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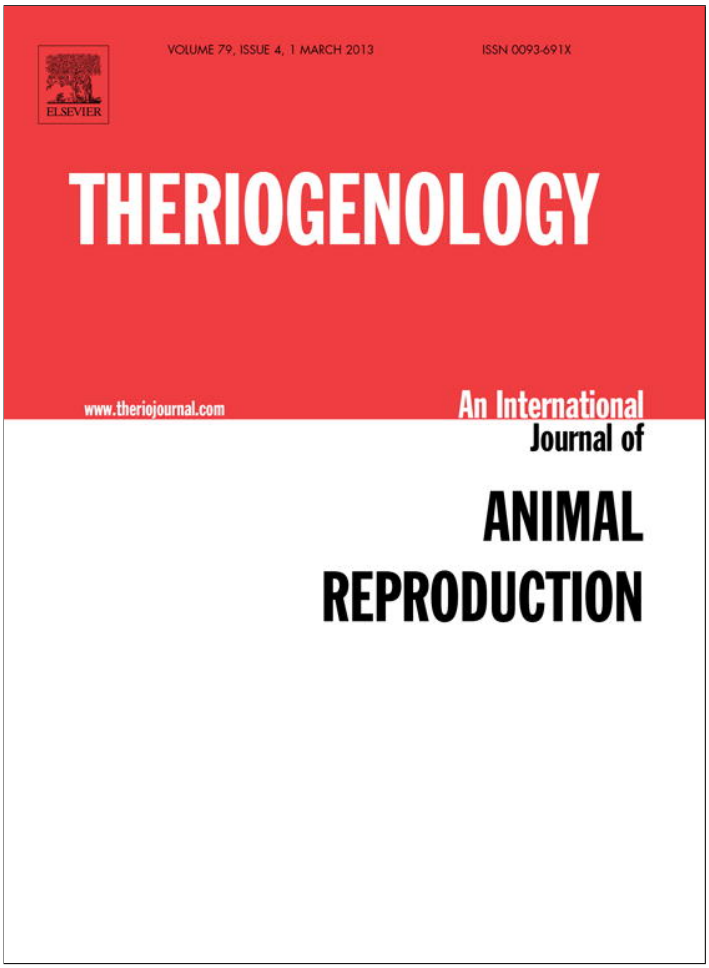

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Linoleic acid stimulates neutral lipid accumulation in lipid droplets of maturing bovine oocytes

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ABSTRACT

Linoleic acid (LA) is a polyunsaturated fatty acid present in high concentrations in bovine follicular fluid; when added to maturation culture media, it affects oocyte competence (depending on the type and concentration of LA used). To date, little is known about the effective level of incorporation of LA and there is apparently no information regarding its esterification into various lipid fractions of the oocyte and its effect on neutral lipid storage. Therefore, the objective was to assess the uptake and subcellular lipid distribution of LA by analyzing incorporation of radiolabeled LA into oocyte polar and neutral lipid classes. The effects of various concentrations of LA on the nuclear status and cytoplasmic lipid content of bovine oocytes matured *in vitro* was also analyzed, with particular emphasis on intermediate concentrations of LA. Neutral lipids stored in lipid droplets were quantified with a fluorescence approach. Linoleic acid at 9 and 43 μM did not affect the nuclear status of oocytes matured *in vitro*, and 100 μM LA inhibited germinal vesicle breakdown, resulting in a higher percentage of oocytes arrested at the germinal state (43.5 vs. 3.0 in controls; $P < 0.05$). Bovine oocytes actively incorporated LA from the maturation medium (83.4 pmol LA per 100 oocytes at 22 hours of incubation; $P < 0.05$) and metabolized it mainly into major lipid classes, e.g., triacylglycerols and phospholipids (61.1% and 29.3%, respectively). Supplementation of the maturation medium with LA increased triacylglycerol accumulation in cytoplasmic lipid droplets at all concentrations assayed ($P < 0.05$). In conclusion, LA added to a defined maturation medium at concentrations that did not alter the nuclear status of bovine oocytes matured *in vitro* (9 and 43 μM) improved their quality by increasing the content of neutral lipids stored in lipid droplets. By directing the free fatty acid (LA) to triacylglycerol synthesis pathways and increasing the degree of unsaturation of membrane phospholipids, the oocyte was protected from lipotoxic effects (with an expectation of improved cryotolerance).

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1. Introduction

Efficient utilization of bovine oocytes for *in vitro* embryo production requires successful maturation. This is important not only for embryo production, but also for tolerating cryopreservation, which preserves oocytes for several biotechnological applications [1]. However, despite many advances, developmental rates of vitrified/warmed bovine oocytes are still low and variable (4%–13%) [2–4]. Cold-induced damage of oocytes occurs mainly as a result of physical changes experienced by lipids at low temperatures [5]. Although the effect of cytoplasmic lipids on the cryopreservation process is considered negative, positive effects of oocyte lipid droplets in acquisition of developmental competence *in vitro* have been reported [6–8]. In that regard, Jeong et al. [7] reported that production of bovine embryos was higher from oocytes with numerous lipid droplets.

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Lipids have important roles in energy metabolism during oocyte maturation [9]. In this respect, the maturation environment has a major influence on the ability of oocytes to develop into blastocysts. Serum added to culture media modifies the lipid composition of bovine oocytes matured *in vitro* [10] and produces embryos with excessive lipid accumulation and reduced cryotolerance [11]. Therefore, many studies have used chemically defined media for all *in vitro* procedures.

The effect of adding fatty acids (FAs) to oocyte maturation culture media on oocyte competence has also been studied; the outcome depends on the type and concentration of FA to which oocytes are exposed [8]. Linoleic acid (LA), an essential long-chain unsaturated FA incorporated by cattle from the diet, is the most abundant FA in follicular fluid [12].

Prostaglandin E_2 is considered a critical mediator of oocyte maturation [13]. Linoleic acid is a precursor of arachidonic acid, a substrate of prostaglandin H synthase and lipooxygenase in synthesis of prostaglandins, thromboxanes, and leukotrienes [14].

The addition of LA to culture media increased the survival rate of frozen/thawed bovine embryos and enucleated oocytes [15–17]. This could be because of membrane fluidization as result of incorporation of an unsaturated FA, thus reducing damage during freezing. Cumulus-oocyte complexes (COCs) maintain their FA profile by selective incorporation of FAs from the follicular fluid [9,18]. Therefore, FA supplementation of diets or LA addition to maturation media does not ensure effective incorporation into the oocyte. Inconsistent findings have been reported on the effects of LA supplementation to culture media on bovine oocyte meiotic competence [12,19], particularly at intermediate concentrations of LA. Although Marei et al. [19] reported no effects on oocyte maturation of oocytes incubated in the presence of LA at 50 μM, Homa and Brown [12] reported that the same concentration had a significant inhibitory effect on germinal vesicle breakdown.

On the other hand, LA supplementation at a high concentration (100 μM) altered molecular mechanisms that regulate oocyte maturation by inhibiting meiotic progression [12,19] and subsequent embryo development [19]. Nonetheless, these effects also seem to depend on the type of LA used. In that regard, Lapa et al. [20] reported that the presence of a conjugated LA isomer (*trans*-10 *cis*-12 conjugated linoleic acid; CLA) in IVM medium at the same concentration (100 μM) did not alter oocyte maturation or embryo production rates, although it increased the quality of bovine embryos. The amount of CLA incorporated by bovine oocytes was estimated using gas chromatography by analysis of the FA profile of oocytes matured in a serum containing medium in the presence of this CLA isomer [20]. To date, there is apparently no information regarding esterification of LA into the various lipid fractions of the oocyte and its effect on neutral lipid storage. Also, mechanisms by which LA exerts its effects on bovine oocytes, and its optimal concentration and level of incorporation, are still unclear.

On the other hand, taking into account LA properties as a polyunsaturated fatty acid with direct effects on membrane fluidity, an increased potential for cryopreservation is expected. Therefore, determining the concentration of LA at which oocyte meiotic competence is not compromised and further understanding how bovine oocytes metabolize it are a starting point for further studies aiming at increasing cryotolerance, and improving developmental competence of vitrified/warmed oocytes. The objectives of the present study were to: (1) further investigate the effect of adding various LA concentrations to a serum-free maturation medium on the nuclear status of bovine oocytes matured *in vitro*, with particular attention to intermediate concentrations; (2) assess the uptake and subcellular lipid distribution of LA by analyzing incorporation of a radiolabeled LA into polar and neutral lipid classes; and (3) determine the effect of LA incorporation on oocyte cytoplasmic lipid content (particularly lipid droplets).

2. Materials and methods

All chemicals and reagents were purchased from Sigma Chemical Company (St. Louis, MO, USA), unless otherwise stated.

2.1. Oocyte collection

Bovine ovaries were collected from a local abattoir and stored in a thermos container at 20 °C during transport to the laboratory within 2 hours after cattle sacrifice. Ovaries were washed several times with PBS supplemented with antibiotics, and COCs were aspirated from follicles ranging from 2 to 10 mm in diameter by a vacuum pump with a 21-ga needle and an aspiration pressure of 120 mm Hg. Cumulus-oocyte complexes with homogeneous ooplasm and more than four complete layers of cumulus cells corresponding to grades 1 and 2 according to de Loos et al. [21] were selected under a stereomicroscope and washed three times in modified M199 supplemented with 0.5% HEPES (wt/vol).

2.2. In vitro maturation

Selected COCs were incubated in culture plates containing five wells (NUNC, Thermo Fisher Scientific, Loughborough, Leicestershire, UK) in groups of 60 per well, with 400 μL of serum-and gonadotropin-free maturation medium: M199 plus 0.1 mg/mL L-glutamine, 2.2 mg/mL $NaHCO_3$ and 10 ng/mL EGF [22] supplemented with 0.15 ng/mL hyaluronic acid, 0.15 mg/mL cysteamine and various concentrations of either LA bound to albumin (9, 43, and 100 μM) or control medium (without LA). Cumulus-oocyte complexes were incubated for 22 hours at 38.5 °C under 5% CO_2 in humidified air.

2.3. Oocyte staining and nuclear maturation stage determination

To assess the stage of nuclear maturation, oocytes were completely denuded of cumulus cells by pipetting in M199-HEPES containing 300 U/mL hyaluronidase (H3506; from bovine testes) for 2 minutes. Denuded oocytes were fixed in 2% glutaraldehyde in M199-HEPES for 10 minutes and stained with 5 μM bisBenzimide Hoechst 33342 for 20 minutes. Oocytes were mounted on a slide with glycerol

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covered with a coverslip. An epifluorescence microscope (Nikon TE-300; Nikon, Tokyo, Japan) using excitation wavelength (380 nm) and barrier filter (420 nm) was used to visualize the fluorescent dye bisBenzimide Hoechst 33342. Nuclear maturation stage was determined with a 40× objective (taking into account nuclear morphology). Oocytes were classified as matured when a metaphase (M) II chromosome set and the chromatin of the first polar body were present. Approximately 30 to 50 COCs were used in each experimental group, with a total of N = 157 (control), N = 189 (9 μM), N = 209 (43 μM), and N = 129 (100 μM) COCs in four independent experiments.

2.4. Radiolabeled LA incorporation assay

Cumulus-oocyte complexes were incubated in the maturation medium supplemented with 9 μM LA in the presence of 0.8 μCi [^{14}C]LA (specific activity: 58.2 mCi/mmol, Perkin Elmer; final concentration of 43 μM, including nonradiolabeled and radiolabeled LA) and randomly separated into three groups of approximately 60 COCs, according to the following incubation intervals: T0 = 0 hours (N = 188), T1 = 1 hour (N = 173), and T22 = 22 hours (N = 252). This experiment was conducted in three independent replicates. After incubation, oocytes were denuded and washed three times by successive immersion in 400 μL final volume of M199-HEPES, M199- HEPES/PBS, and PBS.

2.5. Lipid analysis

Lipid extracts were prepared and partitioned according to Bligh and Dyer [23]. After lipid extraction, organic solvents were evaporated under N_2 and samples were dissolved in chloroform-methanol (2:1, by volume). Radioactivity incorporated into total lipids was measured by scintillation counting using a scintillation counter Wallac1214 RackBeta (Rad α; Wallac Oy, Turku, Finland). Neutral lipids were isolated by monodimensional thin-layer chromatography (TLC) on preparative TLC plates (500 μm, silica gel 60G; Merck) using hexane-diethylether-acetic acid (80:20:1, by volume) and commercial standards. Phospholipids were isolated by two-dimensional TLC on preparative TLC plates (500 μm, silica gel 60H; Merck), according to Rouser et al. [24]. Appropriate nonlabeled pure rat liver lipid extract was added to oocyte lipids as a carrier to ensure enough material for detection by iodine vapors. Lipid spots were scraped off and placed into glass scintillation vials containing 0.2 mL water and 4 mL of 0.4% Omnifluor in 20% Arcopal in toluene. Radioactivity was measured in the isolated lipid fractions by scintillation counting for 5 minutes.

During all procedures (lipid extraction, solvent evaporation, TLC spotting, and drying), lipids were kept in an N_2 atmosphere. All organic solvents were analytical grade.

2.6. Staining of oocyte lipid droplets

Matured COCs in maturation medium supplemented with either LA (9, 43, and 100 μM) or control medium (without LA) were denuded and fixed in 2% glutaraldehyde, 2% formaldehyde in M199-HEPES for 5 minutes and were subsequently washed in M199-HEPES. Oocytes were stained (for 10 minutes) with 1μg/mL Nile Red (Molecular Probes) in PBS.

Fluorescent dye Nile Red was excited by a 450 to 500 nm line; digital photographs of the equatorial part of the oocyte were taken with a 40x objective in an epifluorescence microscope (Nikon TE-300; Nikon, Tokyo, Japan) attached to a Nikon DSfi1 camera. The area and fluorescence intensity from oocytes (arbitrary fluorescence units) were measured using the software Nis Elements Br 3.1 analyzing a total of N = 16 (control), N = 21 (9 μmol/L), N = 21 (43 μM), and N = 27 (100 μM) oocytes randomly selected from each experimental group. The mean fluorescence value of background selections was measured to calculate the corrected total cell fluorescence and to avoid variations in ultraviolet lamp intensity.

2.7. Statistical analysis

All experiments were replicated at least three times. Each batch of ovaries or session was considered a block. Data for nuclear maturation, LA incorporation, and lipid droplet staining were analyzed with SAS 9.1 software (SAS Institute Inc., Cary, NC, USA) using ANOVA with block analysis. When the main treatment effect was significant, the following post hoc tests were used: Bonferroni for the LA incorporation assay, Fisher's least significant difference for LA incorporation into the different lipid classes (for both neutral and polar lipids) and Tukey for nuclear maturation data and lipid droplet staining. Differences were considered significant at $P < 0.05$.

3. Results

3.1. Effect of LA concentration on nuclear maturation stage

The effect of incubation with increasing concentrations of LA (9, 43, and 100 μM) added to a defined maturation medium on oocyte nuclear maturation is shown (Fig. 1). Treatment of COCs with 9 and 43 μM of LA did not affect the percentage distribution of oocytes at the various nuclear stages. In these groups, and in the control group, the highest percentage corresponded to oocytes post germinal vesicle breakdown (GVBD): approximately 20% of the oocytes were in MI and approximately 60% to 70% of oocytes completed the first meiotic division and reached MII. The percentages of oocytes at germinal vesicle (GV) and GVBD stages did not exceed 10%. Conversely, the highest concentration of LA assayed (100 μM) significantly decreased the percentage of MII oocytes and resulted in a significant level of oocytes arrested at the GV state (approximately 40%).

3.2. Incorporation of [^{14}C]LA into bovine oocytes

To determine the level of incorporation of LA in bovine oocytes during *in vitro* maturation, incubations were done in the presence of LA (9 μM) and radiolabeled LA (0.8 μCi per sample) resulting in a final concentration of 43 μM of LA. Radioactivity was measured in the lipid extracts obtained at the three incubation times (T0, T1, and T22). Incorporation of LA into oocytes as a function of time,

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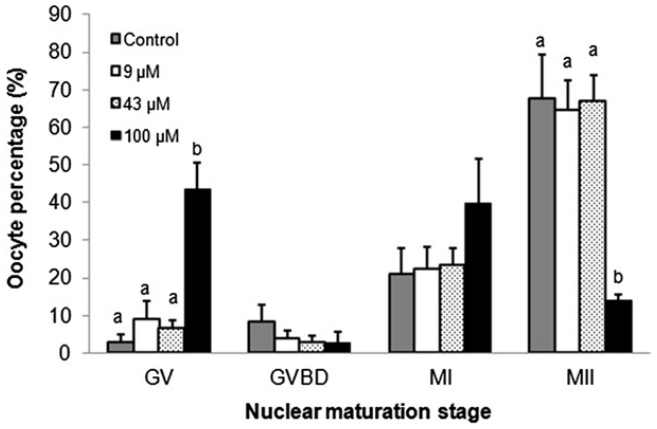

Fig. 1. Effect of *in vitro* maturation in the presence of increasing concentrations of LA on the nuclear stage of bovine oocytes. Results are shown as percentages ± SEM from four independent experiments using 30 to 50 oocytes under each condition. The total number of oocytes used was: control, N = 157; 9 μM, N = 189; 43 μM, N = 209; and 100 μM, N = 129. Characterization of the various stages of bovine nuclear maturation was assessed by Hoestch staining. [a,b] Within a nuclear maturation stage, percentages without a common letter differed ($P < 0.05$). GV, germinal vesicle; GVBD, germinal vesicle breakdown; MI, metaphase I; MII, metaphase II.

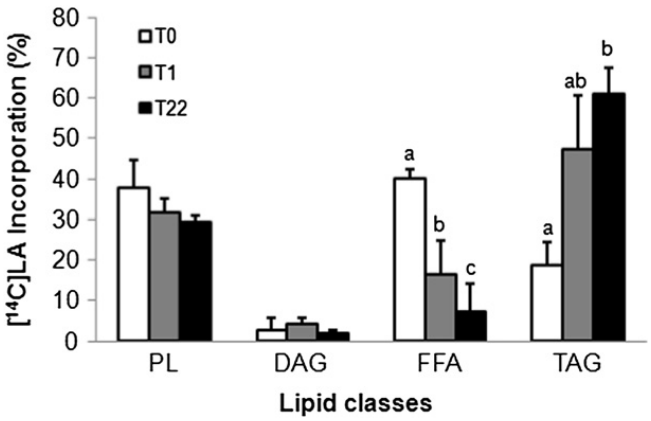

Fig. 2. Incorporation of [^{14}C]LA into the different lipid classes of bovine oocytes as a function of incubation time. Results are shown as percentage of [^{14}C]LA incorporated for each incubation time and represent the mean values ± SEM of three independent experiments using a total of N = 188 (T0), N = 173 (T1), and N = 252 (T22) oocytes. [a–c] Within a lipid class, means without a common letter differed ($P < 0.05$). DAG, diacylglycerols; FFA, free fatty acids; LA, linoleic acid; PL, phospholipids; T0, 0 hours of incubation; T1, 1 hour of incubation; T22, 22 hours of incubation; TAG, triacylglycerols.

estimated through [^{14}C]LA uptake, was 1.18 ± 0.69 pmol LA per 100 oocytes for T0, 12.18 ± 3.67 pmol LA per 100 oocytes for T1, and 83.39 ± 19.61 pmol LA per 100 oocytes for T22. Linoleic acid incorporation significantly increased at 22 hours of incubation, however, there were no significant differences between LA incorporation at T0 and T1.

3.3. Incorporation of [^{14}C]LA into the different lipid classes of bovine oocytes

After lipid extraction of oocytes incubated in the presence of [^{14}C]LA, neutral lipids were separated by monodimensional TLC and radioactivity was measured in isolated lipid fractions (Fig. 2). The percentage of [^{14}C]LA incorporated into the phospholipids (PL) recovered from the TLC plate origin reached approximately 30% of total lipid incorporation and remained constant as a function of time. Linoleic acid incorporated into diacylglycerols (DAG) also remained constant over time and represented 3% of the total. The presence of [^{14}C]LA in PL and in DAG at T0 indicated its rapid incorporation into the plasma membrane. At T0, LA was mainly esterified in PL, triacylglycerols (TAG) (approximately 20%), and a nonnegligible fraction still remained as free fatty acids (FFA) (40%). With increasing incubation time, LA concentrations in PL and DAG remained constant, because of continuous exposure of oocytes to the radiolabeled FA. However, a decrease in the percentage of [^{14}C]LA as FFA (8%) and a concomitant increase in its esterification in TAG were recorded; the latter reached a maximum of 61% at 22 hours of incubation, completely changing the profile present at T0.

Regarding incorporation of [^{14}C]LA into oocyte PL classes (Fig. 3), at 22 hours of incubation, approximately 70% of radiolabeled FA was incorporated into phosphatidylcholine and, to a minor extent, into phosphatidylethanolamine (10%) and phosphatidylinositol (7%). In addition, less than 3% of incorporation was recorded in the other PL classes analyzed (sphingomyelin, cardiolipin, phosphatidylserine, phosphatidic acid, and lysophosphatidylcholine).

3.4. Effect of LA incubation on oocyte lipid droplets

Cytoplasmic lipid droplets in oocytes matured *in vitro* were identified using Nile Red, a lipophilic dye that fluoresces yellow (510–514 nm) with neutral lipids such as TAG (Fig. 4). Fluorescence was restricted to neutral lipid inclusions, with higher fluorescence intensities corresponding to

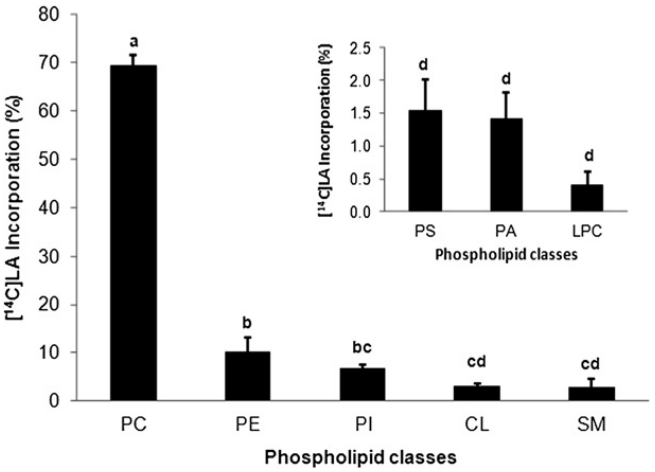

Fig. 3. Incorporation of [^{14}C]LA into phospholipids of bovine oocytes matured *in vitro* for 22 hours. Results are shown as percentage of [^{14}C]LA incorporated by each phospholipid and represent the mean values ± SEM of three independent experiments using a total of 252 oocytes. [a–d] Means without a common letter differed ($P < 0.05$). CL, cardiolipin; LA, linoleic acid; LPC, lysophosphatidylcholine; PA, phosphatidic acid; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PS, phosphatidylserine; SM, sphingomyelin.

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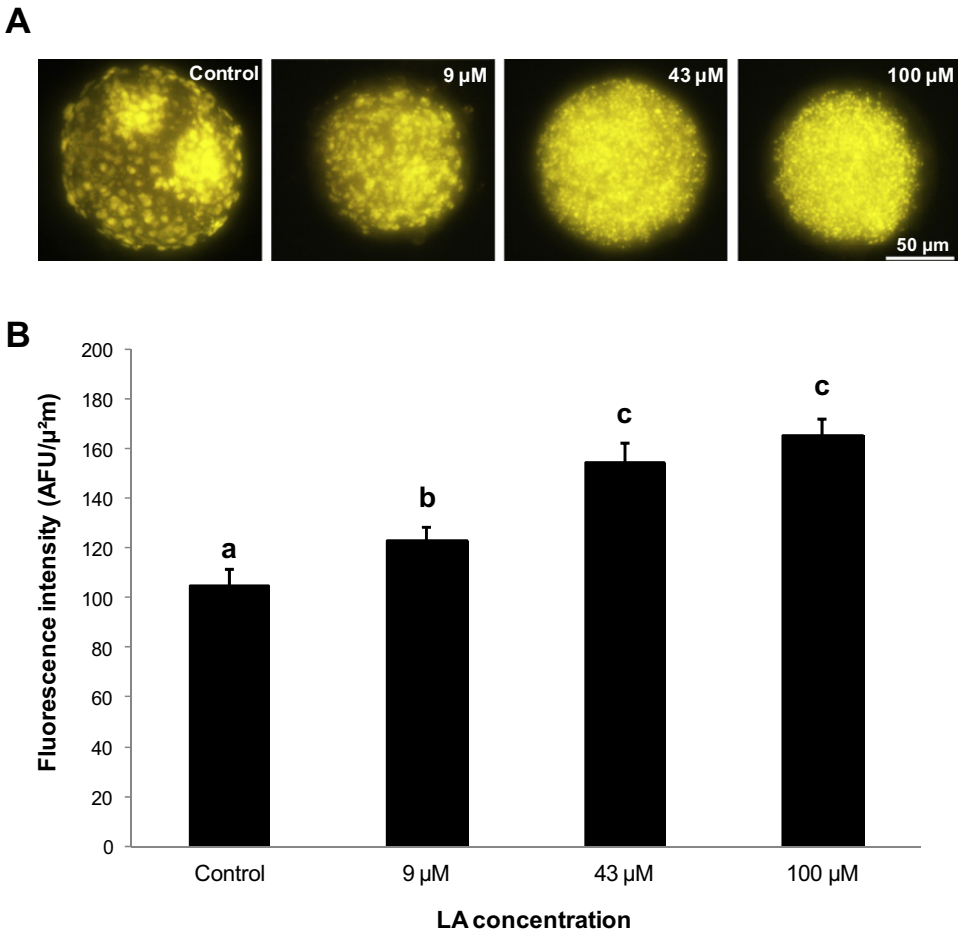

Fig. 4. (A) Bovine oocyte lipid droplets visualized by Nile Red staining. Matured bovine cumulus-oocyte complexes (COCs) in maturation medium supplemented or not with linoleic acid (LA) were fixed and stained with 1 µg/mL Nile Red in PBS. The emitted fluorescence was restricted to lipid droplets (40× objective). (B) Effect of *in vitro* maturation in the presence of LA on fluorescence intensity emitted by bovine oocytes after Nile Red staining. Results are shown as fluorescence intensity mean values ± SEM of three independent experiments analyzing a total of N = 16 (control), N = 21 (9 µM), N = 21 (43 µM), and N = 27 (100 µM) oocytes randomly selected. $^{a-c}$ Means without a common letter superscript differed ($P < 0.05$). AFU, arbitrary fluorescence units; LA, linoleic acid.

higher neutral lipid content, as validated by Barcelo-Fimbres and Seidel [25]. Given that bovine maturing oocytes incorporated LA from the maturation medium and that they actively metabolized this FA, whether exposure to increasing concentrations of LA (9, 43, and 100 µM) affected oocyte lipid storage was also studied. After incubation with Nile Red, identification of lipid droplets was achieved, based on their size, shape, and distribution revealed by emitted fluorescence (Fig. 4A). The distribution pattern of lipid droplets changed in a dose-dependent manner, with droplet clusters in controls and at the lowest concentration of LA assayed (9 µM) and a more homogeneous distribution at higher concentrations (43 and 100 µM). Fluorescence intensity quantification, expressed as arbitrary units of fluorescence relative to the total area of each oocyte observed (arbitrary fluorescence units per μm^2), showed an increase in mean fluorescence at all LA concentrations assayed ($P < 0.05$; Fig. 4B). The increase in fluorescence was higher when incubations were performed at 43 and 100 µM. There were no significant differences in fluorescence intensity between these two concentrations. Results indicate an increase in the amount of TAG stored in lipid droplets of *in vitro* matured oocytes in the presence of LA.

4. Discussion

4.1. Effect of LA concentration on the stage of nuclear maturation

Oocyte maturation is a fundamental process in which the oocyte arrested at prophase I (since fetal life) ultimately resumes meiosis and prepares for subsequent fertilization. One of the purposes of the present study was to analyze the effect of LA supplementation to a serum-free medium on nuclear maturation of bovine oocytes. Under our experimental conditions, at least up to a concentration of 43 µM, LA had no apparent negative effects on bovine oocyte maturation competence. In this respect, LA concentrations of 9 and 43 µM exerted no changes in the distribution pattern of oocyte nuclear maturation. Interestingly, the LA concentrations tested (9, 43, and 100 µM) did not exceed the physiological concentrations reported for bovine follicular fluid: 71 to 710 µM (0.02 to 0.2 mg/mL) [26]. However, and in accordance with previous results [12,19], 100 µM inhibited maturation progression, manifested by a high percentage of oocytes arrested at the GV stage and a concomitant decrease in the percentage of oocytes that

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completed the first meiotic division. Supplementation of the serum-free medium with 100 μM LA affected meiotic competence of bovine oocytes, as acquisition of meiotic competence occurs before GVBD [27]. In addition, although Marei et al. [19] observed no effects on oocyte maturation and cumulus expansion of bovine oocytes incubated in the presence of LA at 50 μM, Homa and Brown [12] reported a significant inhibitory effect on germinal vesicle breakdown using the same concentration. This discrepancy could be partly because of different experimental conditions and the use of different LA sources. Homa and Brown [12] followed a protocol for preparation of FA bound to albumin, whereas in the present study, as well as in that of Marei et al. [19], the same commercially available LA in which FA is already conjugated with albumin was used.

4.2. Linoleic acid incorporation into bovine oocytes

Although previous studies in which low LA concentrations (3 to 9 μM) in cryopreservation had a beneficial effect of LA on oocyte and embryo survival [15–17], the optimal concentration at which LA exerts these positive effects in maturing oocytes, and its incorporation level and subcellular distribution, remain unclear. To our knowledge, this was the first study in which a radiolabeled LA was used to assess uptake and subcellular lipid distribution of this FA. The use of radiolabeled LA enabled confirmation that bovine oocytes actively incorporated LA from the culture medium and metabolized it during *in vitro* maturation. Oocyte incorporation of [^{14}C]LA occurred rapidly, because radioactivity was detected in lipid extracts from oocytes of the T0 group (including 15-minute washing). Furthermore, although PL and DAG are components of cell membranes, the radioactivity in lipid extract fractions corresponding to PL and DAG for T0 was an indicator of the presence of [^{14}C]LA in bovine oocyte plasma membrane. The DAG, membrane lipids with a key role as lipid second messengers, remain in the membrane and recruit cytosolic proteins [28]. Although the percentage of [^{14}C]LA as FFA decreased as a function of time, the percentage of [^{14}C]LA esterified in TAG increased. At 22 hours of incubation, [^{14}C]LA was esterified mainly in TAG and PL, two of the major lipid classes of bovine oocytes [10,29]. Furthermore, given the two double bonds of LA in *cis* configuration, its incorporation into PL could increase membrane fluidity. Subbaiah et al. [30] reported that incorporation of *cis*-9 *trans*-11 CLA into liposomes increased the fluidity of these synthetic membranes. Membrane fluidization and the decrease in phase transition temperature could reduce the formation of ice crystals during cryopreservation, thus explaining the positive effects of LA previously reported [15–17]. Conversely, changes in membrane fluidity seemed to affect cellular signaling cascades as a consequence of alteration of second messengers and effector protein binding [31]. The inhibitory effect on meiotic progression exerted by LA at high concentrations could affect the signaling cascade leading to GVBD.

Isolation of PL classes using two-dimensional TLC enabled determination of the PL in which oocyte LA was incorporated. The [^{14}C]LA was esterified mainly in phosphatidylcholine (PC) (69.8%) and, to a lesser extent, in phosphatidylethanolamine (10.2%) and phosphatidylinositol (6.9%). Phosphatidylcholine is the main structural lipid, representing more than 50% of the PL in most eukaryotic membranes [28]. Hydrolysis of PC generates second messengers such as lysophosphatidylcholine, lysophosphatidic acid, and phosphatidic acid. Moreover, phosphoinositides (phosphorylated derivatives of phosphatidylinositol) are involved in signaling and recruiting proteins, because they can interact with soluble and membrane proteins through electrostatic bonds to positively charged residues [32].

Linoleic acid (18:2n-6) is a precursor of arachidonic acid (20:4n-6) generated by the activity of desaturases and elongases. Apart from its importance as a signaling molecule, arachidonic acid is itself a precursor of eicosanoids (prostaglandins, thromboxanes, and leukotrienes) [14]. McEvoy et al. [29] reported a higher percentage of LA than of arachidonic acid in bovine oocytes and hypothesized that it was more beneficial for oocytes to store LA as the precursor of arachidonic acid, because it has fewer double bonds, thus being less susceptible to peroxidation, resulting in a low risk of oxidative stress for oocytes. Furthermore, as stated above, at 22 hours of incubation, [^{14}C]LA was esterified in high proportions in TAG. These lipids, formed of a glycerol backbone esterified with three FAs, are predominantly saturated [33]. Ferguson and Leese [34] reported a decrease in TAG content after maturation, fertilization, and after the first zygote division, consistent with a metabolic role for these lipids. Furthermore, lipase activity increased during maturation of bovine oocytes *in vitro*, consistent with the hypothesis that catabolism occurs during the maturation process [35]. The incorporation of LA into TAG increased their unsaturation level and made them available as an energy source through β-oxidation during maturation and early development.

4.3. Effect of LA on lipid droplets

The use of Nile Red facilitated the study of the changes in the content of TAG stored in lipid droplets during bovine oocyte maturation in the presence of LA. Lipid droplets are organelles that have recently gained notoriety because of their involvement in diverse cellular processes and their association with certain diseases [36]. Excess FFA in the cytoplasm that is not quickly esterified is toxic, because it can induce cell dysfunction and/or trigger apoptosis [37]. Listenberger et al. [38] studied the protective effect of oleic acid (an unsaturated FA) added to culture medium on the lipotoxicity derived from excess of FFA in the cytoplasm of Chinese hamster ovary cells. They reported a protective effect of oleic acid by directing the FFA to TAG synthesis pathways. Similarly, the increase in the amount of TAG stored in lipid droplets of bovine oocytes matured in the presence of LA could be indicative of a protective effect. Because there were no differences between 43 and 100 μM in Nile Red fluorescence intensity, we inferred that lipid droplets cannot accumulate more TAG when oocytes are incubated with LA at 100 μM. In agreement with this, when LA subcellular lipid distribution was analyzed at 43 μM, esterification in TAG increased with time of incubation, and LA concomitantly decreased as a FFA. Furthermore, at a higher concentration (100 μM) it seems likely that the

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excess of LA incorporated by the oocyte remains as FFA generating toxic effects that can affect bovine oocyte meiotic competence. This could, in turn, be indicative of the fact that the ability of LA to be incorporated into TAG reaches maximal efficiency in lipid droplets. Our results therefore provided new insights regarding the physiology of oocyte lipid droplets and their role in oocyte maturation and early development.

4.4. Conclusions

The present study provided evidence on the effect of LA added to a given maturation medium on bovine oocyte meiotic competence, which depended on the FA concentration used. Bovine oocytes were able to incorporate LA from the maturation medium and metabolize it into major lipid classes (e.g., TAG and PL). Among PL, LA was esterified mainly in PC, the most abundant PL of eukaryotic membranes. Furthermore, the addition of LA to the maturation medium at concentrations that did not alter the nuclear status of bovine oocytes matured *in vitro* (9 and 43 μM) improved their quality by increasing the content of neutral lipids stored in cytoplasmic lipid droplets. This effect was not improved by increasing the amount of LA to greater than 43 μM. Previous studies involving the use of LA in cryopreservation [15–17] were performed using concentrations not exceeding 9 μM. Taken together, based on the current findings, a 43 μM concentration of LA enlarged the range of concentrations that could be used in culture medium for gamete cryopreservation without compromising oocyte meiotic competence.

Acknowledgments

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