Theriogenology 79 (2013) 687–694

Contents lists available at SciVerse ScienceDirect

Theriogenology

journal homepage: www.theriojournal.com

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

M. Carro [a], J. Buschiazzo [a,b,*], G.L. Ríos [a], G.M. Oresti [b], R.H. Alberio [a]

[a] Biotecnología de la Reproducción, Departamento de Producción Animal, INTA, Balcarce, Buenos Aires, Argentina
[b] Instituto de Investigaciones Bioquímicas de Bahía Blanca, Universidad Nacional del Sur–Consejo Nacional de Investigaciones Científicas y Técnicas (UNS-CONICET), Bahía Blanca, Buenos Aires, Argentina

ARTICLE INFO

Article history:
Received 26 June 2012
Received in revised form 24 November 2012
Accepted 24 November 2012

Keywords:
Linoleic acid
In vitro maturation
Lipid droplets
Oocytes
Cattle

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).

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.

* Corresponding author. Tel.: +54 2266 439100; fax: +54 2266 439101.
E-mail address: jbusch@criba.edu.ar (J. Buschiazzo).

0093-691X/$ – see front matter © 2013 Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.theriogenology.2012.11.025

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