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Impact of linoleic acid on bovine oocyte maturation and embryo development

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Abstract

Linoleic acid (LA; 18:2 *n-6*) is the most abundant fatty acid in bovine follicular fluid, and it was previously reported that LA concentration significantly decreases when follicle size increases. This suggests that LA may have a role in the regulation of oocyte maturation. The present study investigated the effect of LA supplementation on bovine oocyte maturation and early embryo development *in vitro*. Treatment of cumulus–oocyte complexes (COCs) with LA significantly inhibited cumulus cell expansion and retarded development of the oocytes to the metaphase II (MII) stage in a dose-dependent manner. This effect was reversible, and the oocytes developed to the MII stage after extended culture in the absence of LA. Treatment of COCs with LA also resulted in a significantly lower percentage of cleaved embryos and blastocyst yield. Furthermore, COCs treated with LA had significant effects compared with controls in i) increasing prostaglandin E_2 concentration in the medium, ii) decreasing intracellular cAMP at 6 and 24 h of maturation and iii) decreasing phosphorylation of the MAPK1 and 3 at 24 h, and AKT at 6 h of maturation. In conclusion, LA supplementation to bovine oocytes during maturation altered the molecular mechanisms regulating oocyte maturation and resulted in decreased percentage of oocytes at MII stage and inhibition of the subsequent early embryo development. These data provide evidence for adverse effects of LA on oocyte development, which can be associated with dietary increased level of LA in the follicular fluid and the decline in fertility in farm animals and human.
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Introduction

Dairy cow fertility has declined in recent decades in part due to continuous selection for high milk production traits, which has promoted a state of negative energy balance (NEB) during the postpartum period leading to a reduction in fertility. Manipulation of the dietary fat content has been used in research and practice aiming to correct the energy balance defect and restore reproductive function. Different types of dietary fats were shown to have differential effect on reproductive function in cattle (see Wathes *et al.* (2007) for review).

In cattle, the effects on reproductive performance by manipulations of dietary polyunsaturated fatty acids (PUFAs) type and content are inconsistent among different studies. In some studies, *n-6* FAs (such as linoleic acid (LA; C18:2 *n-6*)) had a negative effect on fertility compared with *n-3* FAs (such as α-linolenic acid (ALA; C18:3 *n-3*); see Santos *et al.* (2008) for review). In lactating Holstein cows, feeding *n-6* enriched diet resulted in smaller mean diameter of ovulatory follicles and subsequent mean size of corpus luteum, decreased plasma progesterone concentration, lowered conception rate and increased embryo mortality compared with cows fed *n-3* supplemented diets (Petit *et al.* 2001, Ambrose *et al.* 2006, Petit & Twagiramungu 2006). Although both ALA- and LA-enriched diets had no effect on oocyte morphology, cleavage or development when compared with monounsaturated fatty acid diets (Bilby *et al.* 2006), and resulted in lower cleavage rate compared with rumen-inert fats (Fouladi-Nashta *et al.* 2009), they could improve blastocyst quality when compared with palmitic and stearic acids (Thangavelu *et al.* 2007). These inconsistent effects of FAs on oocyte development among different studies resulting from differences in the metabolic status of the experimental animals (Adamiak *et al.* 2006), season (Zeron *et al.* 2001), or other factors make it difficult to dissociate and understand the differential effect of fatty acids on oocyte development. More importantly, in all these studies, oocytes were collected by ovum pickup mainly from small (<3 mm in diameter) and medium-sized (4–10 mm in diameter) ovarian follicles, which do not reflect the microenvironment of preovulatory follicles. Therefore, using *in vivo* collected oocytes, it will not be possible to provide direct evidence for the effects of PUFAs on oocyte maturation and development.

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The effect of dietary PUFAs on reproduction may be partly mediated by changing the microenvironment of the follicular fluid surrounding the oocytes, since the fatty acid profile of the follicular fluid is correlated with the dietary fatty acid type and content (Childs *et al*. 2008, Fouladi-Nashta *et al*. 2009). However, cumulus–oocyte complexes (COCs) maintain their FA composition by selective uptake of saturated FAs at the expense of PUFAs supplemented in diet (Adamiak *et al*. 2006, Fouladi-Nashta *et al*. 2009). Therefore, dietary supplementation of PUFAs does not alter the concentration of relevant PUFAs in the oocytes. Alteration in the FA composition in the follicular fluid, the microenvironment of the oocyte during growth and maturation, may mediate the effects of PUFAs on oocyte development. These changes can influence the level of cumulus cell expansion and the timing of resumption of nuclear maturation in the oocytes, all of which are critical for oocyte development after fertilization. In cattle, high follicular fluid concentrations of palmitic (C16:0) and stearic acids (C18:0) observed in cows during NEB had a negative effect on oocyte maturation, fertilization and cleavage rate, and blastocyst yield when added *in vitro* (Leroy *et al*. 2005). Therefore, dietary FAs may have a direct effect on the oocyte maturation and subsequent early embryonic development.

Both LA and ALA fatty acids are found in the plasma and follicular fluid (Childs *et al*. 2008). We have recently reported that supplementation of bovine oocytes with ALA during *in vitro* maturation resulted in an increased maturation rate and a higher blastocyst yield and production of better quality blastocysts (Marei *et al*. 2009). On the other hand, little is known about the effect of LA on oocyte development. Analysis of the fatty acid content of the bovine follicular fluid collected from different sized follicles showed a significant decrease in the concentration of LA in large follicles as compared with small follicles, whereas no significant difference was observed in ALA concentration (Homa & Brown 1992). This suggests that the physiological decrease in LA concentration in the follicular fluid may be important to oocyte maturation, and that LA may have a role in controlling oocyte maturation.

Bovine oocytes are arrested at the prophase of first meiotic division (germinal vesicle, GV) prior to the surge of LH and development to the metaphase II (MII) stage. Resumption of oocyte nuclear maturation *in vivo* or *in vitro* is controlled by a complex molecular signalling mechanism involving activation of maturation-promoting factor (MPF; Masui & Markert 1971). Many pathways interact to control this process including cAMP-dependent pathways (Dekel 1988, Homa 1988, Downs *et al*. 2002) and MAPKs (Kubelka *et al*. 2000, Tian *et al*. 2002, Ye *et al*. 2003, Fan & Sun 2004). Moreover, activation of AKT (protein kinase B) was found to stimulate transition from metaphase I (MI) to MII stage (Tomek *et al*. 2002). Prostaglandin E_2 (PGE_2) is also considered as a critical mediator of oocyte maturation (Neal *et al*. 1975, Hizaki *et al*. 1999).

The present study aimed to investigate the effect of LA on oocyte maturation. Changes in important molecular events which occur during maturation, namely intracellular cAMP (cAMPi) accumulation, and MAPK1, MAPK3 and AKT phosphorylation in the COCs and production of PGs by oocytes were monitored to investigate the possible mechanisms of action of LA. Subsequent early embryonic development of LA-treated oocytes was also investigated.

Results

Oocyte nuclear maturation and cumulus cell expansion

Supplementation of COCs with different concentrations of LA (50, 100 and 200 μM) for 24 h resulted in a dose-dependent decrease ($P<0.01$) in the percentages of COCs showing full expansion (66±6, 27±4 and 2±2 respectively versus 93±2 in control; Fig. 1) and the percentage of oocytes at MII stage (80±2, 63±3 and 42±7 respectively versus 87±2 in control; Fig. 2). The remainder of the oocytes treated with 100 μM LA were found at telophase (29%), anaphase I (3%), MI (2%) and GV breakdown (GVBD) (3%) stages.

When COCs were initially treated with LA for 24 h and were then washed and transferred to LA-free maturation media, a significant increase ($P<0.05$) in the percentage of oocytes at MII stage was achieved after a further 24-h culture (77±4) to reach a similar maturation rate to the control group (84±2; $P>0.05$; Fig. 3). In addition, when COCs were initially treated with LA for only 6 h and were then transferred to LA-free maturation media for 18 h, no significant difference was detected in maturation rate (85±6) compared with the control group (89±4).

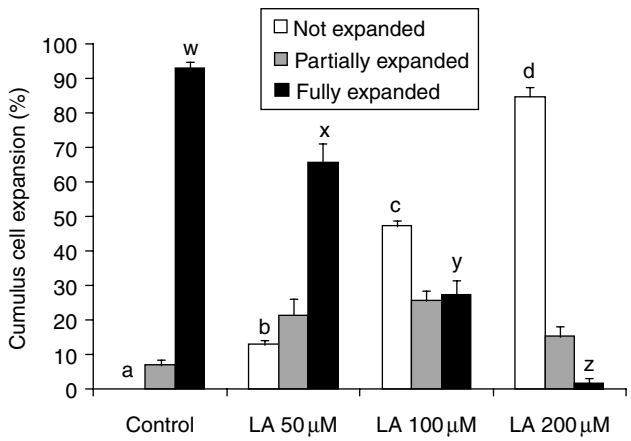

Figure 1 The effect of LA supplementation to the oocyte maturation media on the degree of cumulus expansion after 24 h. Data are shown as percentages ±S.E.M. from five independent experiments. Bars with different superscripts (a–d or w–z) are significantly different ($P<0.01$).

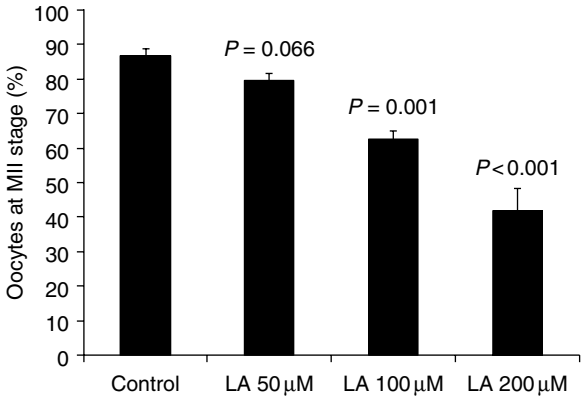

Figure 2 The effect of LA on the percentage of the oocytes in MII stage after 24-h maturation. Data are shown as percentages ±S.E.M. from five independent experiments. *P* values are shown in comparison with the control group.

PG synthesis

Supplementation of oocyte maturation medium with LA at all concentrations did not have any significant effect on the production of $PGF_{2\alpha}$ by COCs as measured in the spent media (Fig. 4A and B). However, supplementation of the COCs with 100 μM LA resulted in a significant increase in the concentration of PGE_2 ($P=0.016$) and PGE_2:$PGF_{2\alpha}$ ratio ($P=0.019$).

Intracellular cAMPi concentration in cumulus cells and oocytes

The cAMPi was measured in intact COCs or in the oocytes after removal of cumulus cells (denuded oocytes; DO) at 0, 6 and 24 h of maturation. The average cAMPi was 1.8±0.1 fmol/COC and 0.2±0.1 fmol/DO at 0 h. As shown in Fig. 5A, LA did not affect the cAMPi level ($P>0.05$) in the DO. In the COCs, cAMPi increased in the control group to a level of 10±3.4 fmol/COC after 6 h and then decreased to a level of 8±1.0 fmol/COC after 24 h. The increase in cAMPi in the LA-treated COCs was significantly lower than the control group at 6 h (7.7±3.2 fmol/COC; $P=0.03$), and at 24 h (2±0.7 fmol/COC; $P=0.005$; Fig. 5B).

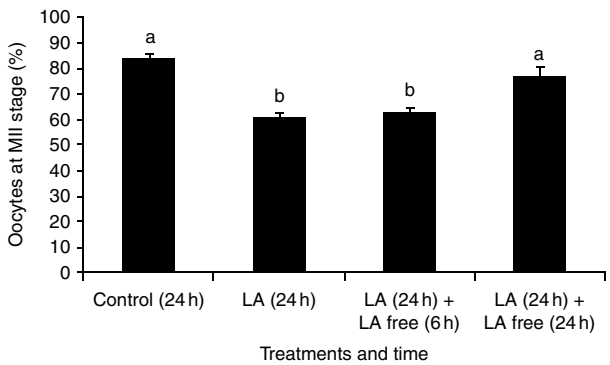

Figure 3 Oocyte nuclear maturation in LA-free maturation media for 6 and 24 h after initial treatment with LA for 24 h. Data are as percentages ±S.E.M. from three independent experiments. Bars with different superscripts are significantly different ($P<0.05$).

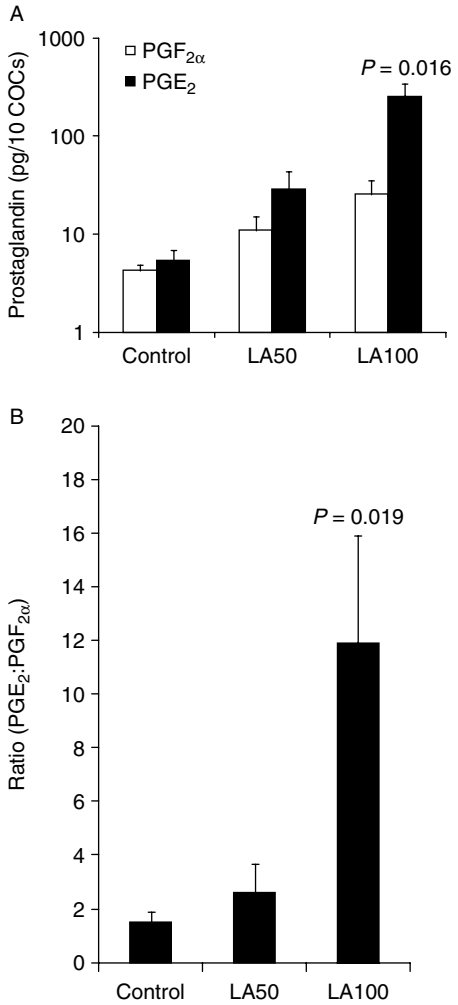

Figure 4 $PGF_{2\alpha}$ and PGE_2 concentration (A) and PGE:PGF ratio (B) in the spent media after maturation for 24 h in the presence of LA (0, 50 and 100 μM). Data are shown as mean±S.E.M. from five independent experiments. *P* values shown as compared with the control group.

MAPK1 and MAPK3 activation

Figure 6A represents western blotting of phosphorylated and total MAPK1 and MAPK3 in COCs supplemented with 0 or 100 μM LA for 0, 3, 6 and 24 h. The immunoreactive bands from four independent experiments were quantified, and the percentages of phosphorylated/total MAPK1 and MAPK3 were analysed (Fig. 6B and C). LA-treated oocytes had a significant decrease in the phosphorylated MAPK1 and MAPK3 percentages at 24 h. No significant differences were noticed in the first 6 h of maturation.

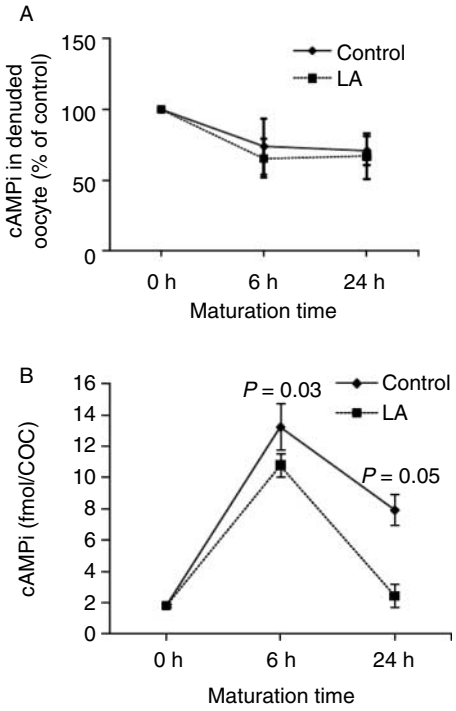

Figure 5 Concentration of cAMPi in (A) DOs and (B) COCs at 0, 6 and 24 h of maturation in control and LA (100 μM)-treated groups. Data are shown as mean±S.E.M. from three independent experiments.

AKT phosphorylation

Western blots of phosphorylated and total AKT (Fig. 7A) from COCs treated with 0 or 100 μM LA were analysed by densitometric quantification of the immunoreactive bands from four independent experiments. The percentage of phosphorylated/total AKT is shown in Fig. 7B. In the control group, LA supplemented COCs tended to have lower AKT phosphorylation with a significant effect at 6 h ($P=0.046$). No significant difference was noticed at 24 h.

Cleavage and embryo development

Oocytes matured in the presence of 100 μM LA had significantly lower cleavage (58±2.8 vs 74±3.9%) and blastocyst rates as a percentage from total cleaved (8±4.0 vs 33±4.0%) compared with control ($P\leq0.001$). Although treatment of the oocytes with 50 μM failed to achieve a statistically significant effect on cleavage rate (63±6.2; $P=0.08$), lower blastocyst/cleaved rate (12±6.9%; $P=0.007$) was observed as compared with control.

Discussion

The main objective of this study was to determine the effect of *n-6* PUFA supplementation with LA to the oocyte maturation media on the developmental potential of bovine oocytes *in vitro*. LA at 100 μM inhibited oocyte nuclear maturation and cumulus expansion. This effect was associated with higher PGE_2 synthesis, lower cAMPi concentrations and lower MAPK1, MAPK3 and AKT phosphorylation in the COCs, and was also reflected in a subsequent decrease in embryo development.

LA is the most abundant fatty acid constituting about 30% of the total fatty acids of the follicular fluid.

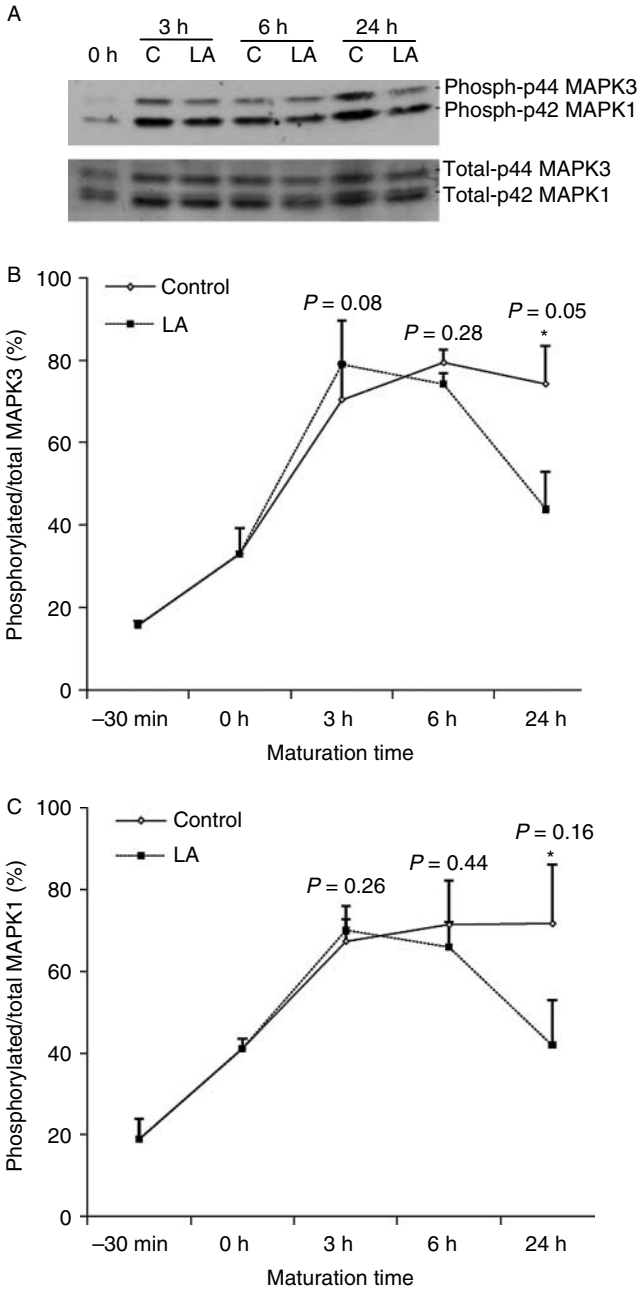

Figure 6 (A) Western blotting of phosphorylated and total MAPK3 and MAPK1 at 0, 3, 6 and 24 h of maturation in the presence or in the absence of 100 μM LA. (B and C) The percentage ±S.E.M. of phosphorylated/total MAPK3 and MAPK1. Data are from four independent experiments (15 COCs per treatment/time point per experiment).

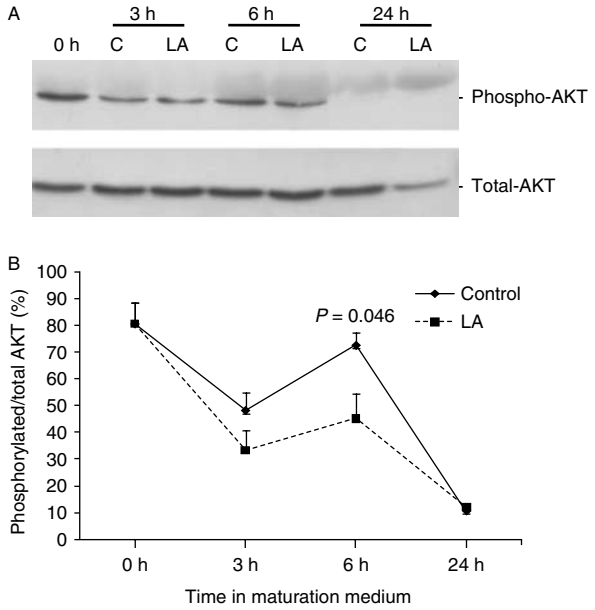

Figure 7 The effect of LA (100 μM) on AKT phosphorylation. (A) Representative immunoreactive bands from western blotting phosphorylated and total AKT. (B) The percentage ±S.E.M. of phosphorylated/total AKT. Data are from four independent experiments (15 COCs per treatment/time point per experiment). *P* value is shown as compared with the control group.

It was previously reported that the LA concentration significantly decreases as the follicle size increases (Homa & Brown 1992). This suggests that LA may have a role in the regulation of oocyte maturation, and that high LA concentration can be a factor contributing to the arrest of the oocyte at GV stage. In addition, LA concentration in the follicular fluid is associated with *n-6* fatty acids content of the diet (Fouladi-Nashta *et al.* 2009), and may alter the oocyte maturation process and affect its further development. In cattle, the concentration of LA was reported to be 0.118–0.475 mg/ml (670–1629 μM) in plasma and 0.02–0.2 mg/ml (71–710 μM) in follicular fluid depending on the FA content of the diet (Adamiak *et al.* 2006, Childs *et al.* 2008). In the present study, the concentrations of LA used were in the range from 0 to 200 μM and thus reflected the normal physiological condition.

Results of the experiment 2 showed that supplementation of the serum-free maturation media with LA (100 μM or more) significantly inhibited oocyte nuclear maturation. Similar results were reported previously, where LA significantly inhibited GVBD (35% in LA versus 81% in control; Homa & Brown 1992). This inhibition of oocyte nuclear maturation was proved to be reversible after extended culture of the oocytes in the absence of LA for either 6 or 24 h. In addition, treatment of the oocytes with LA in the later stage of culture did not have any effect on oocyte maturation. These findings indicate that the effect of LA occurs at the early stages of culture prior to GVBD, which influence its meiotic progression towards MII stage. In addition, it suggests a non-toxic and specific effect of LA in the regulation of oocyte nuclear maturation to MII stage.

Fatty acids are necessary components of cell membranes, and they are evident for diverse effects on different cultured cells. One of these effects can be caused by altering the membrane fluidity and thus affecting any process that is mediated via the membrane (Grammatikos *et al.* 1994*a*). In this context, inhibition of oocyte maturation in LA-treated COCs may be mediated by altering the cumulus cell membrane response to gonadotrophin stimulation.

Cumulus cell expansion is an important marker for oocyte maturation, and is also induced by gonadotrophin stimulation *in vivo* or *in vitro* leading to massive production of mucoid extracellular matrix protein (mainly hyaluronan; Chen *et al.* 1990). At least in pigs, volumetric expansion of the COCs correlates with the outcome of oocyte maturation (Qian *et al.* 2003), where hyaluronan, through the effect on its receptor CD44, regulates the disruption of gap junctions in the COCs and concurrently controls the incidence of meiotic resumption (Sato & Yokoo 2005). However, in cattle, inhibition of cumulus cell expansion was shown to be independent from nuclear maturation, but essential for fertilization and subsequent cleavage and blastocyst development (Gutnisky *et al.* 2007). Thus, inhibition of cumulus cell expansion observed in the study presented here may contribute to the reduced developmental potential of these oocytes.

Through conversion to arachidonic acid by delta5- and delta6-fatty acid desaturases (FADS1 and FADS2) and elongase enzymes, LA is a precursor of two-series PGs (Abayasekara & Wathes 1999). The ability of different types of cells to elongate and desaturate *n-6* and *n-3* fatty acids is diverse (Grammatikos *et al.* 1994*b*). However, these enzymes have been shown to be expressed in cumulus cells, and are involved in follicular growth and oocyte competence in rat (Moreau *et al.* 2006), human (Feuerstein *et al.* 2007) and zebrafish (Ishak *et al.* 2008). In the mouse, deletion of *Fads2* gene expression impaired the gap junction network between granulosa cells of ovarian follicles and resulted in sterility (Stoffel *et al.* 2008).

In the study presented here, LA supplementation (100 μM) resulted in a marked increase in the production of PGE_2 and $PGF_{2\alpha}$ in the spent media with an increased PGE:PGF ratio. This suggests the ability of bovine cumulus cells to metabolize LA and produce PGs. These results are in accordance with a previous study using endometrial cells of late gestation ewes, where the PGE_2:$PGF_{2\alpha}$ ratio increased 2–3 times when cells were supplemented with the linoleic, γ-linolenic or arachidonic acid (Cheng *et al.* 2004). PGE is an autocrine/paracrine mediator of oocyte maturation and cumulus expansion. It acts through G-protein coupled receptors (PTGER) on cumulus cells (Calder *et al.* 2001).

mRNA for PTGS2 and PGE_2 receptors (*PTGER2* and *PTGER3*) are expressed in bovine COCs (Nuttinck *et al.* 2002, 2008), and PGE_2 was also shown to induce moderate expansion of cumulus cells (Calder *et al.* 2001). This suggests that PGE may have a role in oocyte maturation. In the studies presented here, increased PGE_2 production could not overcome the inhibitory effect of LA on cumulus cell expansion and oocyte maturation. However, we have recently shown that a less marked increase in PGE_2 ($233 \pm 41\%$ as a percentage of control) observed in the spent media of COCs matured in the presence of ALA indirectly mediated the stimulatory effect of ALA on oocyte nuclear maturation (Marei *et al.* 2009). On the contrary, very high levels of PGE_2 ($>10\times$ compared with control) produced from LA-treated COCs may have opposite effects on oocytes maturation.

The resumption of oocyte meiotic maturation and GVBD after gonadotrophin stimulation is mainly mediated through increasing cAMPi concentrations in the cumulus cells (Dekel 1988), but not in the oocytes which lack gonadotrophin receptors. PGE receptors also require an intracellular increase in cAMP as a second messenger (Coleman *et al.* 1994). In the current study, the cAMPi was measured in COCs and DO matured in the presence or in the absence of LA. In the control group, peak level of cAMPi was observed in COCs at 6-h maturation, followed by a decrease in cAMPi at 24 h. In the DO, the opposite was noticed, where cAMP level dropped after 6 h of maturation. A similar pattern of cAMP on bovine COCs was previously reported (Luciano *et al.* 2004), where a peak of cAMP was observed between 3 and 7 h. This diverse pattern of changes in cAMP levels is necessary for maturation, and results from a selective regulation of phosphodiestrase enzyme (PDE) through the inhibition of PDE type 4 in granulosa cells and activation of PDE type 3 in the oocyte (Tsafriri *et al.* 1996, Nogueira *et al.* 2003).

The present study showed that LA-treated COCs had significantly lower levels of cAMPi at 6 h. High levels of cAMPi are necessary for good quality oocyte maturation. It was previously reported that low cAMPi in bovine COCs resulted in premature interruption of cumulus–oocyte gap junction communication and inhibition of cumulus cell expansion, leading to defective oocyte maturation and development (Modina *et al.* 2001). These findings suggest that the lower cAMPi in LA-treated COCs may have a major role in mediating the inhibitory effect on oocyte maturation and cumulus expansion. The mechanism by which LA decreases cAMPi level in COCs needs more investigation; however, it might be through altering the adenylate cyclase activity in the cumulus cell membranes. LA did not have any significant effect on cAMPi in the DO.

Phosphorylation of MAPK in cumulus cells is an essential mediator for gonadotrophins during meiotic resumption and cumulus cell expansion (Kubelka *et al.* 2000, Su *et al.* 2002, 2003, Ye *et al.* 2003, Fan & Sun 2004, Fan *et al.* 2009). In bovine oocytes, MAPK activation occurs during the peri-GVBD (6 h) stage, and is associated with MPF activation and reaches its maximum level at 24 h of maturation (Wehrend & Meinecke 2001). In the present study, in the control group, MAPK1 and MAPK3 (the main two types of MAPKs in COCs) followed the same pattern. LA did not have any significant effect on MAPK phosphorylation during the first 6 h of maturation, but significantly decreased the level of phosphorylated MAPK1 and MAPK3 at 24 h. Sustained high levels of MAPK activity at 24 h are essential for maintaining the oocyte in MII arrest because of their involvement in the regulation of microtubule organization and meiotic spindle assembly (Tian *et al.* 2002) and its inactivation after that is a prerequisite for pronuclear formation after fertilization (Fan & Sun 2004). Thus, low levels of MAPK phosphorylation at 24 h in the LA-treated group may cause of failure of the chromosomes to arrange in MII stage.

AKT is an important protein kinase which stimulates transition of bovine oocytes from MI to MII stage (Tomek & Smiljakovic 2005). Analysis of AKT phosphorylation in COCs showed that AKT is abundant in the COCs at all stages of oocyte maturation with increased phosphorylation status around the time of GVBD and MI stages (Tomek & Smiljakovic 2005). In the present study, LA resulted in significantly lower level of AKT phosphorylation at 6 h, which may mediate its action in inhibition of oocyte maturation by blocking transition from MI to MII stage. AKT is also important regulator of translation and protein synthesis in bovine oocytes (Tomek *et al.* 2002). Inhibition of AKT can result in decreased protein accumulation, and have a negative impact on the further developmental potential of these oocytes, which is the case in the LA-treated group.

The quality of oocyte maturation is determinant for its developmental potential after fertilization. The mRNAs and proteins acquired by the oocyte during its growth and final maturation allow the zygote to go through the early stages of embryo development up until the moment when the embryo produces these factors on its own (Vigneault *et al.* 2004). In the present study, maturation of oocytes in the presence of LA at 50 μM (which has no significant inhibitory effect on oocyte maturation and cumulus expansion) and 100 μM significantly decreased the cleavage and blastocyst rate. In a previous study, similar effects were reported when mouse pronuclear and two-cell stages were treated by LA *in vitro* (Nonogaki *et al.* 1994). These authors also showed that the addition of antioxidants to embryo culture media could attenuate the rate of developmental blockage, suggesting that the effect was caused by damage of the cells by lipid peroxidation. However, in the present study, LA was not added to the embryo culture media, and therefore, the negative effects of LA observed is carried over from oocyte maturation stage.

In conclusion, LA supplementation in oocyte maturation medium can induce molecular changes associated with oocyte maturation leading to decreased oocyte developmental potential. This may also be of importance to cattle fertility, given that many housed animals receive feed with a higher *n-6* content than would be obtained from a more natural grass-based diet.

Materials and Methods

Chemicals and reagents

All chemicals and reagents were purchased from Sigma Chemical Company unless otherwise stated.

Experimental design

Water soluble LA (10 mM stock solution) was added to the oocytes in serum-free maturation medium (M-199) supplemented with fatty acid-free BSA as a carrier. In this study, a total of 2025 COCs (grade 1; with homogenous ooplasm and more than four complete layers of cumulus cells) were used.

In experiment 1, to study the effect of increasing concentrations of LA on oocyte nuclear maturation, cumulus cell expansion, and PG synthesis, a total of 600 COCs were used in five independent repeats, allocating about 25–30 oocytes per treatment (0 μM (Control), 50, 100 and 200 μM LA). After 24 h of culture, cumulus cell expansion was recorded, and oocytes were fixed and stained to assess the stage of nuclear maturation. Spent media were collected for PG assay. A proportion of the oocytes were also labelled with TUNEL to assess apoptosis in cumulus cells (method described below). Based on oocyte maturation results obtained, LA was used at a concentration of 100 μM in all future experiments unless otherwise stated.

Experiment 2 aimed to rule out the non-specific and toxic effect of LA on oocyte maturation by assessing nuclear maturation of the oocytes after extended culture in the absence of LA. In Experiment 2A, COCs were incubated for an additional period of 6 or 24 h in LA-free medium after initial treatment with 100 μM LA for 24 h. A total of 260 COCs were used in three independent repeats, allocating about 20 COCs per treatment per time point. In Experiment 2B, COCs were incubated for 18 h in LA-free medium after initial treatment with 100 μM LA for 6 h. A total of 120 COCs were used in three independent repeats, allocating 20 COCs per treatment. At each time point, a proportion of COCs were randomly taken from the culture to determine the stage of nuclear maturation by aceto-orcein staining.

Experiment 3 was designed to determine the effect of LA on cAMPi in COCs and DOs. A total of 630 COCs were cultured in three independent repeats, allocating 10 COCs or 25 DOs per treatment (0 μM (Control) and 100 μM LA) per time point (0, 6 and 24 h). cAMPi was then measured by EIA.

Experiment 4 investigated the effect of LA on MAPK1, MAPK3 and AKT phosphorylation. A total of 480 COCs were used in four independent repeats, allocating 15 COCs per treatment (0 and 100 μM LA) per time point (0, 1, 3, 6 and 24 h). Changes in total and phosphorylated MAPK1, MAPK3 and AKT were then assessed by western blotting.

Finally, in experiment 5, to study the effect of LA supplementation in maturation media on early embryo development, a total of 315 COCs were used in three independent repeats, allocating at least 30 COCs per treatment (0, 50 and 100 μM LA). All oocytes were fertilized as described below. Cleavage rate was recorded on day 2 (4-plus-cell embryos), and blastocysts were counted on day 8.

Collection of oocytes

Bovine ovaries were collected from a local abattoir and transported to the laboratory in a thermos container in PBS at 37 °C within 2 h after killing, and they were washed in fresh PBS immediately after arrival. COCs were retrieved from antral follicles 3–8 mm in diameter with a 19-gauge needle mounted on a 10-ml syringe. The grade 1 COCs were selected under a stereomicroscope and washed two times in M-199 supplemented with 20 mM HEPES and 0.4% (w/v) BSA.

In vitro *maturation*

Selected COCs were cultured in four-well dishes (NUNC, Thermo Fisher Scientific, Loughborough, Leicestershire, UK) containing 20 μl/COC serum-free maturation medium (M-199 supplemented with 0.6% (w/v) fatty acid-free BSA, 10 μg/ml FSH (Follitropin; Bioniche Animal Health, Belleville, Ontario, Canada), 10 μg/ml LH (Leutropin; Bioniche Animal Health), 1 μg/ml oestradiol and 50 μg/ml gentamycin). COCs were incubated for 24 h at 38.5 °C under 5% CO_2 in humidified air.

Assessment of cumulus cell expansion

The degree of cumulus expansion was assessed under a stereomicroscope after 24-h maturation subjectively as not expanded, partially expanded (the outer layer of cells was loosened) or fully expanded (all cumulus cells were loosened).

Oocyte staining and determination of the stage of nuclear maturation

To assess the stage of nuclear maturation at the end of the maturation time, oocytes were stained with aceto-orcein staining as described previously (Marei *et al.* 2009). Briefly, DO were overlaid with a coverslip supported by four droplets of a vaseline/paraffin mixture (40:1) and placed in acetic acid:methanol fixative (1:3 v/v) for at least 48 h. Oocytes were then stained with aceto-orcein (1% orcein in 45% acetic acid) and examined under phase contrast microscopy (Leica, Milton Keynes, UK). The stage of nuclear maturation was determined according to the morphology of the nuclear material.

PGE_2 and $PGF_{2\alpha}$ RIAs

Spent maturation media were collected at 24 h of culture and kept at −20 °C awaiting assay. Concentrations of PGE_2 and $PGF_{2\alpha}$ were quantified using charcoal–dextran-coated RIA

methods as described previously (Cheng *et al*. 2001). Briefly, for the PGE_2 assay, standards (range 0.08–10 ng/ml) or samples were mixed with anti-PGE_2 serum (a kind gift from Dr N L Poyser; University of Edinburgh, Edinburgh, UK), and tritiated tracer ([5, 6, 8, 11, 12, 14, 15 (n)-3H]-PGE_2; Amersham International plc, Amersham) in duplicate. After overnight incubation at 4 °C, dextran-coated charcoal suspension containing 0.4% dextran (T-70; Amersham Pharmacia Biotech) and 2% neutralized charcoal was added to all tubes except the total count. They were incubated at 4 °C for 10 min and centrifuged at 2000 ***g*** for 10 min. The supernatant was removed into 6 ml scintillation vials containing 4 ml scintillant (Ultima gold; Packard Bioscience BV, Pangbourne, Berks, UK) and counted for 2 min.

The RIAs for $PGF_{2\alpha}$ were carried out using the same procedures as described for PGE_2, but using the appropriate standards, antisera and tracer ([5, 6, 8, 9, 11, 12, 14, 15 (n)-3H]-$PGF_{2\alpha}$) for $PGF_{2\alpha}$. The concentrations of PGE_2 and $PGF_{2\alpha}$ were calculated using a semi-logarithmic plot. The limit of detection was 2 pg/tube for PGE_2 and 1 pg/tube for $PGF_{2\alpha}$. The intra-assay coefficients of variation were 3.5 and 4.1%, while the inter-assay coefficients were 6.3 and 9.6% respectively.

Determination of intracellular concentration of cAMP

cAMPi content in COCs and DOs was determined by a competitive enzyme immunoassay system (Biotrak; GE Healthcare, Chalfont St Giles, Bucks, UK) with acetylation protocol for highest test sensitivity (2 fmol/well) as described before (Marei *et al*. 2009). Briefly, at each time point, 10 COCs or 25 DOs were removed from the culture and washed twice in H-M199 containing 0.5 mM isobutylmethylxanthine (a non-specific phosphodiesterase inhibitor). Zona pellucida was then removed from DOs by incubating in H-M199 containing 5 mg/ml pronaze for few seconds. Finally, COCs and DOs were transferred in a minimum volume of the washing buffer (3–5 μl) to a 0.5 ml microcentrifuge tube, snap frozen in liquid nitrogen and stored at −20 °C until assayed. Lysed oocytes and the peroxidase-labelled cAMP standards (range 2–128 fmol) were acetylated using a mixture of triethylamine and acetic anhydride (2:1 v/v) for 5 min before initiation of the competitive reaction against the anti-cAMP antiserum. At the end of the procedures, concentrations of cAMPi were calculated by measuring the optical density of samples in a plate reader at 450 nm within 30 min. Each sample was tested in duplicate in each experiment.

Western blotting to detect total and phospho-MAPK1, MAPK3 and AKT

Total and phospho-MAPK1, MAPK3 and AKT were detected in COCs as described previously (Marei *et al*. 2009). Briefly, COCs were lysed and loaded into 12% polyacrylamide gels. The separated proteins were then transferred onto Immobilon-PVDF membrane using a Mini Trans-Blot electrophoretic transfer cell (Bio-Rad Laboratories). Active MAPK1 and MAPK3 were probed by incubating blocked membrane blots with anti-phospho-MAPK1 and MAPK3 antibody (mouse, 1:1000; Santa Cruz Biotechnology, Heidelberg, Germany) in TBS–T overnight. The blots were then incubated with HRP-conjugated goat anti-mouse IgG antibody (1:10 000; New England Biolabs, Hitchin, Hertfordshire, UK) for 2 h at room temperature. Membranes were developed using the ECL detection system (GE Healthcare, Amersham). After washing in stripping buffer, total MAPK was then detected on the same membranes by probing with anti-MAPK1 and MAPK3 (rabbit, 1:1000; Cell Signalling, New England Biolabs Ltd) and HRP-conjugated goat anti-rabbit IgG antibody (1:10 000; New England Biolabs). For detecting AKT, anti-phospho-AKT (rabbit, 1:500; Cell Signalling) and then anti-total-AKT (rabbit, 1:5000; Cell Signalling) were used following the same procedure. Densitometric quantification of the immuno-reactive bands was carried out using Quantity-one software (Bio-Rad Laboratories). The phosphorylation of MAPK1, MAPK3 and AKT were calculated as a percentage from the total MAPK1, MAPK3 and AKT proteins respectively.

IVF and embryo culture

For experiments designed to assess the effects of LA in maturation medium on the developmental potential of oocytes, *in vitro*-matured oocytes (in the presence of 0, 50 or 100 μM LA) were fertilized with frozen semen (gift from Genus ABS, Nantwich Cheshire, UK) from a single bull as previously described (Fouladi-Nashta & Campbell 2006). Briefly, motile sperm were selected by swim up for 45 min in calcium-free medium followed by centrifugation at 300 ***g*** at room temperature and re-suspension of the pellet in fertilization medium (TALP supplemented with 0.6% (w/v) fatty acid-free BSA, 1 μg/ml heparin, 50 ng/ml adrenaline and 50 ng/ml hypotaurine). The COCs were gently pipetted to remove adhering granulosa cells and to break up aggregated COCs. Groups of about 30 disaggregated COCs were then washed once in oocyte wash medium and transferred into 400 μl of fertilization medium containing 1×10^6 sperm/ml and cultured for 18 h at 38.5 °C in a humidified incubator of 5% CO_2 in air. Presumptive zygotes were denuded from cumulus cells by gentle pipetting and cultured in 500 μl of synthetic oviductal fluid medium containing amino acids, sodium citrate and myoinositol (SOFaaci; Holm *et al*. 1999) supplemented with 0.4% (w/v) fatty acid-free BSA and cultured at 38.5 °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 was recorded on day 2 (fertilization is day 0), and blastocysts were counted on day 8.

Statistical analysis

In all the experiments, the data were from at least three independent repeats. Binominal data from cumulus expansion, oocyte nuclear maturation, cleavage and blastocyst rates were converted into percentages. All data were then analysed in SPSS v16 (SPSS Inc., Chicago, IL, USA) using one-way ANOVA with randomized block analysis (Linear mixed model) taking the different batches of ovaries into consideration as a random effect. If the main treatment effect was significant, Bonferroni post-hoc tests were performed. Differences of P values ≤ 0.05 were considered as significant.

Declaration of interest

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

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