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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).

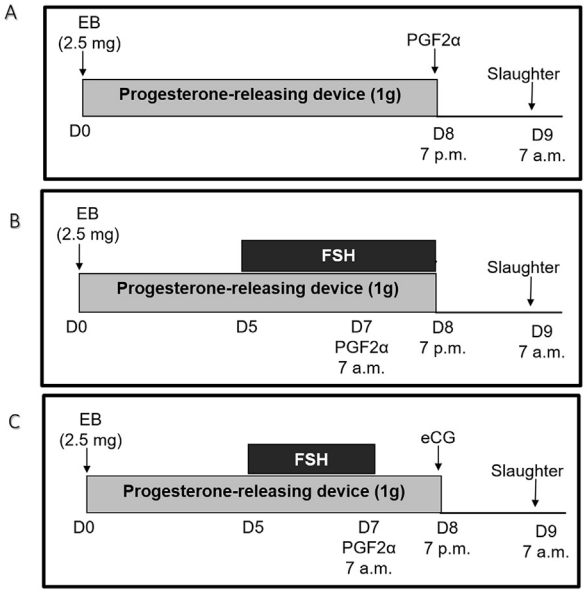

Fig. 1. Experimental design of ovarian superstimulatory protocols in cows. Panel (A): control group, non-superstimulated cows. Panel (B): P-36 protocol. Panel (C): P-36/eCG protocol. EB: Estradiol benzoate, PGF2α: Prostaglandin F2 alpha, D: Day.

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.

After that, the ions were analyzed by a time-of-flight (ToF) type analyzer.

In the present work, the ions analyzed by MS were protonated $[M + H]^+$. Moreover, depending on the matrix being worked on, sodium and/or potassium adducts may occur, in which case the molecules present as sodium adduct, $[M + Na]^+$ or potassium adduct $[M + K]^+$.

Each spectrum was acquired from 500 laser shots on each spot and recorded on a mass range of *m/z* 700 to 900 in the positive ion mode. The laser power range was adjusted to 30–50%, and the measurements were performed under the following operating conditions: ion source 1 = 19.00 kV, ion source 2 = 16.72 kV, lens voltage = 8.30 kV, reflector voltage = 21.00 kV, reflector voltage 2 = 9.70 kV, pulsed ion extraction time = 10 ns, suppression = 500 Da.

Tandem MS experiments were performed using MALDI-LIFT-MS. The tandem MS data were manually acquired by increasing the collision energy until extensive dissociation of the precursor ion was observed.

Lipids are labeled by class abbreviation (PC, SM, PE) followed by the total number of carbons and double bonds attached to the glycerol backbone, separated by a colon.

2.4. Spectra processing and statistical analysis

Raw spectra were processed using the software Flex Analysis 3.3 (Bruker Daltonics) for background subtraction, smoothing and peak centroiding. Each peak list was copied to a spreadsheet software (Microsoft Excel, 2007) and submitted to the online software MetaboAnalyst 3.0 for peaks alignment. Spectral data were then processed using the same software. All zero-values were replaced by the half of the minimum positive value in the data obtained from the preprocessing procedure. The intensity values of each peak across multiple spectra were auto-scaled (mean-centered and divided by the standard deviation of each variable) in order to give the same importance to every *m/z* value.

The most abundant ions that were clearly distinct from noise, after the exclusion of isotopic peaks, were submitted to statistical analysis. Principal component analysis (PCA) and partial least square discriminant analysis (PLS-DA) were performed using the MetaboAnalyst 2.0 [31] to show the relationship between variance in the data and differences among treatments.

The effect of ovarian superstimulation (P-36 and P-36/eCG) on the relative PL abundance of the detected ions that most explain variance in the follicular fluid (i.e. greatest variable importance in projection - VIP score) was also tested by ANOVA, and mean comparisons were performed using the Tukey test. Data are presented as the means ± S.E.M. These analyses were performed using the JMP software (SAS Institute, Cary, NC, USA). Differences were considered significant at $P < 0.05$.

PL structures were attributed based on our MALDI-LIFT-MS data, a literature search [20,28,29,32–35] and a database search (http://lipidsearch.jp or www.lipidmaps.org).

3. Results

The follicular fluid extracts of the control, P-36 and P-36/eCG groups were analyzed and their PL compositions characterized by MALDI-MS and MALDI-MS/MS are demonstrated by Fig. 2 and Table 1. Initially, 105 ions were analyzed by principal component analysis (PCA), which explained the maximum variance of all ions. It should be noted that this analysis does not take into consideration the experimental group, but the characteristics of each individual according to score plot chart (Fig. 3a). After this, partial least squares discriminant analysis (PLS-DA) was performed to determine the variance among the maximum number of ions associated

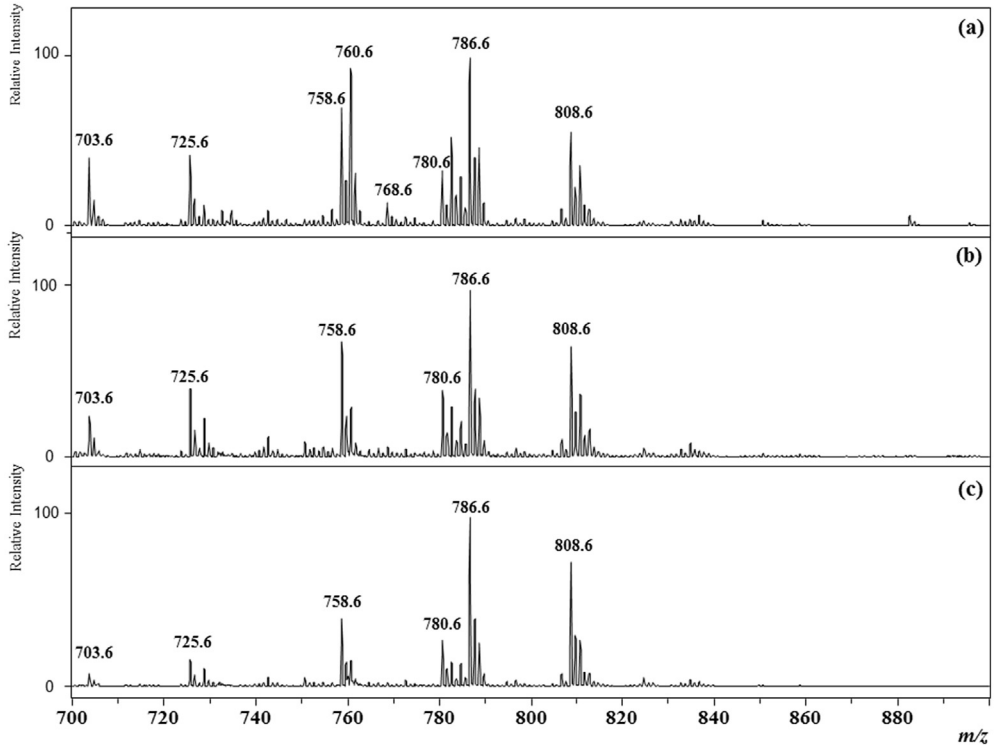

Fig. 2. Representative MALDI -MS phospholipid profiles in bovine follicular fluid. (a) Non superstimulated cows. (b) P-36 ovarian superstimulatory protocol. (c) P-36/eCG ovarian superstimulatory protocol. The *x-axis* represents the *m/z* values of lipid ions whereas the *y-axis* reports the relative abundance of each ion expressed as a percentage to the most abundant ion.

Table 1
Phospholipids (PL) identified[a] in bovine follicular fluid via MALDI(+)-MS.

Experimental *m/z*	Lipid ion (CN:DB)[a]	References[b]
703.6	$[SM\ (16:0) + H]^+$	1,3,5
725.6	$[SM\ (16:0) + Na]^+$	1,2,3,5
728.6	$[PC\ (32:3) + H]^+$, $[PE\ (36:2) + H]^+$	2, 3, 6
758.6	$[PC\ (34:2) + H]^+$,	2, 3, 4, 5
760.6	$[PC\ (34:1)] + H]^+$	3,4,5
768.6	$[PCp\ (36:3) + H]^+$, $PCe\ (36:4) + H]^+$, $[PC\ (35:4) + H]^+$	2, 3, 4, 6
780.6	$[PC\ (34:2) + Na]^+$, $[PC\ (36:5) + H]^+$,	2, 3, 4, 5
782.6	$[PC\ (36:4)] + H]^+$, $[PC\ (34:1) + Na]^+$	2,3,4,5
784.6	$[PC\ (36:3) + H]^+$, $[PC\ (34:0) + Na]^+$	3, 5
786.6	$[PC\ (36:2) + H]^+$	1, 2, 3, 5
788.6	$[PC\ (36:1) + H]^+$	1, 2, 3, 4, 5
806.6	$[PC\ (38:6) + H]^+$, $[PC\ (36:3) + Na]^+$	3, 5, 6
808.6	$[PC\ (38:5) + H]^+$, $[PC\ (36:2) + Na]^+$	1, 2, 3, 4,5, 6
810.6	$[PC\ (38:4) + H]^+$, $[PC\ (36:1) + Na]^+$	1, 2, 3, 4, 5, 6

[a] CN:DB carbon number/double bound; PC: phosphatidylcholines; SM: sphingomyelins; PE: phosphoethanolamines. PC with a lower case "e" or "p" refer plasmanyl and plamenyl (alkyl ether and plamogens) subspecies respectively.
[b] Identification is based on earlier studies and MS/MS data. For references, see: 1- [32]; 2- [33]; 3- [35]; 4- [34]; 5- [20]. 6- [29].

with each group within each component (Fig. 3b).

Although an overlap between groups was demonstrated (Fig. 3a), the visual inspection of such a multitude of spectra is certainly quite challenging and may be subject to bias. For a more accurate interpretation of the MALDI-MS data, a statistical analysis was beneficial. ANOVA was therefore performed, and indeed, significant differences could be perceived in the PL PC (34:2), a phosphatidylcholine, which was less abundant ($P < 0.05$) in non-superstimulated cows compared with cows superstimulated by the P-36 or P-36/eCG treatments (Fig. 4). Additionally, the PL SM (16:0), PC (36:4) and/or PC (34:1) were found to be significantly less abundant in the P-36 group compared with non-superstimulated cows and the P-36/eCG group ($P < 0.05$, Fig. 4).

Major ions were also submitted to MALDI-MS/MS for more definite characterization. The MALDI-MS/MS data display characteristic neutral losses of 59 Da (trimethylamine) and 124 Da, which are known to involve the cyclophosphane ring. The sodiated cyclophosphane (*m/z* 147.0) and the choline polar head group (*m/z* 184.0) ion were also observed as main fragments (Fig. 5) [20,32–35].

4. Discussion

To maximize female reproductive potential, various protocols have been developed. Both for women and cattle, the success of in vitro fertilization (IVF) and other reproductive biotechnologies are highly dependent on the effectiveness of the superstimulatory protocols and consequently on oocytes, as reviewed by Ref. [12]. We have herein presented, for the first time, the PL composition of the follicular fluid, including phosphatidylcholines and sphingomyelins, from cows submitted to ovarian superstimulation as revealed by MALDI-MS.

Recently, (Cataldi, Cordeiro, Costa, et al., 2013) found lipid biomarkers of follicular fluid from young women who were good or poor responders when submitted to ovarian stimulation protocols. The author proposed a correlation between higher concentrations

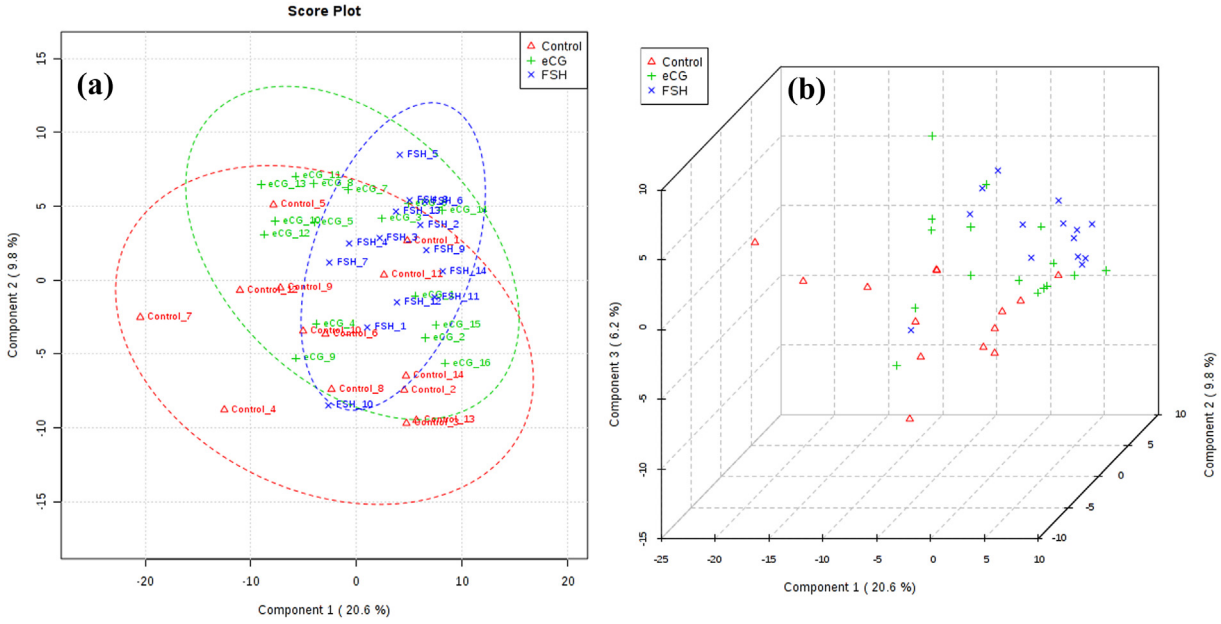

Fig. 3. (A) PCA 2D *score plot*: variance among groups according to Principal Component 1 without considering experimental group. (B) PLS-DA 3D shows the individual scores considering the experimental groups. Control group = non-superstimulated cows; FSH = P-36 protocol and eCG = P-36/eCG groups.

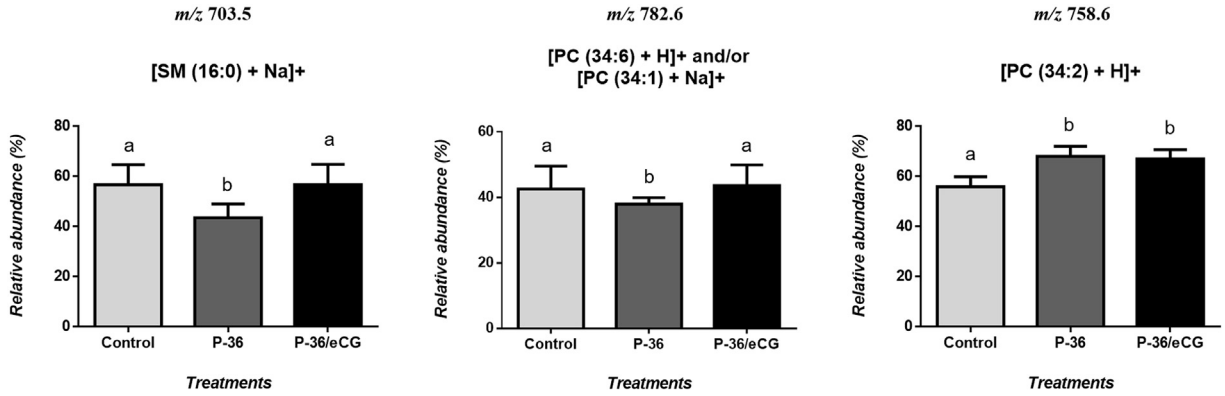

Fig. 4. Changes in the relative abundances of lipids in follicular fluid from cows submitted (P-36 or P-36/eCG protocols) or not to ovarian superstimulation as monitored by MALDI-MS. Letters a and b indicate significant differences ($P < 0.05$). Control group, non-superstimulated cows (n = 14 follicles); P-36 protocol (n = 14 follicles); and P-36/eCG protocol (n = 16 follicles). Data are presented as the mean ± S.E.M.

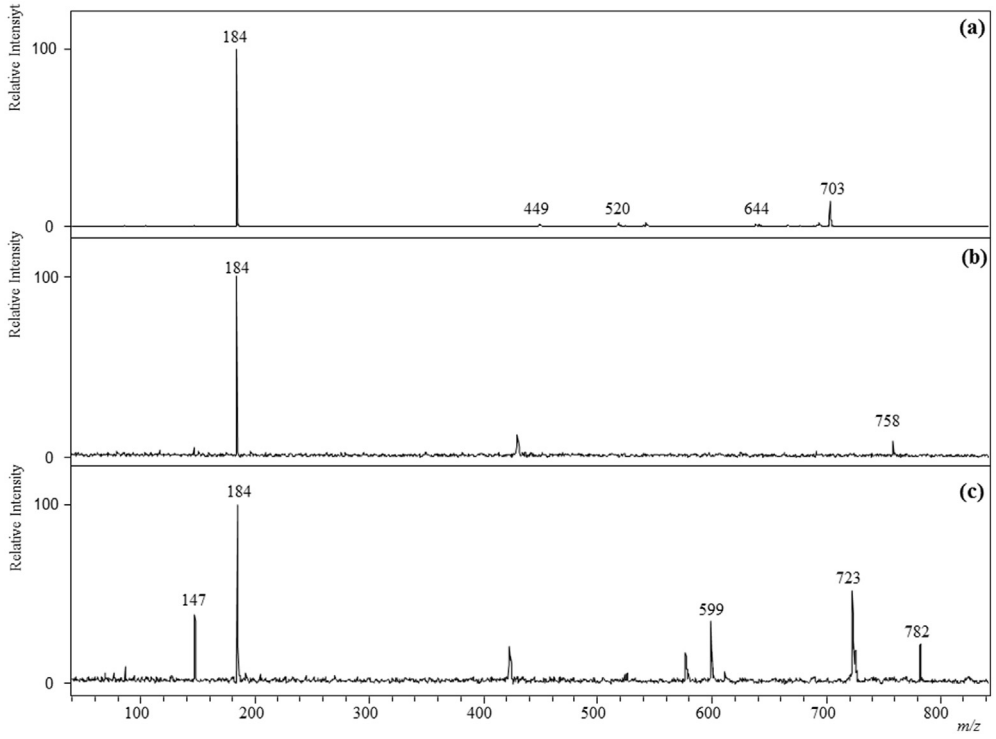

Fig. 5. MALDI-MS/MS for the ions (a) SM (16:0). (b) PC (34:2) and (c) PC (36:4) and/or PC (34:1).

of PCs in women's follicular fluid and the maintenance of cell proliferation and differentiation. We therefore also hypothesize that the characterization of the changes in the lipid profile of the follicular liquid of cows submitted to ovarian superstimulation might aid in the development of new protocols able to improve the follicular microenvironment, thereby influencing the quality and competence of oocytes and subsequently, the competence of embryos [11–13,36].

Although modulation of different PLs was observed, the most important and statistically validated finding in both superstimulatory treatments was the higher relative abundance of the PC (34:2) in the follicular fluid from superstimulated cows. Interestingly, the overrepresentation by the PC (34:2) was also observed by electrospray ionization mass spectrometry (ESI-MS) in the follicular fluid of women with polycystic ovary syndrome (PCOS) and a hyper-response to gonadotropin [37].

Several reports have also indicated the involvement of this PC in embryo quality, for instance, the PC (34:2) was described in both Nelore and Simmental bovine blastocysts and was suggested as a prospective biomarker for embryo quality and cryopreservation success in cattle [21]. Additionally, this ion was increased at bovine embryos of 8–16 cell stage, suggesting a potential association with the embryonic genome activation [28]. Interestingly, the PC (34:2) was also observed by Ref. [38] in the implantation site of the rat uterus. The author show, among other PC ions, a significant increase in the PC (34:2) abundance on day 5 after implantation, with accumulation in the stroma immediately surrounding the implanting embryo.

LH plays a key role in controlling physiological processes in the ovary, such as in the development of antral follicles, ovulation and

luteal development and maintenance, acting through the LH receptor [39]. The eCG shows double activity in FSH and LH receptors [40]. The lower levels of the PL PC (36:4) and/or PC (34:1) in the P-36 group compared with cows submitted to the P-36/eCG protocol demonstrates that, specifically in the control of the bovine follicular fluid microenvironment, LH activity seems to be more important than FSH activity in the PCs content.

Moreover, the lower levels of the PL SM (16:0), also seems to be influenced by LH activity as demonstrated to PCs. Phosphatidylcholines represent the most important phospholipid subclass presenting in eukaryotic cells and play a key role in the structure of membranes and cell signaling [41]. Previous study [42] described an increase in PC abundance of lipid profiles obtained from cumulus cells from pregnant patients undergoing IVF due to the induction of an LH surge by the administration of hCG. Additionally, higher levels of serum LH in woman with PCOS or hyper response to gonadotropins has also been correlated with an increase of PCs abundance in follicular fluid [37].

About the origin of these phospholipids, we believe they could be structural units of functional membranes founded in follicular fluid microenvironment. Recent data of our group (data not published) demonstrate the presence of exosomes, an extracellular vesicle (EV), on follicular fluid of cows submitted to ovary superstimulation. EVs are composed by a lipid bilayer, similar to cell membranes and there are two main subtypes: exosomes and microvesicles. It is believed that EVs are cellular communication mediators to contain proteins, RNA fragments, miRNAs and lipids [43].

There are reports of alterations in EVs compositions, causing enrichment of important components to physiological and pathological responses [44–47]. These studies lead us to believe that superstimulation could to alter the lipid membrane of EVs, may also modify its components. However, more studies are necessary to identified the origin of these PLs, even more, to investigate the effects of isolated PLs and their impact on regulation of follicular microenvironment as well the role on oocyte and embryo competence.

5. Conclusion

In summary, bovine follicular fluid contains important phosphatidylcholines and sphingomyelins, and ovarian superstimulation seems to modulate the content of these phospholipids in bovine follicular fluid. Furthermore, the presence of some PLs in bovine follicular fluid demonstrated in embryo, uterus and human follicular fluid, suggest the role of these PL with reproductive processes as ovary superstimulation response, embryo development and implantation in mammals.

Acknowledgments

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