may be due to stimulation of LH by improved energy status, although studies have shown this to be energy independent (e.g. Robinson *et al*. 2002). Cholesterol, insulin and IGF1 have been suggested as possible mediators of the effect of FAs on follicle development (Staples *et al*. 1998, Argov *et al*. 2004). Cholesterol was not measured in the current study, but insulin and IGF1 were unaffected by FA source. In the current study, and that of Fouladi-Nashta *et al*. (2007), regular sessions of follicular aspiration would inevitably have altered the endocrine milieu (Petyim *et al*. 2001) and truncated follicle development, so that observed effects might have differed from studies of cyclic animals. In the current study, there was no treatment effect on follicle numbers, whereas in the previous study a higher intake of FAs decreased follicle numbers. It may be that the total amount of FAs in a diet has a greater influence than individual FAs on ovarian follicular development, but this remains to be tested.

Oocyte quality and development

In a study by Zeron *et al*. (2002), small changes in FA composition of oocytes were reported, and higher numbers of grade 1 COC were collected from ewes supplemented with PUFA. Diet did not affect the proportion of COC in each grade in the current study. However, both diet and COC grade had an effect on cleavage. The proportion of inseminated oocytes that

cleaved was greater for RIF than for PUFA diets (Table 5) and was negatively related ($P=0.027$) to proportion of grade 4 COC (data not presented). When the effects of diet were examined after allowing for proportion of grade 4 COC, the difference between RIF and PUFA diets was still significant ($P=0.025$). To test for carry over effects between periods, data were analyzed with previous diet as the main treatment factor. Cleavage was lower ($P<0.05$) when the previous diet had been RIF, but there was no carry over effect of other diets, and blastocyst yield was not affected. This confirms the superiority of RIF over PUFA diets, in terms of cleavage, and also suggests that the effects are relatively short term (2–4 weeks). This is consistent with previous studies in our laboratory and elsewhere (McNamara *et al.* 2003, Adamiak *et al.* 2006, Fouladi-Nashta *et al.* 2007). In an experiment with similar treatments to the current study, Thangavelu *et al.* (2007) examined effects of PUFA on *in vivo* embryo development 7 days after insemination. In agreement with the current study, they found no effect of treatment on fertilization rate or number of embryos, although PUFA increased total cell numbers in embryos at the expanded blastocyst stage, which the authors speculated might be due to higher circulating progesterone concentrations in cows offered PUFA. In the current study, where treatment effects were not confounded by progesterone concentration during blastocyst development, total cell numbers were not influenced by PUFA. Furthermore, the lack of effect on ICM to TE ratio suggests that PUFA do not affect embryo quality. It has to be acknowledged that the maturation medium used in the current study included FCS in order to maximize developmental potential of oocytes. Although FCS contains some lipids, which might have moderated the effects of dietary FAs on oocyte developmental potential, the same culture system was utilized in our previous study (Fouladi-Nashta *et al.* 2007) where we reported beneficial effects of increased level of dietary FAs on oocyte quality and development. Furthermore, the lack of effect on number of embryos is in agreement with the *in vivo* results of Thangavelu *et al.* (2007).

The small effect on cleavage and lack of effect on blastocyst yield are commensurate with the absence of dietary effects on metabolic hormones (Table 4) and major FAs in granulosa cells (Table 3). Interestingly, the only FAs in granulosa cells that differed statistically were some of the long-chain PUFA with putative biological activity (Abayasekara & Wathes 1999). The biological significance of these differences is likely to be minimal.

The fact that several trans FAs were elevated in plasma of cows fed soya or linseed might be important because trans FAs reduce the fluidity and permeability of biological membranes (Roach *et al.* 2004); however, few of these trans FAs could be detected in granulosa cells. Furthermore, Bilby *et al.* (2006) found no effect of supplementary trans FAs on oocyte quality, when compared to monounsaturated FAs in dairy cows.

Conclusions

Whereas short-term feeding of different dietary FA sources to high-yielding dairy cows modifies n-3 and n-6 FAs in plasma and milk, the FA composition of granulosa cells is largely unaffected. The ovary would seem to be particularly effective in moderating the uptake of individual plasma FAs to present a relatively constant profile of FAs to the oocyte. Consequently, although previous studies have demonstrated that total concentration of dietary FAs can influence oocyte quality, the current study has clarified this by further demonstrating that dietary fat source exerts only modest effects on oocyte quality, defined as the proportion of oocytes that cleave following insemination, and has no effect on development to the blastocyst stage in lactating dairy cows.

Materials and Methods

Animals and experimental design

A total of 12 Holstein dairy cows were allocated to three groups on the basis of days in milk (40–46), parity (1–3) and body condition score in a 3×3 Latin-square experimental design. Each group received a total mixed ration containing either RIF, soyabean or linseed as the main FA source for three periods of 25 days, with a 2-day adaptation period between diets. Treatment diets were formulated to have similar contents of metabolizable energy, crude protein and oil, but to vary in FA profile (Table 6). The RIF (Megalac) contained predominantly palmitic and oleic acids, soyabean was high in linoleic acid, and linseed was high in linolenic acid. The full-fat soyabean was toasted and the full-fat linseed was extruded to reduce rumen degradation of protein and thereby provide some degree of protection against biohydrogenation of FAs. Within each period, cows were fed experimental diets using a robotic system for two weeks before start of OPU. Estrous cycles were synchronized using a progesterone releasing implant (CIDR, InterAg, Hamilton, New Zealand) on day 2 followed by injection of $PGF_{2\alpha}$ on day 10 and removal of CIDR on day 11. OPU sessions started on day 13 of each feeding period and were repeated four times on each animal with a 3–4 day interval between each OPU (Fig. 1).

Immediately prior to each OPU, blood samples were obtained from each cow. These were analyzed for plasma concentrations of FAs, amino acids, and metabolic hormones (insulin, glucagon, IGF1, GH, and leptin). In addition, milk samples were collected on each OPU day for fat, protein, and FA analysis.

Collection of oocytes and granulosa cells by OPU

OPU was performed using an ultrasound scanner (Medison Sonovet 600, Marl, Germany) fitted with a 6.5 MHz transvaginal probe. Before collection, cows were premedicated with 0.8 ml acepromacine (i.v.) and an epidural injection of 6–10 ml of 2% lignocaine. Follicular aspiration was performed

Table 6 Diet ingredients and composition.

	Treatment		
	Rumen inert fat	Soya	Linseed
Ingredients (kg/t DM)			
Grass silage	397	395	395
Maize silage	132	132	132
Molassed sugar beet pulp	214	213	213
Soya bean meal	82		
Rapeseed meal	61	61	61
Wheat	75	74	74
Mins/vitamins[a]	11	11	11
Rumen inert fat[b]	28		
Soya full-fat, toasted		114	
Linseed full-fat, extruded			114
Composition			
Dry matter (g/kg)	460	462	461
Metabolizable energy (MJ/kg DM)	12.4	12.3	12.5
Crude protein (g/kg DM)	173	174	165
NDF (g/kg DM)	340	344	352
Starch (g/kg DM)	99	102	122
Oil[b] (g/kg DM)	49	49	51
Major fatty acids (g/100 g total)			
C16:0	39	18	10
C18:0	4	2	3
C18:1c9	31	21	17
C18:2c9,c12 (n6)	17	49	20
C18:3c9,c12,c15 (n3)	7	8	48

[a]Bibby HiPhos, ABN Ltd, Peterborough, UK; calcium 18%; phosphorus 10%; magnesium 5%; salt 17%; copper 2000 mg/kg; manganese 5000 mg/kg; cobalt 100 mg/kg; zinc 6000 mg/kg; iodine 500 mg/kg; selenium 25 mg/kg; vitamin A 400 000 IU/kg; vitamin D3 80 000 IU/kg; vitamin E 1000 mg/kg. [b]Megalac, Volac International, Royston.

with a 60 cm long, 18 G needle (Arnolds Veterinary Products, Arnolds, Harlescott, UK) at 60 mmHg vacuum pressure.

Follicular fluid was collected into flushing medium (0.25 mg/ml heparin in PBS supplemented with 5% heat inactivated FCS (HIFCS)). The contents of the collection tubes were passed through an embryo filter (Em-Con, 75 μm pore size; Cook, Brisbane, Queensland, Australia) and washed with flushing medium. All collected oocytes were graded (grades 1–4) morphologically based on the number and intensity of the cumulus cells and homogeneity of the ooplasm, as was previously described (de Loos *et al.* 1989).

Following oocyte recovery, the follicular fluid and flushing media were centrifuged for 10 min at 805 ***g*** and 4 °C. The cell pellets were then suspended in 9 ml distilled water and mixed thoroughly to lyse any red blood cells. To restore isotonicity, 1 ml 10×PBS (Invitrogen) was added and the mixture was centrifuged at 805 ***g*** for 5 min at 4 °C. The supernatant was removed and granulosa cells were re-suspended in 1 ml PBS to wash the cells and cells were again centrifuged at 805 ***g*** for 5 min at 4 °C (cell washing was repeated 2–3 times). Before cell suspensions were centrifuged for the final time they were transferred to eppendorfs. Cell suspensions were micro-centrifuged at 8765 ***g*** for 90 s and the supernatant was removed. Granulosa cells were snap frozen in liquid nitrogen (in minimum volume of PBS) and were stored at −80 °C until used for FA analysis.

In vitro *maturation, fertilization, and embryo culture*

Prior to culture, oocytes were washed twice in oocyte washing medium (tissue culture medium 199 (TCM199) with Earle's salts (Gibco), 75.0 mg/l kanamycin monosulfate (Sigma), 7.08 g/l Hepes (pH 7.8, osmolarity 279 mOsmol/kg H_2O)) supplemented with 10% HIFCS. Groups of oocytes recovered from each OPU for each cow were cultured separately. All oocytes were cultured for 24 h in 35 mm culture dishes (Nunc, Roskilde, Denmark) containing 10 μl maturation medium per oocyte (TCM199 supplemented with 10 μg FSH/ml (follitropin; Bioniche Animal Health, Bellevile, Ontario, Canada), 10 μg LH/ml (leutropin; Bioniche Animal Health) 1 mg estradiol/ml, 50 μg gentamicin/ml, and 10% HIFCS under mineral oil in a humidified atmosphere of 5% CO_2 in air at 39 °C. *In vitro*-matured oocytes were fertilized with frozen sperm from a single bull, as described previously (Fouladi-Nashta & Campbell 2006). Briefly, motile sperm were prepared after 45 min of swim up in calcium-free medium followed by centrifugation at 300 ***g*** at room temperature and re-suspension of the pellet in fertilization medium. The COCs were gently pipetted to remove adhering granulosa cells and to break up aggregated COCs. Disaggregated COCs were then washed once in oocyte wash medium and transferred into 45 μl microdrops of fertilization medium containing sperm (10^6 sperm/ml) and cultured for 24 h at 39 °C in a humidified incubator of 5% CO_2 in air. After 24 h, all presumptive zygotes were denuded from cumulus cells and cultured in 5 μl/embryo of synthetic oviductal fluid medium supplemented with amino acids, sodium citrate, and myoinositol (SOFaaci; Holm *et al.* 1999) supplemented with 4 mg/ml of FA-free BSA and cultured at 39 °C in a humidified incubator with 5% O_2, 5% CO_2, and 90% N_2. The culture was continued up to day 8, and medium was renewed every 2 days. The number of cleaved embryos and development to the blastocyst stage were recorded.

Differential staining of blastocysts and detection of apoptotic nuclei

Day 8 blastocysts were stained using a differential staining combined with TUNEL labeling technique for counting the number of healthy and apoptotic cells in ICM and TE compartments as previously described (Fouladi-Nashta *et al.* 2005). Briefly, TE cells were

Figure 1 Experimental design and timetable of the experiment. Ovum pick-up (OPU) was carried out with an interval of 3–4 days. Mondays (M), Fridays (F), and Tuesdays (T).

permeablized and stained by incubating embryos in a 0.2% solution of Triton X-100 in SOF–BSA containing 30 µg/ml propodium iodide for 5 min. Immediately after, the embryos were washed twice in SOF–BSA culture medium and fixed in 4% paraformaldehyde containing 10 µg/ml bisbenzimide (Hoescht 33342) for 20 min at RT. This allows fixation of embryos as well as staining total cell nuclei. Embryos were washed thoroughly and then incubated in small droplets of *In Situ* Cell Death Detection Kit solution (Roche) for 45 min at 37 °C according to the manufacturer's instructions. Next, embryos were washed in SOF culture medium and mounted in small droplets of Vectashield Mounting medium (Vector Laboratories Ltd, Peterborough, UK) on glass slides and examined under a Leica epiflourescent microscope (Leica Microsystems, Milton Keynes, UK). Differentially stained blastocysts are visualized with distinct TE (red) and ICM (blue) compartments and apoptotic cells are green (Fig. 2). A higher number of apoptotic nuclei characterises a degrading blastocyst and a low ratio of ICM/TE cell number is a further marker of a poor quality embryo.

Hormone assays

Plasma insulin concentrations were measured by RIA based on the method of Starr *et al.* (1979) modified according to Sinclair *et al.* (2000). Assay sensitivity was 2.2 mIU/l and intra- and inter-assay coefficient of variation (CV) were 4.0 and 8.4% respectively. Plasma glucagon was measured by using a RIA kit supplied by Linco Research Inc. (St Charles, MO, USA). The sensitivity of the assay was 40.5 pg/ml and the intra- and inter-assay CV were 8.8 and 8.3% respectively.

IGF1 as measured after removal of IGF-binding protein by size-exclusion HPLC, as described by Gutierrez *et al.* (1997). The sensitivity of the assay was 0.11 ng/ml and the intra-assay CV was 3.8%.

GH was measured as described by Lovendahl *et al.* (1991). The sensitivity of the assay was 1.2 ng/ml. The inter-assay CV was 11.3% and the mean intra-assay CV was 3.9%.

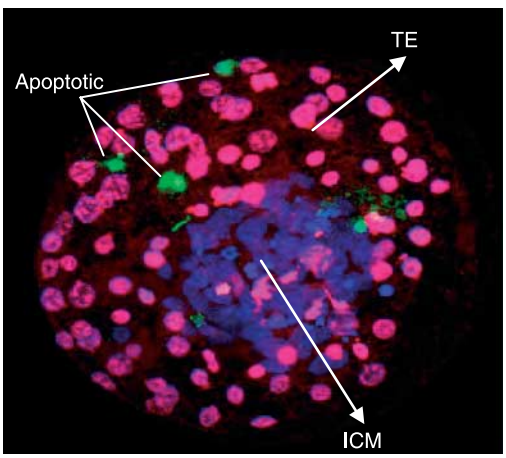

Figure 2 Example of a blastocyst after DST staining. Blastocysts were stained with differential staining combined with TUNEL labeling for counting total cell number and the number of inner cell masses (ICMs), trophectoderms (TEs), and apoptotic cells.

Plasma leptin concentration was determined using the method of Blache *et al.* (2000). The primary antibody, normal emu serum, and sheep anti-emu serum were both provided by Dr Blache (University of Western Australia, Perth, Australia). Ovine leptin (supplied by Dr Keisler, University of Missouri, Columbia, MO, USA) was iodinated in-house. The detection limit for leptin was 0.2 ng/ml, and the interassay CV for low, medium, and high controls were 30.4, 3.9, and 15.3% respectively. The corresponding intra-assay CV were 9.1, 12.2, and 13.7% respectively.

Analysis of plasma FAs and amino acids

Plasma FAs were extracted by the method of Bligh & Dyer (1959), and transesterified by the method of Christie (1982). FA methyl esters were quantified by gas chromatography following the procedures described by Feng *et al.* (2004).

Amino acid analysis was performed as described by Fouladi-Nashta *et al.* (2007).

Analysis of milk composition

One 20 ml aliquot of each milk sample was stored at 4 °C with preservative (30 mg potassium dichromate; Lactab MkIII tablet, Thomson and Capper Ltd, Runcorn, Cheshire, UK) until analyzed for fat and protein by infrared analysis at the National Milk Records Laboratory, Harrogate, Yorkshire, UK, using reference method AOAC (1990) method no. 972.16. Another 20 ml aliquot was stored without preservative at −20 °C for determination of milk FAs. Milk FAs were extracted by centrifugation and individual FAs were determined by gas chromatography following the procedures described by Feng *et al.* (2004).

FA analysis of granulosa cells

Granulosa cells were thawed over ice and together with 100 µl (200 µg/ml) of internal standard (pentadecanoic acid; C15:0) were extracted using a 2:1 mixture of chloroform:methanol, based on the method of Folch *et al.* (1957) and methylated using methanolic HCl (5% v/v). FA methyl esters were reconstituted in hexane and 2 µl injected in splitless mode into an Agilent GC6890 gas chromatograph equipped with a flame ionized detector (Agilent Technologies, Cheadle, UK). The column used was a 100 m Varian CP7489 capillary column (internal diameter 0.2 µm; Varian Scientific, Oxford, UK). Oven temperature was increased from 59 °C to a final temperature of 240 °C over 1 h. Individual FAs were identified by comparison with a standard containing 37 FAs (Supelco, Poole, UK). Twenty-five FAs (from C15:0 upwards) were detected.

Statistical analysis

All data were analyzed using the Genstat 9.0 statistical package (Lawes Agricultural Trust, Rothamstead, UK). Food intake, milk yield and composition, plasma, milk and granulosa-cell FAs, and plasma hormones, metabolites and amino acids, were

compared by ANOVA. Dietary FA source was the independent variable; feeding periods, OPU collection within periods, and cows were random effects. Proportions of oocyte cleavage and blastocyst development from IVF or cleaved oocytes were analyzed by Generalized Linear Model (GLM) regression with a binomial distribution and logit transformation. Dietary FA source (fed during the current period or the previous period) was the independent variable; feeding periods, OPU collection within periods, and cows were random effects. Proportions of cell types in stained blastocysts were analyzed by GLM regression with a normal distribution for TE and ICM cells, and a Poisson distribution for apoptotic cells. Dietary FA source was the independent variable and cows were random effects.

None of the data differed between feeding periods or OPU collection within periods, so only dietary treatment effects are reported.

Declaration of interest

There is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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