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Lipid profiles of follicular fluid from cows submitted to ovarian superstimulation

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ABSTRACT

Ovarian superstimulation with exogenous gonadotropins has been extensively used to produce *in vivo*-derived embryos for embryo transfer in cattle. This process modifies the antral follicle microenvironment and affects oocyte and embryo quality as well the differentiation of granulosa cells. Lipids play significant roles in the cell, such as energy storage, cell structure, and fine-tuning of the physical properties and functions of biological membranes. The phospholipid (PL) contents as well as the effects of superstimulatory treatments on the PL profile of follicular fluid from cows, however, remain unknown. Therefore, to gain insight into the effects of superstimulation with follicle-stimulating hormone (FSH; P-36 protocol) or FSH combined with equine chorionic gonadotropin (eCG; P-36/eCG protocol) on the profile and abundance of PL from cows submitted or not submitted to superstimulatory protocols, were treated with these two superstimulatory protocols. As a control, non-superstimulated cows were only submitted to estrous synchronization. The follicular fluid was aspirated, the remaining cells removed and the follicular fluid stored at −80 °C until extraction. The lipid screening was performed by matrix-assisted laser desorption ionization mass spectrometry (MALDI-MS) and this technique allowed the identification of sphingomyelins (SM) and phosphatidylcholines (PC) and phosphoethanolamines (PE). The relative abundance of the ions observed in the three experimental groups was analyzed by multivariate and univariate statistical models. The phospholipid SM (16:0) and PC (36:4) and/or PC (34:1) were less ($P < 0.05$) abundant in the P-36 group compared to the control or P-36/eCG groups. However, the PC (34:2) was more ($P < 0.05$) abundant in both group of superstimulated cows compared to the control. In summary, ovarian superstimulation seems to modulate the PL content of bovine follicular fluid with a significant increase in PC (34:2), which jointly with others PC and SM, seems to offer a suitable biomarker involved with reproductive processes successful as ovary superstimulation response and embryo development.

1. Introduction

A better understanding of follicular dynamics (for review, see Refs. [1–10]) may facilitate the control of follicular development through hormonal treatment protocols. Toward this goal, [11] designed an ovarian superstimulation protocol termed P-36, in which the progesterone-releasing device is left in place for up to 36 h after the administration of prostaglandin F2 alpha ($PGF_{2\alpha}$), and ovulation is induced by the administration of exogenous LH 12 h after the withdrawal of the progesterone device (i.e., 48 h after $PGF_{2\alpha}$ administration). Greater success of the P-36 protocol is also observed when the final two doses of FSH are replaced by the administration of eCG (the protocol called P-36/eCG).

Recent studies [12–14] have shown that these protocols modify the antral follicle microenvironment, regulating and increasing the expression of genes involved with oocyte and embryo competence,

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as well as the expression of genes involved with ovulatory capacity in granulosa cells and the oviductal physiology of cows. However, there is no information about the changes in lipid profile of the bovine follicular fluid of cows submitted to ovarian superstimulation.

The phospholipids (PL) are the most abundant lipids in the cell membrane, and phosphatidylcholines (PC) and sphingomyelins (SM) are structural units of functional membranes, where changes in PC and SM composition are used to control the fluidity and permeability of eukaryotic cells [15,16]. As it is well known that lipid exchanges occur between cells and extracellular fluid compartments, it is anticipated that the lipid composition and metabolism of female genital tract fluids may also play regulatory roles in sperm motility, capacitation and acrosome reactions, as well as in later reproductive stages such as embryo development and implantation [17]. Several studies have demonstrated the ability of MALDI-MS to analyze PL and define their roles as possible biomarkers in oocytes, embryos, follicular fluids, oviduct and uterus with minimal sample preparation [18–29]. Thus, to gain insight into the modifications of the follicle microenvironment after ovarian superstimulation, the changes in the PL compositions of follicular fluid of cows submitted or not to the superstimulatory P-36 and P-36/eCG protocols were monitored by MALDI-MS.

2. Materials and methods

2.1. Ovarian superstimulation

This study was conducted on a farm located in Santa Cruz do Rio Pardo (São Paulo, Brazil, latitude 22° 53′ 56″; longitude 49° 37′ 57″; altitude 467 m). The cattle were maintained on pasture (*Brachiaria brizantha*), with *ad libitum* access to water and a mineral supplement. Nelore non-lactating multiparous cows ranged from 5 to 7 years of age, and some cows with body condition scores ranging from 2.0 to 3.5 were submitted to P-36 (n = 14) or P-36/eCG (n = 16) ovarian superstimulatory protocols with a control group of 14 cows (Fig. 1). At a random stage of the estrous cycle, all animals received progesterone-releasing vaginal inserts (1.0 g, PRIMER®, Tecnopec, São Paulo, Brazil) and estradiol benzoate (2.5 mg, i.m., Estrogin®, Farmavet, São Paulo, Brazil) on day 0. The P-36 protocol was performed using pFSH (Folltropin-V®, Bioniche Animal Health, Belleville, ON, Canada), administered twice daily (AM & PM) from days 5–8 in decreasing doses of 40% (day 5), 30% (day 6), 20% (day 7) and 10% (day 8) of the total amount used (200 mg). All cattle were given 150 mg of d-cloprostenol (Prolise®, Tecnopec, São Paulo, SP, Brazil) i.m. twice on day 7 (7 a.m. and 7 p.m.). Progesterone-releasing vaginal inserts were removed at 7 p.m. on day 8, and the cows were slaughtered on day 9 at 7 a.m. For P-36/eCG treatment, the final two doses of FSH were replaced by two eCG (total dose = 400 IU, i.m., Novormon®, Syntex, Buenos Aires, Argentina; Fig. 1). The local *Ethics Committee on Animal Use* from the Institute of Biosciences [University of São Paulo State (UNESP), Botucatu, São Paulo, Brazil] approved the experiments (protocol number: 379).

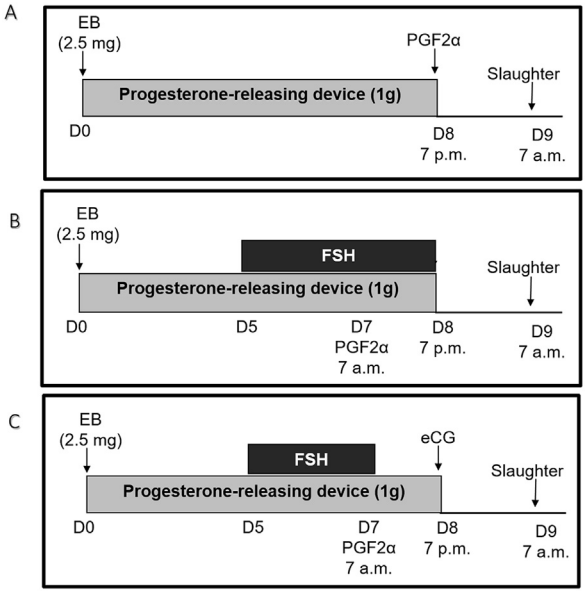

Fig. 1. Experimental design of ovarian superstimulatory protocols in cows. Panel (A): control group, non-superstimulated cows. Panel (B): P-36 protocol. Panel (C): P-36/eCG protocol. EB: Estradiol benzoate, PGF2α: Prostaglandin F2 alpha, D: Day.

2.2. Follicular fluid recovery

The ovaries were collected and transported to the laboratory in saline solution (0.9%) at 4° C and evaluated for the presence of *corpora lutea* or previous ovulations. The average diameter of each follicle, as measured by the average of two lines of measurement approximately perpendicular to one another, was ascertained using a caliper. For non-superstimulated cows (a control group), only the dominant follicle was dissected, whereas the the largest follicles were dissected from cows submitted to ovarian superstimulation. The dominant follicles (n = 14) from non-superstimulated cows and the largest follicle from P-36 group cows (n = 14) and for P-36/eCG group cows (n = 16) was submitted to follicular fluid recovery. For all cows, the diameter of follicle ranged 11–14 mm.

These follicles were previously detected in an ovarian ultrasonography performed 12 h before slaughter. Additionally, blood samples were collected from the jugular vein on day 8 (7 p.m.) and day 9 (7 a.m.) to quantify the plasma concentration of LH and to ensure that no cow had undergone an endogenous LH surge.

The follicular fluid was aspirated and the remaining cells removed by centrifugation at 1000×*g* for 1 min to guarantee only follicular fluid for the lipid analysis, and the samples were stored at −80 °C.

2.3. Lipid analysis by MALDI-MS

For the lipid analysis, follicular fluid from each antral follicle (Control group, non-superstimulated cows, n = 14 follicles; P-36 protocol, n = 14 follicles; and P-36/eCG protocol, n = 16 follicles) were separately submitted to total lipid extraction adapted by Ref. [30]. Thus, to 150 μL of each analyzed sample, it was added 150 μL of water, 190 μL of methanol and 370 μL of chloroform. After continual vortexing for 2 min, each sample was centrifuged for 5 min at 13000×*g*. The apolar lower layer of solution was separated and dried for 40 min in a vacuum concentrator (Concentrator plus, Eppendorf) with subsequent evaporation of the solvents. The samples were kept frozen under −80 °C until the moment of analysis.

For MALDI-TOF-MS analysis, the dried samples were re-suspended in 50 μL of a MeOH:$CHCl_3$ solution (1:1, v/v). A volume of 1 μL of the re-suspended extract was spotted on the MALDI plate, allowed to dry and then covered with 1 μL of 2,5-dihydroxybenzoic acid (DHB) matrix (30 $mg.mL^{-1}$ in MeOH). The experiments were performed using a MALDI-TOF/TOF Autoflex III (Bruker Daltonics, Germany) instrument equipped with a λ 332 nm laser.

Matrix assisted laser desorption ionization (MALDI-MS) generates ions and the produced ions can now be accelerated to the bound mass spectrometer and analyzed based on their *m/z* ratio.