Dietary unsaturated fatty acids influence preovulatory follicle characteristics in dairy cows

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Abstract

Dietary unsaturated fatty acids (UFAs) have been implicated in several reproductive processes in dairy cows through a variety of mechanisms. This study examined the effects of periparturient supplementation of rumen bypass fats low or high in proportion of UFAs (oleic and linoleic) on preovulatory follicle characteristics. Forty-two 256-day pregnant dairy cows were divided into three groups and were fed a control diet ($n=14$) or supplemented with fats either low (LUFA; $n=14$) or high (HUFA; $n=14$) in UFAs. At 14–15 days following behavior estrus, the cows received a prostaglandin $F_{2\alpha}$ injection and 48 h later >7 mm follicles were aspirated. Progesterone (P_4), androstenedione (A_4), and estradiol (E_2) were determined in the follicular fluid. Out of 75 follicles, 37 follicles that were aspirated between 55 and 70 days *post partum* were regarded as E_2 -active follicles (E_2/P_4 ratio >1) and subjected for further analysis. The diameter of preovulatory follicles was greater in cows fed HUFA than in those fed control or LUFA. The concentrations and content of A_4 and E_2 in follicles and E_2/P_4 ratio were higher in the HUFA group than in the other two groups. The P450 aromatase mRNA expression in granulosa cells that were collected from the aspirated preovulatory follicles was also higher in the HUFA cows than in the other groups. A significant correlation was observed between E_2 concentrations in preovulatory follicles and E_2 concentrations in plasma at aspiration. In conclusion, dietary UFA increased the size of and elevated steroid hormones in preovulatory follicles, which may be beneficial to consequent ovarian function.

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Introduction

Dietary fat has been shown as beneficial to the reproductive system in dairy cows (Staples *et al.* 1998). Recently, it has been accepted that the composition of fatty acids (FAs) in the supplemented fat has a crucial role in determining the effect on reproduction (Mattos *et al.* 2000, Lucy 2001). There is some evidence that dietary unsaturated FAs (UFAs) positively affect ovarian function in dairy cows, although the precise mechanism is unknown (Staples *et al.* 1998, Robinson *et al.* 2002). Leroy *et al.* (2005) demonstrated that the saturated FAs palmitic (C16:0) and stearic (C18:0) had reduced the cleavage rate and the development rate of blastocysts *in vitro*.

UFAs are essential components of all cell membranes and the proportion of different UFAs in tissues of the reproductive tract reflect dietary consumption (Wathes *et al.* 2007). UFAs can influence reproductive processes through a variety of mechanisms; they provide the precursors for prostaglandins (PGs) synthesis and can modulate the expression patterns of many key enzymes involved in both PG and steroid metabolism (Wathes *et al.* 2007). It has been shown by Elmes *et al.* (2004) in pregnant ewes that increased consumption of linoleic acid (C18:2) elevated the proportion of arachidonic acid (C20:4)

in maternal plasma and fetal tissues and enhanced the placental PGs production. Supplementation of rumen bypass polyunsaturated FA to ewes increased the number of follicles and oocytes in the ovaries and enhanced the number of high-quality oocytes (Zeron *et al.* 2002). Dietary linolenic acid (C18:3) has been shown to increase follicle diameter and elevate plasma estradiol (E_2) concentrations during the follicular phase (Robinson *et al.* 2002). These results suggest that dietary UFA may have a pivotal role in modulating follicular development, PGs secretion, and steroidogenesis.

Our hypothesis was that specific dietary FAs might have a role in regulation of steroidogenesis in preovulatory follicles. The objective of the present study was to compare the effects of periparturient supplementation of two rumen bypass dietary fats containing either a high or low proportion of UFAs (oleic and linoleic) on the characteristics of preovulatory follicles in dairy cows.

Results

Milk production and dry matter intake

The average milk production during the first 70 days *post partum* was 5% higher in the high proportion of UFAs (HUFA) and low proportion of UFAs (LUFA) groups as

compared with control; 41.2, 43.3, and 43.1 kg/day for control, LUFA, and HUFA respectively (pooled S.E.M.=0.6; $P<0.02$), with no significant differences in 3.5% fat-corrected milk production. Daily average dry matter intake until 70 days *post partum* was similar between groups; 23.6, 23.2, and 23.6 kg for control, LUFA, and HUFA groups respectively (pooled S.E.M.=0.2). No differences between groups were observed in the calculated net energy balance of the cows until 70 days in lactation. The interval between calving and time of positive energy balance was longer in the HUFA group than in the control group ($P<0.04$) but not from the LUFA group (26.9, 41.6, and 44.2 days for control, LUFA, and HUFA groups respectively; pooled S.E.M.=5.7).

Preovulatory follicles characteristics and hormones

The average plasma progesterone (P_4) concentration was 5.12 ng/ml at the day of PGF_{2α} injection and 0.11 ng/ml at the day of aspiration with no differences between groups. Three cows that had a different pattern of plasma P_4 concentrations from the expected (according to the experimental schedule) were excluded from the analysis. Aspirations that were conducted between 55 and 70 days *post partum* were subjected to further analysis.

Follicles were regarded as E_2 active whenever the E_2/P_4 ratio in FF was >1 and regarded as E_2 inactive whenever the E_2/P_4 ratio was ≤ 1 (Ireland & Roche 1982). Thirty-seven out of seventy-five follicles larger than 7 mm ($\sim 50\%$) that were aspirated were defined as E_2 active and these follicles were used for further analysis. The average number of E_2 -active follicles that were aspirated was 1.3 per cow in the control and LUFA groups, and 1.0 in the HUFA group with no significant differences between groups. The average diameter of the follicles aspirated from the HUFA group was larger than in the control and LUFA groups ($P<0.03$; Table 1). The volume of the follicles in the HUFA group tended to be larger than in the control and LUFA groups ($P<0.06$; Table 1).

The concentrations of P_4 , androstenedione (A_4), and E_2 in FF of E_2 -active follicles are presented in Fig. 1. The P_4 concentrations and content in FF did not differ between groups (Table 1; Fig. 1). The A_4 concentrations in the HUFA group were 69% higher than in the control group ($P<0.04$), and 4.8-fold higher than in the LUFA group ($P<0.001$). The A_4 content (Table 1) was 3-fold higher in the HUFA group than in the control ($P<0.001$), and 11.5-fold higher than in the LUFA group ($P<0.001$). The A_4 concentrations were lower in LUFA as compared with the control ($P<0.04$), but the A_4 content of the FF did not differ between these two groups.

The FF concentrations of E_2 were 40% higher in the HUFA group than in the control group ($P<0.01$), and threefold higher than in the LUFA group ($P<0.001$;

Table 1 Characteristics of preovulatory follicles from cows supplemented with fat high or low in unsaturated fatty acids (UFAs) proportion.

	Treatments ^c			S.E.M.
	Control	LUFA	HUFA	
<i>n</i> , cows ^d	12	9	9	
<i>n</i> , follicles	16	12	9	
Follicles per cow	1.3	1.3	1.0	0.2
Diameter (mm)	11.8 ^b	10.5 ^b	15.4 ^a	1.0
Volume (ml)	1.1	1.0	2.2	0.4
Progesterone (ng)	136.9	136.8	211.6	75.7
Androstenedione (ng)	136.9 ^b	36.0 ^b	416.7 ^a	70.6
Estradiol (ng)	1490.5 ^b	867.6 ^b	3523.6 ^a	539.0
NEFA (μEq/l)	219.8	211.8	178.9	39.0
NEFA (μEq)	198.3	228.5	416.5	87.6
Insulin (pg/ml)	101.7	86.3	115.9	22.9
Insulin (pg)	96.7	95.7	225.3	53.3

^{a,b}Within rows, means with different superscript letters are statistically different ($P<0.05$). NEFA, non-esterified fatty acids.

^cTreatments were: cows fed from 256 days of pregnancy a dry cow ration and *post partum* fed a lactating cow diet (CTL) or supplemented with a fat either containing a low (LUFA) or high (HUFA) proportion of unsaturated fatty acids. ^dCows with at least one E_2 -active follicle.

Fig. 1). The E_2 content in HUFA was 2.4-fold higher than in the control ($P<0.01$) and 4.1-fold higher than in the LUFA group ($P<0.003$; Table 1). The E_2 concentrations in the LUFA group were 53% lower than in the control ($P<0.001$), with no differences in E_2 content among these groups. The E_2/P_4 ratio was 47% higher in the HUFA group than in the control ($P<0.03$) and 2.5-fold higher than in the LUFA group ($P<0.001$). The E_2/P_4 ratio was 41% lower in LUFA than in control ($P<0.05$).

Concentrations and content of non-esterified fatty acids (NEFA) and insulin in FF are shown in Table 1. NEFA and insulin concentrations in FF did not differ between groups, but NEFA and insulin contents in FF tended to be higher in HUFA as compared with control ($P<0.09$).

Plasma E_2 concentrations at the day of follicle aspiration

The concentration of E_2 in plasma at the day of follicle aspiration was examined in a subgroup of 14 randomly selected cows (four to five cows from each treatment). The concentration of E_2 in plasma at the day of follicle aspiration was numerically but not significantly higher in HUFA cows than in the control and LUFA cows (3.5, 4.9, and 5.1 pg/ml for control, LUFA, and HUFA groups respectively; pooled S.E.M.=0.1).

A positive correlation between E_2 concentrations in plasma at the day of follicle aspiration and E_2 concentrations in FF was observed ($r=0.55$, $P<0.01$). Furthermore, the correlation between E_2 concentrations in plasma and the E_2 content (ng) in FF was also significant ($r=0.48$, $P<0.03$). No significant correlation was found between the diameter of the follicle and E_2 in plasma ($r=0.32$, $P<0.19$).

Figure 1 Concentrations of (A) progesterone, (B) androstenedione, (C) estradiol and (D) estradiol/progesterone ratio in FF of cows fed from 256 days of pregnancy a dry cow ration and *post partum* fed a lactating cow diet (CTL) or supplemented with a fat either containing a low (LUFA) or high (HUFA) proportion of unsaturated fatty acids. ^{a,b,c}Means with different superscript letters are statistically different ($P < 0.05$).

P450 aromatase mRNA expression in granulosa cells

The mRNA expression of P450 aromatase in granulosa cells was determined and the relative expression is presented in Fig. 2. The P450 aromatase mRNA expression in granulosa cells was similar between control and LUFA and was increased by one order of magnitude in HUFA cows in comparison with control ($P < 0.016$).

FA profile in plasma and follicular fluid (FF)

As shown in Table 2, the percentage of linoleic acid (C18:2) in plasma was higher in the HUFA group than in the LUFA group ($P < 0.05$) and similar to the control group. The total percentage of PUFA (C18:2 and longer FAs) was higher in HUFA than in LUFA (Table 2; $P < 0.05$), and similar to the control group ($P < 0.12$).

Figure 2 P450 aromatase mRNA relative expression in granulosa cells obtained from preovulatory follicles of cows fed from 256 days of pregnancy a dry cow ration and *post partum* fed a lactating cow diet (CTL) or supplemented with a fat either containing a low (LUFA) or high (HUFA) proportion of unsaturated fatty acids. ^{a,b}Means with different superscript letters are statistically different ($P < 0.05$).

The total percentage of saturated FAs tended to be higher in LUFA compared with HUFA (Table 2; $P < 0.08$).

The FA profile in FF was analyzed in 30 out of 37 E_2 -active follicles, since not all follicles had sufficient volume of FF for analysis. As shown in Table 3, the FA profile in FF did not differ between treatments, except for the percentage of arachidonic acid (C20:4), which was lower in the LUFA group than in the control group ($P < 0.05$), but was not different from that in the HUFA group.

Relationship between FF hormones, insulin, and NEFA

Across treatments, correlation coefficients between FF hormones, insulin, and NEFA are presented in Table 4. The content of P_4 , A_4 , and E_2 in FF was positively correlated with insulin and NEFA contents in FF. Furthermore, E_2 content in FF was positively correlated with P_4 and A_4 contents in FF. E_2 concentrations in FF was positively correlated with NEFA, A_4 content, and P_4 concentrations

Table 2 Fatty acid profile in plasma at the day of follicle aspiration.

	Treatments ^c			S.E.M.
	Control	LUFA	HUFA	
<i>n</i>	12	9	9	
C14:0	1.66	1.53	1.49	0.09
C14:1	0.72	0.71	0.68	0.08
C16:0	13.31	13.91	13.14	0.41
C16:1	1.23	1.46	1.27	0.19
C18:0	12.56	13.35	12.29	0.47
C18:1	8.65	8.99	8.57	0.39
C18:2	55.36 ^{a,b}	53.68 ^b	56.24 ^a	0.87
C18:3	1.80	1.73	1.71	0.10
C20:3	2.35	2.54	2.55	0.16
C20:4	2.18	2.10	2.05	0.22
Saturated fatty acids	27.53 ^{a,b}	28.79 ^a	26.92 ^b	0.69
Polyunsaturated fatty acids	61.69 ^{a,b}	60.05 ^b	62.55 ^a	0.88

^{a,b}Within rows, means with different superscript letters are statistically different ($P < 0.05$).

^cTreatments were: cows fed from 256 days of pregnancy a dry cow ration and *post partum* fed a lactating cow diet (CTL) or supplemented with a fat either containing a low (LUFA) or high (HUFA) proportion of unsaturated fatty acids.

Table 3 Fatty acid profile in follicular fluid of estradiol (E₂)-active follicles.

	Treatments ^c			S.E.M.
	Control	LUFA	HUFA	
<i>n</i>	11	10	9	
C14:0	2.40	2.80	2.86	0.28
C14:1	1.12	2.50	1.10	0.15
C16:0	15.52	14.03	16.24	0.86
C16:1	1.74	1.65	1.70	0.15
C18:0	11.43	11.46	11.46	0.42
C18:1	9.43	9.78	9.15	0.39
C18:2	51.74	52.47	51.99	0.95
C18:3	1.67	1.57	1.66	0.09
C20:3	1.88	1.96	1.97	0.10
C20:4	2.25 ^a	1.79 ^b	1.87 ^{a,b}	0.17
Saturated fatty acids	26.95	25.49	27.70	1.03
Polyunsaturated fatty acids	57.55	57.78	57.48	0.97

^{a,b}Within rows, means with different superscript letters are statistically different ($P < 0.05$).

^cTreatments were: cows fed from 256 days of pregnancy a dry cow ration and *post partum* fed a lactating cow diet (CTL) or supplemented with a fat either containing a low (LUFA) or high (HUFA) proportion of unsaturated fatty acids.

in FF. The NEFA content in FF was positively correlated with insulin content and P₄ concentrations in FF. Other correlations were not statistically significant.

Relationship between follicular fluid FA concentrations and hormones

Correlations were examined between follicular fluid P₄, A₄, and E₂ concentrations and specific FA concentrations (Table 5). A positive correlation was found between P₄ and E₂ concentrations in FF and concentrations of C16:0, C18:1, C18:2, C18:3, and total FA in FF ($P < 0.05$). A₄ concentrations in FF had a positive correlation with the concentrations of C16:0, C18:2, and total FA in FF ($P < 0.05$). The concentration of C20:4 in FF was positively correlated with the concentrations of P₄ ($P < 0.03$).

NEFA and insulin in E₂-active or E₂-inactive follicles

A total of 75 follicles were aspirated; 37 follicles were regarded as E₂ active and 38 follicles were regarded as E₂ inactive. Across treatments, analysis showed that insulin concentration was 4.2-fold ($P < 0.06$) and insulin content

was 2.9-fold ($P < 0.0001$) higher in FF from E₂-active follicles as compared with E₂-inactive follicles (Table 6). Furthermore, NEFA concentrations and content were 3.3- and 4.9-fold higher in E₂-inactive follicles than in E₂-active follicles respectively (Table 6; $P < 0.0001$).

Discussion

To our knowledge, this is the first study in which dietary UFA had an effect on preovulatory follicle characteristics in dairy cows. In the present study, the concentrations and content of A₄ and E₂ in preovulatory follicles of cows supplemented with fat that contained high proportion of UFA were enhanced compared with control or cows that were supplemented with fat that contained low proportion of UFA. The mRNA expression of P450 aromatase was also increased in granulosa cells obtained from HUFA cows than in the control or LUFA cows.

In the current study, no significant correlation was observed between preovulatory follicles size and E₂ concentrations in plasma ($r = 0.32$; $P < 0.19$), which is in agreement with a report by Wiltbank *et al.* (2006). However, a significant correlation was detected between E₂ concentrations in plasma and E₂ concentrations or E₂ content in follicles. The concentration of E₂ in plasma has been positively correlated with estrus duration and behavior in cattle (Mondal *et al.* 2006, Wiltbank *et al.* 2006). Pregnancy rates were related to the diameter of the preovulatory follicles and E₂ concentration in plasma in dairy cows (Lopes *et al.* 2007). It was also demonstrated by Botero-Ruiz *et al.* (1984) that follicles aspirated from women who conceived after *in vitro* fertilization had significantly higher E₂ concentrations than similar follicles from women who failed to conceive. Furthermore, a relationship between peak serum E₂ concentrations and pregnancy rate after embryo transfer on day 5 was also observed in human (Chen *et al.* 2003). In an *in vitro* study, Tesarik & Mendoza (1995) demonstrated a direct effect of E₂ on fertilization and cleavage rate of mature oocytes. They also suggested a model in which E₂ impacts the cell surface by increasing the intracellular free [Ca²⁺], which serves as a second messenger and contributes to capacitation and early post-fertilization development (Tesarik & Mendoza 1995). Collectively, these findings indicate positive effects of E₂ concentrations in FF on

Table 4 Across treatments ($n = 37$) correlation coefficients between follicular fluid hormones, NEFA and insulin in E₂-active follicles.

Item	P ₄ (ng)	A ₄ (ng)	E ₂ (ng)	NEFA (μEq)	Insulin (pg)	E ₂ (ng/ml)
P ₄ (ng)	–	NS	0.76 [†]	0.88 [†]	0.40*	NS
A ₄ (ng)	NS	–	0.77 [†]	0.40*	0.34*	0.53 [†]
E ₂ (ng)	0.76 [†]	0.77 [†]	–	0.79 [†]	0.60 [†]	–
NEFA (μEq)	0.88 [†]	0.40*	0.79 [†]	–	0.53 [†]	NS
NEFA (μEq/l)	NS	NS	NS	–	NS	0.31*
P ₄ (ng/ml)	–	NS	0.45 [†]	0.49 [†]	NS	0.41*

P₄, progesterone; A₄, androstenedione; E₂, estradiol; NEFA, non-esterified fatty acid. * $P < 0.05$; [†] $P < 0.0001$.

Table 5 Across treatments ($n=30$) correlation coefficients between follicular fluid fatty acids concentrations and hormones.

Item	C16:0	C18:1	C18:2	C18:3	C20:3	C20:4	Total FA
P ₄ (ng/ml)	0.49 [†]	0.35*	0.45*	0.34*	NS	0.40*	0.46*
A ₄ (ng/ml)	0.47 [†]	NS	0.32*	NS	NS	NS	0.35*
E ₂ (ng/ml)	0.58 [†]	0.39*	0.43*	0.36*	0.49 [†]	NS	0.48 [†]

P₄, progesterone; A₄, androstenedione; E₂, estradiol; FA, fatty acid. * $P < 0.05$, [†] $P < 0.008$.

estrus characteristics in cattle and fertilization, cleavage rate and pregnancy rate in human. These findings make the enhancement of E₂ concentrations in FF of special interest, since it may increase the potential of successful pregnancy.

There is little information on the effects of dietary UFA on ovarian steroidogenesis. It has been found in two studies that feeding UFA increased E₂ levels in plasma; Robinson *et al.* (2002) supplemented dairy cows with fats containing either high concentrations of C18:3 or high concentrations of C18:2 and observed higher E₂ concentrations in plasma in the C18:3 treatment than in the control, with intermediate values in the C18:2 group. Lammoglia *et al.* (1997) also found increased E₂ concentrations in plasma throughout the first, but not the second estrous cycle after fat supplementation using rice bran (rich in C18:2). There is some evidence that dietary fat could increase E₂ content or concentrations in FF. Cows that were supplemented with fat that contained calcium salts of FA (6.5% C18:2) had higher E₂ content in E₂-active follicles as compared with the control group (Moallem *et al.* 1999). In our previous report that focused on the follicular development in early lactation, a tendency for higher concentrations of E₂ in FF from E₂-active follicles that were aspirated on day 12 *post partum* was observed in cows that were supplemented with HUFA as compared with LUFA or control cows ($P < 0.1$; Moallem *et al.* 2007). In the current study, cows that were supplemented with HUFA that contained 33.6% of C18:1 and 30.5% of C18:2 had higher concentrations and content of E₂ in follicles than control or LUFA supplemented cows.

We also observed higher E₂/P₄ ratio in follicles from the HUFA group than in the control and LUFA groups (1.5- and 2.5-fold higher respectively). It is well established that the E₂/P₄ ratio is one of the most precise indicators of follicles health (McNatty *et al.* 1979, Ireland & Roche 1982). Andersen (1993) demonstrated a significant correlation between the pregnancy potential of human oocytes after *in vitro* fertilization and the E₂/androgen ratio in FF. The enhanced E₂ concentrations and content and the higher E₂/P₄ ratio in preovulatory follicles of the HUFA cows in the current study may contribute to an increased pregnancy potential as explained previously.

Although we did not observe differences in the percentage of C18:2 in FF between groups, across treatment data showed a positive correlation between the C18:2 and E₂ concentrations in FF ($r=0.43$; $P < 0.02$). The mechanism behind the enhanced E₂ synthesis when feeding UFA is still unclear, however,

several explanations have been suggested. Steroid synthesis requires increased expression of StAR protein, which mediates transfer of cholesterol from the cytosol to the inner mitochondrial membrane (Stocco & Clark 1996). Linoleic acid can be converted to arachidonic acid that increases the expression of StAR (Wang *et al.* 2000). Wathes *et al.* (2007) suggested that arachidonic acid and its metabolites may indirectly affect the steroidogenic machinery via PGs. It was demonstrated by Wang *et al.* (2003) that inhibition of specific PGs endoperoxide synthase-2 (PTGS2) was associated with increased StAR expression and steroid output, which could be caused by a decrease in PGF_{2 α} that inhibits StAR protein expression. However, in the current study, we did not observe differences in P₄ concentrations and content between groups, which may not support this theory of elevated StAR activity. Elmes *et al.* (2004) and Cheng *et al.* (2005) reported that feeding ewes a diet with high C18:2 increased the *in vitro* and *in vivo* synthesis of PGE₂ in placental membranes. This finding indicated that C18:2 plays a role in regulation of PGs synthesis and thus could affect steroidogenesis. It was reported by Sarel & Widmaier (1995) that C18:2 stimulated the adrenal corticosterone synthesis, which demonstrated the effects of UFA on steroidogenesis in a variety of tissues. It was also suggested by Wathes *et al.* (2007) that UFA may alter the function of transcription factors and thus affect cellular enzymes that regulate PGs and steroids synthesis. Indeed, the expression of P450 aromatase mRNA was higher in granulosa cells of UFA cows compared with the control, which supports this theory. Although there is some evidence for the implication of UFA in steroidogenesis regulation, the mechanism behind this regulation is not clear and further research is required to elucidate this issue.

Preovulatory follicles from the HUFA group were larger and tended to have larger volume compared with

Table 6 Across treatments insulin and non-esterified fatty acids (NEFAs) in estradiol (E₂)-active and E₂-inactive follicles.

	E ₂ active	E ₂ inactive	S.E.M.
<i>n</i> , follicles	37	38	
Insulin (pg/ml)	91.97 ^a	21.64 ^b	11.92
Insulin content (pg)	117.55 ^a	41.12 ^b	28.43
NEFA (μ Eq/l)	206.81 ^b	682.72 ^a	36.94
NEFA content (μ Eq)	260.12 ^b	1280.18 ^a	194.7

^{a,b}Within rows, means with different superscript letters are statistically different ($P < 0.05$). NEFA, non-esterified fatty acids.

the control and LUFA follicles. There are some other reports in which increased diameter of preovulatory follicles were observed as a result of fat supplementation in dairy cows (Lucy *et al.* 1991, Moallem *et al.* 1999). It was shown by Bilby *et al.* (2006) that cows fed a diet enriched with C18:2 had larger follicles, which is in agreement with the current study.

Lower concentrations of A_4 and E_2 in preovulatory follicles of the LUFA cows than in the control was observed in the current study, with no differences in A_4 and E_2 contents. The E_2/P_4 ratio was also lower in LUFA than in control. Vanholder *et al.* (2005) showed that saturated FA had a toxic effect on bovine granulosa cell growth and function *in vitro*, and similar effects were observed in human granulosa cells (Mu *et al.* 2001). It was also reported by Mu *et al.* (2001) that supplementation of arachidonic acid antagonized the saturated FA-induced apoptosis in granulosa cells. Leroy *et al.* (2005) demonstrated that *in vitro* addition of saturated FA (C16:0 and C18:0) to oocytes during maturation had negative effects on maturation, fertilization, cleavage rate, and blastocysts yield. These findings indicate that saturated FA may have an adverse effect on granulosa cells, which might partly explain the lower concentrations of A_4 and E_2 in the preovulatory follicles of the LUFA cows. In the current study, we indeed found that the percentage of saturated FA in plasma was higher in the LUFA group than in the HUFA group, but not than that of the control. When examining the FA profile in FF, we also observed lower percentage of arachidonic acid (C20:4) in FF of LUFA than in control. As mentioned above, arachidonic acid has a positive effect on steroidogenesis and it might be that the LUFA follicles had insufficient arachidonic acid and consequently lower steroidogenic hormones compared with the control.

Cell culture studies demonstrated a dependence of bovine granulosa cells on the presence of physiological concentrations of insulin (Gutierrez *et al.* 1997). Armstrong *et al.* (2002) observed a correlation between circulating insulin concentrations and E_2 production in cultured granulosa cells. In the current study, we observed a tendency for higher content of insulin in HUFA follicles that had higher concentrations of E_2 compared with the control follicles. Insulin content was also positively correlated with P_4 , A_4 , and E_2 contents in FF. Our results confirm these previous findings in which insulin plays a significant role in follicle function.

Data from the present study also showed that the E_2 -inactive follicles had 3.3-fold higher concentration and 4.9-fold higher content of NEFA than E_2 -active follicles. The content of the FF is assumed to be derived from the vasculature in the surrounding thecal layers (Clarke *et al.* 2006). However, the NEFA concentration in plasma of cows at that stage of lactation (55–70 days post-calving) is generally lower than that observed in the E_2 -inactive follicles in the current study (Leroy *et al.* 2005). Moreover, Leroy *et al.* (2005) did not observe a constant

association between NEFA concentrations in plasma and FF. In advanced stages of atresia, the granulosa cells have degenerated, and the FF is filled with cellular debris (Van Wezel *et al.* 1999). Therefore, we suggest that the increase in NEFA observed in E_2 -inactive follicles may be due to the disintegration of granulosa cells, which causes a leakage of NEFA and other cellular content into the FF. However, when examining the E_2 -active follicles in the current study, we observed a positive correlation between NEFA and P_4 , A_4 , and E_2 contents, which indicates that NEFA is not necessarily a negative indicator for steroidogenesis potential of the follicle.

In conclusion, dietary UFA increased the diameter and tended to increase the volume of preovulatory follicle that were aspirated between 55 and 70 d *post partum*. No differences were observed between groups in P_4 concentrations and content in follicles, however, A_4 and E_2 concentrations and content were higher in cows that were supplemented with HUFA than control or LUFA. Higher P450 aromatase mRNA expression was also demonstrated in granulosa cells that were collected from the aspirated follicles. The findings of the current study indicate beneficial effects of dietary UFA on the preovulatory follicles size and steroidogenesis, although the mechanism is not fully clear.

Materials and Methods

Cows and treatments

The experimental protocol of the study was approved by the Volcani Center Animal Care Committee and was conducted at the Volcani Center experimental farm in Bet Dagan, Israel. The study was conducted from September to April to avoid heat stress effects. Forty-two multiparous Israeli-Holstein dry dairy cows, 249-day pregnant (average live body weight = 648 ± 8 kg), were group-housed in covered loose pens with adjacent outside yards, which were equipped with a real-time electronic individual feeding system. The cows were stratified into three groups on the basis of previous lactation milk and fat, parity, body weight, and body condition score. All treatments commenced *prepartum* at 256 days of pregnancy as follows: (1) Control were fed a dry cow diet and *post partum* were fed a lactating cow diet according to National Research Council (NRC 2001) recommendations ($n=14$); (2) LUFA were supplemented with 230 g/day per cow of a rumen-protected fat that contained a low proportion of UFAs (Energy Booster 100, Milk Specialties, Dundee, IL, USA), until 100 days *post partum* ($n=14$) and (3) HUFA were supplemented with 215 g/day per cow of a rumen-protected fat that contained a high proportion of UFAs (Megalac-R, Church and Dwight, Princeton, NJ, USA) until 100 days *post partum* ($n=14$). The composition and content of the pre- and postpartum diets are presented in Tables 7 and 8 respectively. The cows were fed *ad libitum* pre- and *post partum*. The FA profile of Energy Booster 100 was 28.2% palmitic acid (C16:0), 51.2% stearic acid (C18:0), 8.4% oleic acid (C18:1), 1.5% linoleic acid (C18:2), 0.1% linolenic acid (C18:3), and 10.6% other FAs (measured as a percentage of total FA). The FA profile of Megalac-R was 17.4%

Table 7 Ingredients and chemical composition of diets.

Ingredients	Treatments ^a		
	Control	LUFA	HUFA
Percentage of dry matter			
Corn grain ground	12.9	4.9	4.8
Barley grain	6.9	5.9	5.8
Rapeseed meal	0.4	0.3	0.3
Gluten meal	1.2	1.0	1.0
Soybean meal	1.9	1.6	1.6
Sunflower meal	0.7	0.6	0.6
Gluten feed	2.9	2.4	2.4
Cottonseed	1.7	1.5	1.5
Wheat silage	4.6	3.9	3.9
Corn silage	4.5	3.8	3.7
Dried distillers grain	1.4	1.2	1.2
Legume hay	0.8	0.7	0.7
Oats hay	59.0	69.6	70.0
Salt	0.5	0.4	0.4
Calcium bicarbonate	0.2	0.2	0.2
Vitamins and minerals	0.04	0.03	0.03
Supplements			
Energy Booster 100		1.9	
Megalac-R			1.7
Chemical composition			
NE _L , Mcal/kg	1.48	1.49	1.49
Crude protein	12.0	12.0	12.0
Ether extract	1.5	3.0	2.9
Crude NDF	45.0	48.0	49.0
Ca	0.6	0.6	0.8
P	0.3	0.3	0.3

NE_L, net energy for lactation; NDF, neutral detergent fiber.

^aTreatments were: cows fed from 256 days of pregnancy a dry cow ration and *post partum* fed a lactating cow diet (CTL) or supplemented with a fat either containing a low (LUFA) or high (HUFA) proportion of unsaturated fatty acids.

palmitic acid (C16:0), 2.1% stearic acid (C18:0), 33.6% oleic acid (C18:1), 30.5% linoleic acid (C18:2), 2.4% linolenic acid (C18:3), and 14% other FAs. The diets were formulated to be isoenergetic. The energy contents of the supplements that were used in this study were calculated according to the manufacturer's specifications, which were 6.0 and 6.6 NE_L Mcal/kg DM for Energy Booster 100 (LUFA) and Megalac-R (HUFA) respectively. Cows were milked thrice daily and milk production was recorded electronically (SAE, Kibbutz Afikim, Israel). The cows were weighed automatically thrice daily after each milking with a walking electronic scale. The individual daily energy balance was calculated as described previously (Moallem *et al.* 2007).

Follicular fluid aspiration

At 38–40 days *post partum*, ovaries of all cows were examined once for the presence of corpus luteum (CL) by ultrasonography (Scanner 200; Pie Medical, Maastricht, The Netherlands). Cows with ovaries that had a CL were injected with 2.5 ml PGF_{2α} analog (Estrumate; Coopers Animal Health Ltd, Berkhamsted, UK) to facilitate estrus. Cows without a CL were injected with 5 ml gonadotropin-releasing hormone analog (0.02 mg buserelin; Receptal, Intervet International B.V. Boxmeer, Holland) to induce ovulation of large follicles, followed 7 days later by injection of 2.5 ml PGF_{2α}. Cows that were observed for signs of

Table 8 Basal lactating cow diet ingredients and chemical composition.

Ingredients	Treatments ^a		
	Control	LUFA	HUFA
Percentage of dry matter			
Corn grain ground	18.0	15.3	15.4
Barley grain rolled	18.2	18.5	18.5
Rapeseed meal	1.0	1.0	1.0
Corn gluten meal	3.1	3.2	3.2
Soybean meal	5.0	5.1	5.2
Sunflower meal	1.7	1.8	1.8
Corn gluten feed	7.5	7.6	7.6
Cottonseed	4.6	4.7	4.7
Wheat silage	12.1	12.4	12.4
Corn silage	11.7	11.9	11.9
Dried distillers grain	3.8	3.9	3.9
Molasses	0.6	0.6	0.6
Vetch hay	2.1	2.2	2.2
Oats hay	8.6	8.8	8.8
Soybean oil	0.1	0.1	0.1
Salt	1.4	1.4	1.4
Calcium bicarbonate	0.5	0.5	0.5
Vitamins and minerals	0.1	0.1	0.1
Energy Booster 100		1.0	
Megalac-R			0.9
Chemical composition			
NE _L (Mcal/kg)	1.78	1.78	1.78
Crude protein	17.2	17.0	17.0
RUP	6.0	6.0	6.0
ADF	19.4	19.4	19.4
NDF	31.7	31.7	31.7
Ether extract	3.55	4.45	4.55
Ca	0.9	0.9	0.9
P	0.4	0.4	0.4

NE_L, net energy for lactation; RUP, rumen undegradable protein;

ADF, acid detergent fiber; NDF, neutral detergent fiber.

^aTreatments were: cows fed from 256 days of pregnancy a dry cow ration and *post partum* fed a lactating cow diet (CTL) or supplemented with a fat either containing a low (LUFA) or high (HUFA) proportion of unsaturated fatty acids.

estrus visually or by pedometers (Computerized Dairy Management Systems, SAE AFIKIM, Afikim, Israel), received 14–15 days later a second injection of 2.5 ml PGF_{2α} to cause luteolysis and to enable preovulatory follicular development. Forty-eight hours after the second PGF_{2α} injection, aspiration of FF was conducted using the ovum pick up procedure (Moallem *et al.* 1999). The FF aspirations were performed as follows: cows were sedated with an i.m. injection of 1 ml of 2% Rompun (XYL-M2 Veterinary, xylazine base 20 mg/ml; VMD, Arendonk, Belgium) and were given a local anesthesia of 5 ml of 2% lidocaine HCl (2% esracain, 200 mg/10 ml; Rafa Laboratories, Jerusalem, Israel) injected epidurally between the last sacral and first caudal vertebrae. Ovaries were examined and the diameters of the large follicles were measured; follicles ≥ 7 mm were aspirated. Each follicle was aspirated into a single tube, centrifuged, and the FF was stored at –18 °C until analysis. After the separation of the FF, 1 ml RNAlater (Sigma–Aldrich Inc.) was added to the residue of each tube and then they were frozen at –18 °C until determination of mRNA of P450 aromatase in the granulosa cells.

Blood samples for determination of P₄ were taken at the day of the second PGF_{2α} injection (48 h prior to follicular

aspiration) and at the day of follicle aspiration from the jugular vein into vacuum tubes with lithium heparin (Becton Dickinson Systems, Cowley, England). Plasma was separated immediately from blood samples and stored at -18°C until analysis.

Chemical analysis

Total mixed rations were sampled weekly and dry matter, crude protein, neutral detergent fiber (NDF), acid detergent fiber (ADF), calcium, and phosphate were determined. Feed samples were dried at 65°C for 24 h and then ground to pass through 1.0 mm screen (Retsch S-M-100). The ground samples were dried at 100°C for 24 h and analyzed for N (AOAC, 1990; method 984.13; Kjeltac Auto 1030 Analyzer, Tecator, Hoganas, Sweden), Ca (AOAC, 1990; method 935.13), P (AOAC, 1990; method 964.06), and NDF and ADF contents were determined with Ankom equipment (Ankom Technology, Fairport, NY, USA; NDF, using α -amylase and sodium sulfite). Net energy for lactation (NE_L) values was calculated using the NRC (2001) values except for the fat supplements. The rumen undegradable protein (RUP) values of most of the feedstuffs were from Arieli *et al.* (1989). The NRC (2001) RUP values were used for feedstuffs that were not examined in Arieli *et al.* (1989).

Concentrations of P_4 and E_2 in FF, and plasma P_4 were determined by RIA (Diagnostic Products, Los Angeles, CA, USA) as well as FF A_4 concentrations (Diagnostic Systems Laboratories, Webster, TX, USA). Concentrations of non-esterified FA (NEFA) in FF were determined by a NEFA kit (Wako NEFA C test kit; Wako Chemicals GmbH, Neuss, Germany). Concentrations of insulin in FF were determined by RIA (Diagnostic Products).

To determine the concentrations of plasma E_2 , 1 ml plasma samples were extracted with diethyl ether (HPLC, Bio Lab Ltd, Jerusalem, Israel) and then E_2 was determined by RIA kit (Third Generation Estradiol kit, DSL-39100; Diagnostic Systems Laboratories).

FAs in plasma and FF were extracted (Moallem *et al.* 1999) and analysis of FA was performed with 5890 series 2 gas chromatograph (Hewlett-Packard) equipped with a capillary column (30 m $\times$ 0.53 mm, 0.5 μm ; Agilent Technologies, Santa Clara, CA, USA) and an FID detector. The column was maintained at 160°C isothermal. Nitrogen was used as carrier gas with a linear velocity of 22 cm/s; injection volume was 2 μl . The injection port was maintained at 230°C and the detector at 235°C . Detector air flow was 400 ml/min and the hydrogen flow was 33 ml/min. A known amount of C17:0 was added to the samples prior to extraction in order to determine the FA concentrations in the FF.

P450 aromatase mRNA expression in granulosa cells

Total RNA isolation

Total RNA was isolated from granulosa cells obtained from the aspirated preovulatory follicles using TRI reagent (10 ml/g tissue) according to the manufacturer's protocol (MRC

Molecular Research Center, Cincinnati, OH, USA). RNA quality and quantity were assessed by spectrophotometric measurements at 260 and 280 nm. Only high-purity (260–280 ratio between 1.75 and 2) RNA was used.

mRNA analysis

First-strand cDNAs were synthesized from 5 μg total RNA from each follicle using oligo(dT)₁₈ as the primer in the presence of MLV reverse transcriptase (Fermentas Inc., Hanover, MD, USA) for 1 h at 42°C . The cDNA was purified from the PCR mix using High Pure PCR Product Purification kit (Roche Diagnostics GmbH).

Real-time PCR

Expression of P450 aromatase mRNA in preovulatory follicles was determined by real-time PCR carried out following the manufacturer's specifications using a GeneAmp 5700 sequence detection system (Applied Biosystems, Foster City, CA, USA). mRNA samples were reverse transcribed as described in the mRNA analysis section and 5 μl cDNA was used in 50 μl final volume for the PCR. cDNA was amplified by qPCR Mastermix for SYBR Green I, using primers designed by the Primer Express version 2.0.0 software (Applied Biosystems) as follows: forward 3'-GCCAAGAGCAACAAGCA-TATCAG-5' and reverse 3'-CTTGGAAAATTCATCATCTGTTGA-5'.

Relative mRNA expression of P450 aromatase was determined by the $\Delta\Delta C_T$ quantification method as described previously by Livak & Schmittgen (2001), using the relative expression of GAPDH mRNA as a reference. C_T stands for the threshold cycle, that is, the PCR cycle in which an increase in reporter fluorescence above a baseline signal can first be detected.

Statistical analysis

The continuous variables (milk and dry matter intake) were analyzed as repeated measurements using Proc Mixed of SAS software (version 8.1, SAS User's Guide 2000). The final model used was

$$Y_{ijklm} = \mu + T_i + L_j + C(T \times L)_{ijk} + \text{DIM}_{ijkl} + \text{DIM}_{ijkl} \times \text{DIM}_{ijkl} + \text{DIM}_{ijkl} \times \text{DIM}_{ijkl} \times \text{DIM}_{ijkl} + E_{ijklm}$$

where μ =overall mean, T_i =treatment effect, $i=1-3$, L_j =parity, $j=2$ or >2 , $C(T \times L)_{ijk}$ =cow_k nested in treatment_i and cow nested in parity_j, DIM_{ijkl} =day in milk as continuous variable, and e_{ijklm} =random residual. Whenever the quadratic or cubic effects were not significant, they were excluded from the model and the model was rerun. Milk production was analyzed using the previous lactation data as co-variable. Dry matter intake was analyzed using the pre-treatment body weight as co-variable. The follicle diameter, volume, hormones, NEFA, and FA profile and concentrations were

analyzed using the general linear models procedure of SAS (2000). Across treatments correlations analysis were performed using the Proc REG procedure of SAS software (2000). The analysis of mRNA expression was carried out using the JMP (version 5.0.1, SAS Institute, Cary, NC, USA). Gene expression levels were compared using one-way ANOVA.

Least squares means and adjusted s.e.m. are presented in the tables and $P < 0.05$ was accepted as significant unless otherwise stated.

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