Biochemical and Fatty Acids Composition of Water Buffalo (Bubalus Bubalis) Follicular Fluid

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Abstract: Aim of this study was to characterize the biochemical and fatty acids composition of follicular fluid collected from follicles of different sizes and in different phases of ovarian cycle in water buffalo farmed in Italy. Ovaries were collected at slaughterhouse during the breeding season; follicular fluid was aspirated dividing samples in small and large follicles (< 6 mm and > 6 mm respectively) and in luteal and follicular phase. Biochemical analysis and gas-chromatography were performed. Biochemical and fatty acids composition were greatly influenced by both follicular dimension and phase of ovarian cycle. Biochemical composition and its variations were in agreement with previously study conducted in buffalo and other species. This is the first report of the fatty acids composition of buffalo follicular fluid. Twenty-two fatty acids were identified in follicular fluid; nine were saturated fatty acids, six monounsatured fatty acids and seven polyunsatured fatty acids. The most dominant fatty acids were linoleic acid, oleic acid, palmitic acid, stearic acid and arachidonic acid. All the identified fatty acids concentrations vary at least because of follicle dimension or phase, with the exception of γ-linoleic acid and arachidonic acid which concentrations remain stable in all classes.

Keywords: Water buffalo, follicular fluid, biochemestry, gas-chromatography, fatty acid.

INTRODUCTION

Reproductive technology in water buffalo has not developed as it has been in cattle [1] as medium composition were reported to be non-physiological in *in-vitro* maturation systems [2]. Knowledge on composition of follicular fluid (FF) may be useful in the selection of oocytes [3] and may be used as a guide for the formulation of specific media for *in-vitro* production [4, 5].

FF is in part a trasudate of serum and also partially composed of products of the metabolic activity of follicular cells [6; 7]. Metabolic activity and blood-follicle barrier properties change during follicle development, influencing the composition of the FF [8-10]. FF plays a major role in autocrine and paracrine regulation and also in physiological, biochemical and metabolic aspects of nuclear and cytoplasmatic maturation of oocyte and in the process of ovulation [11]. Because of his critical role in determining oocyte quality, in the last years, the studies on FF composition has been focused in establishing the relation between a single component and the competence of the oocyte coming from a specific follicle [9].

In buffalo, FF composition has been demonstrated to be influences by the phase of the ovarian cycle [12], though in another study no differences were found between FF collected in luteal and follicular phase [13]. Factors affecting FF composition are: reproductive acyclicity [8, 14] and above all follicular dimension. Nandi *et al.* reported that glucose and lactate concentration in FF are affected by follicles size [2]. The influences of follicle dimension on FF composition were also described in another study for glucose and cholesterol [8]. FF composition is also affected by the cystic status of the follicles [15]. No reports are available on the fatty acids composition of FF in buffalo. Moreover, there is no information on the FF composition of buffalo farmed in intensive system in Italy.

Aim of this work is to investigate the effect of follicular dimension on the biochemical and fatty acids composition in water buffalo.

MATERIALS AND METHODS

Ovaries were collected at a local abattoir in the month of November 2012, during the buffalo reproductive season in Italy. Ovaries were maintained at 4°C in saline solution and transported to the laboratory within 40 min.

Follicles diameter was measured using a divider compass and metric scale, and follicles were classified in small and large size (< 6 mm and > 6 mm respectively). FF was also divided on the base of the presence or absence of the corpus luteum in at least

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one of the ovaries of each animal. Samples were so divided in four groups: FF from small follicles of an animal in follicular phase (small FFF); FF from large follicles of an animal in follicular phase (large FFF); FF from small follicles of an animal in luteal phase (small CLFF); FF from large follicles of an animal in luteal phase (large CLFF).

FF was aspirated using a 18G needle connected to a syringe. After collection FF was centrifuged at 1500 x g for 10 min and the supernatant was stored at -80°C.

Biochemical analyses were performed using an automatic biochemistry analyzer BT1500, Biotecnica Instruments SpA, Rome, Italy. Fatty acids were measured by gas chromatography according to Carnielli *et al.* [16]. Samples were directly methylated inside a glass tube using a 3N HCl-Methanol solution (Supelco, Sigma-Aldrich Group, Germany) containing 1 mg/ml of nonanoic acid and 1mg/ml of heptadecanoic acid as internal standard. After flushing with nitrogen, tubes were capped and incubated for 45 minutes at 100° C. After methylation, the solution containing the fatty acids methyl esters (FAME) was placed on ice, and was supplemented with a 10 % solution of K_2CO_3 up to reach a pH of 7. A solution containing hexane (Merck) and B-idrossi-toluene was added before to centrifuge at 3000 rpm for 10 minutes. After centrifuge, 1 µl of the supernatant containing hexane and the FAME was injected into a HP 5890 Gas-Chromatography (Hewlett Packard, CA, USA) equipped with Omegavax 30 m 0.25 mm x 0.25 microm (Supelco, Sigma-Aldrich Group, Germany). The gas chromatograph was set using the following temperature program: 70° C for 3 minutes, thereafter the temperature was raised by 20° C/min until 205° C and this temperature was held this for 15 minutes. After 15 minutes, the temperature was increased again at 0.4° C/min to reach 213° C and then this temperature was maintained for 10 minutes. The temperature was further increased at 5° C/min up to 240° C. Temperature of 240°C was held for 7 minutes. Peak areas were calculated by HP-Chem station software using known concentrations of nonanoic acid and heptadecanoid acid as internal standard.

Data were analyzed using the GLM procedure of the software SIGMASTAT 2.03. Two-way ANOVA was performed to investigate the effects of the ovarian stage, follicles size and their interactions on the parameters considered in this study. Significant differences were considered with $P<0.05$.

RESULTS

A total of fifty-six FF samples were collected and divided in the four classes: small FFF (N=16), large FFF (N=16), small CLFF (N=12) and large CLFF (N=12).

Table **1** reported the results of biochemical analysis of the FF in the different groups.

Urea was the only parameter that did not differed statistically between any of the groups. Bilirubin, magnesium, phosphorus chloride, GOT-AST concentrations were affected by follicular dimensions ($P<0.05$), but not by the phase of the estrous cycle. On the other hand, cholesterol, creatinine, globulin, total protein, sodium and CK were affected only by the phase of estrous cycle. Both follicular dimensions and phase of the cycle affected the other parameters.

Mean fatty acids compositions of buffalo FF are reported in Table **2**. Twenty-two fatty acids were identified. The most common fatty acid was linoleic acid (C18:2n6), followed by oleic acid (C18:1n9), palmitic acid (C16:0), stearic acid (C18:0) and arachidonic acid (C20:4n6).

Caprylic acid (C8:0), caprinic acid (C10:0), lauristic acid (C12:0), linoleic acid, α – linoleic acid (C18:3n6) and trienoic acid (C20:3n9) concentrations were affected both by follicular dimensions and phase of estrous cycle. Concentrations of nine fatty acids were affected only by the phase of the estrous cycle, among them, palmitic acid and oleic acid. Concentrations of four fatty acids were affected only by follicular dimensions, among them, stearic acid. Concentrations of γ-linoleic acid (C18:3n3) and arachidonic acid did not different between classes.

DISCUSSION

Since FF is in contact with oocyte and granulosa cells, changes in concentration of different components may reflect the requirements of follicular structure, and they may represent a marker of oocyte quality. While other studies described the biochemical composition of FF in buffalo, to our knowledge this is the first report of the fatty acids composition. Moreover, there is no information about the FF composition of Mediterranean water buffalo farmed in Italy. The existing studies on FF composition in buffalo reported variability in relation to the phase of ovarian cycle [12] reproductive acyclicity [8, 14] and follicles size [2].

Table 1: Biochemical Analysis of Follicular Fluid Collected from Small (< 6 mm) and Large (> 6 mm) Follicles in Follicular and Luteal Phase in Water Buffalo (mean ± SD)

	Small FFF	Large FFF	Small CLFF	Large CLFF
Glucose (mg/dl)	51.0 ± 9.31^{b}	96.1 ± 3.87^{d}	36.3 ± 6.33^{a}	81.9 ± 7.89^{c}
Triglycerides (mg/dl)	27.2 ± 1.95^{b}	15.3 ± 1.53^{a}	32.3 ± 4.49^{c}	17.3 ± 2.18^{a}
Cholesterol (mg/dl)	63.6 ± 28.9^{b}	44.0 ± 16.2^{a}	67.2 ± 18.0^{bc}	83.1 ± 34.3^{cd}
Total protein (mg/l)	70.4 ± 3.18^{b}	66.8 ± 4.32^{a}	73.5 ± 1.07^{c}	75.9 ± 0.71^{d}
Albumin (mg/l)	29.5 ± 1.41^{a}	30.9 ± 1.02^{b}	31.1 ± 1.14^{c}	32.4 ± 0.11^{d}
Globulin (mg/l)	39.4 ± 2.57^{b}	37.2 ± 2.68^{a}	42.2 ± 0.13^{c}	43.9 ± 0.33^{d}
Urea (mg/dl)	45.8 ± 9.21	48.8 ± 11.8	47.4 ± 4.59	48.2 ± 4.40
Bilirubin (mg/dl)	0.42 ± 0.08^{b}	0.33 ± 0.09^{a}	0.38 ± 0.03^{ab}	0.34 ± 0.04^{ab}
Creatinine (mg/dl)	1.52 ± 0.17^{a}	1.51 ± 0.10^{a}	1.67 ± 0.36^{ab}	1.76 ± 0.37^{b}
Calcium (mg/dl)	9.31 ± 0.43^{b}	8.67 ± 0.29^{a}	9.71 ± 0.44^{c}	9.69 ± 0.63^{c}
Phosphorus (mg/dl)	12.4 ± 2.14^{b}	8.61 ± 0.82^{a}	13.3 ± 0.50^{b}	9.04 ± 0.02^{a}
Magnesium (mg/dl)	2.93 ± 0.33^{b}	2.36 ± 0.34^{a}	2.92 ± 0.26^{b}	2.54 ± 0.19^{a}
Chloride (mmol/l)	120.3 ± 1.43^{b}	91.3 ± 3.30^{a}	112.2 ± 0.22^{b}	78.9 ± 49.8^{ab}
Potassium (mmol/l)	15.5 ± 0.26^{d}	6.8 ± 0.07^{a}	9.8 ± 0.09^{c}	7.5 ± 0.43^{b}
Sodium (mmol/l)	151.8 ± 2.67^{ab}	151.3 ± 14.2^{a}	156.3 ± 2.15^{ab}	158 ± 3.07^{b}
SAP (u/l)	154.4 ± 64.1^{a}	142.1 ± 29.4^{a}	227.1 ± 60.4^{b}	134.7 ± 70.2^{a}
CK (u/l)	198.4 ± 43.8^{b}	201.1 ± 57.9^{b}	184.7 ± 77.4^{ab}	144.7 ± 76.4^{a}
GGT (u/l)	20.9 ± 1.00^{a}	22.4 ± 4.68^{a}	45.9 ± 18.8^{b}	21.9 ± 0.81^{a}
GOT-AST (u/l)	241.4 ± 2.27^{c}	185.3 ± 15.4^{b}	275.2 ± 8.75^{d}	167.0 ± 25.1^{a}
GPT-ALT (u/l)	101.2 ± 7.02^{b}	57.8 ± 3.72^{ab}	127.9 ± 14.1^{c}	45.0 ± 19.2^{a}

Different letters (a,b,c,d) between columns indicate a statistical difference (P<0.05). SAP: Alkaline phosphatase; CK: Creatine Kinase; GGT: Gamma glutamil transpeptidase; GOT-AST: Aspartate aminotransferate; GPT-ALT: Alanine transaminase; Small FFF: follicular fluid from buffalo cows in follicular phase and follicles <6mm; Large FFF: follicular fluid from buffalo cows in follicular phase and follicles >6mm; Small CLFF: follicular fluid from buffalo cows in luteal phase and follicles <6mm; Large CLFF: follicular fluid from buffalo cows in luteal phase and follicles >6mm.

Table 2: Fatty Acids Composition (mg/dl) of Follicular Fluid Collected from Small (< 6 mm) and Large (> 6 mm) Follicles in Follicular and Luteal Phase in Water Buffalo (mean ± SD)

	Mean	Small FFF	Large FFF	Small CLFF	Large CLFF
Caproic ac.	0.10 ± 0.01	0.09 ± 0.01^{a}	0.09 ± 0.01^{a}	0.10 ± 0.02^{ab}	0.11 ± 0.01^{b}
Caprylic ac.	0.03 ± 0.02	0.02 ± 0.02^{a}	0.02 ± 0.02^{a}	0.03 ± 0.02^{a}	0.05 ± 0.00^{b}
Caprinic ac.	0.06 ± 0.02	0.05 ± 0.01^{a}	0.05 ± 0.01^{a}	0.08 ± 0.02^{b}	0.05 ± 0.03^{a}
Lauristic ac.	0.06 ± 0.02	0.06 ± 0.01^{b}	0.05 ± 0.01^{a}	0.08 ± 0.02^{c}	0.06 ± 0.01^{a}
Miristic ac.	0.61 ± 0.13	0.58 ± 0.13^{a}	0.52 ± 0.04^{a}	0.67 ± 0.18^{b}	0.65 ± 0.08^{b}
Miristoleic ac.	0.10 ± 0.13	0.04 ± 0.02^{a}	0.08 ± 0.02^{a}	0.12 ± 0.17^{ab}	0.20 ± 0.24^{b}
Palmitic ac.	11.85 ± 1.55	11.50 ± 2.16^{a}	10.93 ± 1.85^{a}	12.12 ± 1.04^{ab}	13.20 ± 0.34^{b}
Hexadecenoic ac.	0.55 ± 0.13	0.54 ± 0.15^{b}	0.42 ± 0.11^{a}	0.61 ± 0.11^{b}	0.64 ± 0.05^{b}
Palmitoleic ac.	1.28 ± 0.43	1.40 ± 0.49^{a}	1.21 ± 0.28^{a}	1.46 ± 0.07^{a}	0.84 ± 0.91^{b}
Stearic ac.	7.87 ± 1.80	7.06 ± 1.80^{a}	8.35 ± 1.51^{ab}	7.42 ± 0.97^{a}	9.25 ± 3.79^{b}
Oleic ac.	16.96 ± 3.04	16.13 ± 3.64^{a}	15.26 ± 3.85^{a}	17.83 ± 1.84^{a}	19.00 ± 3.61^{b}
Vaccenic ac.	0.67 ± 0.69	0.42 ± 0.73^{a}	0.34 ± 0.29^{a}	0.85 ± 0.99^{ab}	1.18 ± 0.09^{b}
Linoleic ac.	20.86 ± 9.77	17.29 ± 6.11^{a}	15.25 ± 3.13^{a}	20.28 ± 11.19^{a}	35.79 ± 0.67^{b}
α - linoleic ac.	0.65 ± 0.23	0.55 ± 0.21^{a}	0.57 ± 0.16^{a}	0.63 ± 0.24^{a}	0.96 ± 0.16^{b}
Linolenic ac.	1.30 ± 1.25	0.79 ± 0.36	0.99 ± 0.40	1.64 ± 1.71	1.86 ± 2.37
γ – linolenic ac.	1.03 ± 0.25	0.91 ± 0.19^{a}	1.08 ± 0.23^{ab}	0.97 ± 0.20^{a}	1.23 ± 0.48^{b}
Arachidonic ac.	2.60 ± 1.52	2.19 ± 0.45	1.97 ± 0.44	3.62 ± 2.47	2.09 ± 0.24
Trienoic ac.	0.31 ± 0.10	0.27 ± 0.10^{a}	0.26 ± 0.08^{a}	0.39 ± 0.12^{b}	0.28 ± 0.01^{a}
Docosanedioc ac.	0.11 ± 0.09	0.12 ± 0.03^{b}	0.02 ± 0.04^{a}	0.13 ± 0.11^{b}	0.18 ± 0.10^{b}
Decosatetraenoic ac.	0.34 ± 0.09	0.38 ± 0.13^{b}	0.28 ± 0.04^{a}	0.36 ± 0.11^{b}	0.33 ± 0.05^{ab}
Tetracosanoic ac.	0.16 ± 0.17	0.10 ± 0.08^{a}	0.05 ± 0.09^{a}	0.16 ± 0.15^{a}	0.41 ± 0.21^{b}
Erucic ac.	0.20 ± 0.15	0.20 ± 0.11^{b}	0.04 ± 0.07^{a}	0.24 ± 0.09^{b}	0.39 ± 0.19^{c}

Different letters (a,b,c,d) between colums indicate a statistical difference (P<0.05). Small FFF: follicular fluid from buffalo cows in follicular phase and follicles <6mm; Large FFF: follicular fluid from buffalo cows in follicular phase and follicles >6mm; Small CLFF: follicular fluid from buffalo cows in luteal phase and follicles <6mm; Large CLFF: follicular fluid from buffalo cows in luteal phase and follicles >6mm.

Ovaries of buffalo cows were collected at slaughterhouse during the breeding season. FF was collected from follicles by aspiration and samples were divided in function of follicles dimension and presence/absence of corpus luteum at slaughtering time.

Comparing our results about the biochemical composition to the reports available in literature, most of the parameters agree with those already reported with exception of glucose that seems to be higher in this study [8, 12, 13] although it is similar to Nandi *et al.* [2].

Biochemical composition was greatly influenced either by follicular dimension or by phase of ovarian cycle. Glucose concentration was higher in FF collected from animals in follicular phase than FF from animals in luteal phase, in according to Eissa [12]. Moreover, glucose was more concentrated in large follicles compared to small follicles, in agreement to the results reported in bovine, ovine and dromedary camel [6, 9, 10]. Different hypothesis have been formulated to explain the higher concentration of glucose in the large follicle: 1) a less intensive glucose metabolism in large follicles; 2) a relative larger volume of FF in comparison to the amount of granulosa cells that consumes glucose for their metabolism; 3) an increased permeability of the blood-follicle barrier during follicular growth [9]. Tryglicerides, K and P were more concentrated in small follicles than in large follicles in agreement with previously studies [6, 7, 9, 10]. The difference in the concentration of triglycerides may be explain by the fact that triglycerides probably do not pass through the follicular membrane because they are transported by the Very Low-Density Lipoproteins, that are too large to pass the blood-follicle barrier [17]. The origin of triglycerides in the FF may be local and a dilution effect could be observed at the increasing of follicle size [9]. The decreased concentration of K in FF with follicular development could be related to the use of glucose during the developmental process that leads to the transfer of K from extracellular sites to the intracellular sites [7]. The P levels decreased with the increasing of follicles size in agreement to earlier reports in goat [18] and cattle [7]. Overall, the biochemical composition was extremely variable between groups. Indeed, urea was the only parameters that did not differed among groups.

Although no information was available about the slaughtered buffalo cows, the analogy of biochemical composition of FF in our study to the results of previous works makes us think that our population was not abnormal.

The most dominant fatty acids present in the buffalo follicular fluid were linoleic acid, oleic acid, palmitic acid, stearic acid and arachidonic acid. The same fatty acids were reported to be the most concentrated also in human follicular fluid [19] and dairy cows [20], but in different order. Palmitic acid, stearic acid and

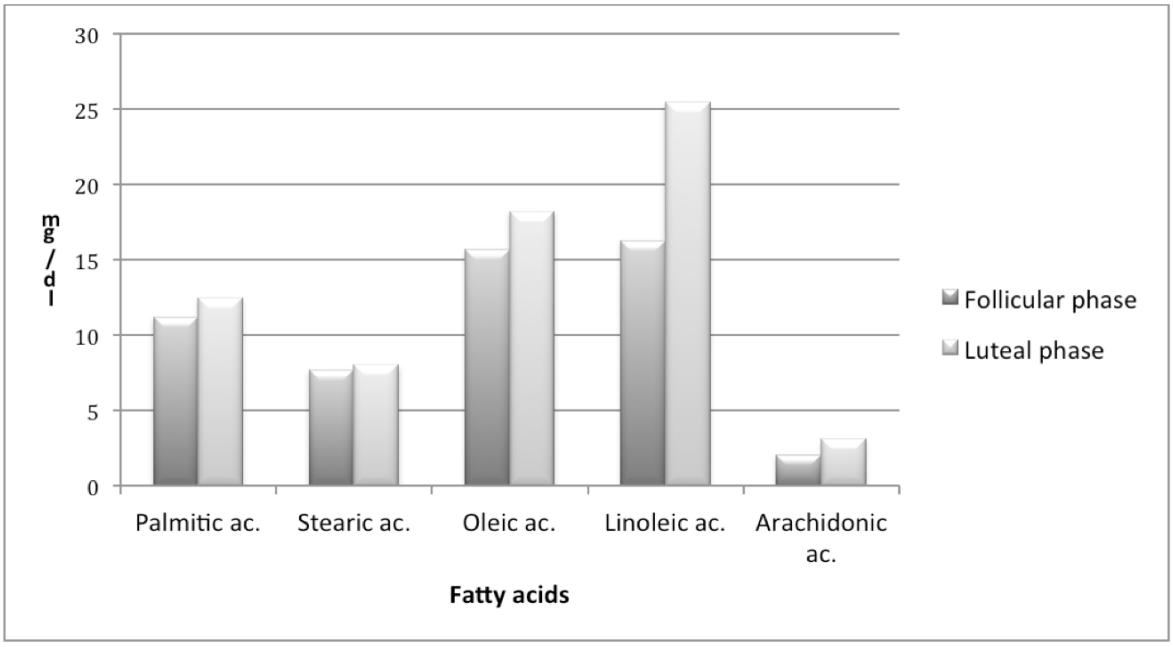

Figure 1: Effects of the phas eof ovarian cycle on the concentration of the most respresentative fatty acids in buffalo follicular fluid.

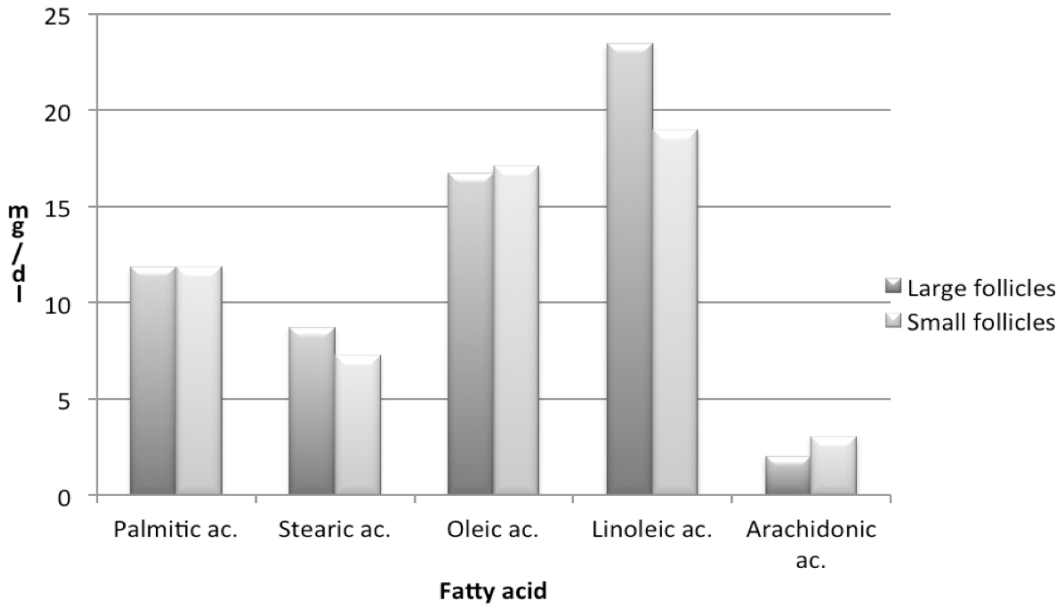

Figure 2: Effects of follicle dimensions on the concentration of the most representative fatty acids in buffalo follicular fluid.

arachidonic acid seems to be less concentrated in buffalo FF than human and dairy cows FF, while oleic acid seems to be more concentrated in buffalo FF [19, 20]. Linoleic acid concentration seems to be higher in buffalo FF than human FF [19] and lower than dairy cows FF [20].

Overall, the fatty acids composition of FF was different depending on the follicular dimensions and phase of ovarian cycle. Of the five most commons fatty acids, stearic acid was affected by the follicular dimensions, two fatty acids by the phase of ovarian cycle (palmitic acid and oleic acid), one was affected by both (linoleic acid) and one did not differ (arachidonic acid) (Figures **1** & **2**).

From the twenty-two fatty acids identifies, nine were saturated fatty acids (SFA), six monounsatured fatty acids (MUFA) and seven polyunsatured fatty acids (PUFA). Only ɣ-linoleic acid and arachidonic acid concentrations remain stable in all classes.

Linoleic acid concentrations were extremely variable, though the only significant difference was found in large CLFF that shown higher concentration than other groups. Different studies reported that linoleic acid affected negatively the oocyte competence. In particular, his concentration was higher in FF from inactive follicles [20], moreover Homa and Brown reported that linoleic acid inhibits resumptions of meiosis in a dose-dependent manner [21]. Palmitic acid is the most abundant fatty acid in human FF and was found to be significantly increased in the FF where the oocytes fertilized but failed to cleave [19]. Excess of palmitic acid has been shown to induce apoptosis in human and bovine granulosa cells and affected steroidogenesis, which is critical in supporting follicular development and oocyte maturation [22, 23]. In our study palmitic acid concentration was affected by the presence of the corpus luteum. In particular the large CLFF samples had higher concentration of palmitic acid. Linoleic acid and palmitic acid concentrations seem to suggest that the oocytes coming from follicle grown under the influence of a CL may have less possibility to develop. Moreover, elevated levels of palmitic acid and stearic acid were reported to impair post-fertilisation development [24]. In contrast, a subsequent study reported that stearic acid was found to be significantly increased in the FF from follicles which oocytes undergo cleavage [19], probably because stearic acid can be converted in oleic acid, which is reported to be more concentrated in good-quality oocyte [25]. In our study stearic acid and oleic acid were higher concentrated in large CLFF samples. These finding suggest that while stearic acid may affect developmental potential of the oocytes, on the other hand oleic acid may have beneficial effect. Arachidonic acid is the major precursor for prostaglandins and protects granulosa cells from palmitic and stearic acid effect [22]. Its concentration did not change between inactive and active follicles FF in bovine [20], while it was more concentrated in human FF from follicles which oocytes undergo cleavage [19]. In our study his concentration did not statistical differed between groups.

Because FF composition can give information on the requirement of oocytes and follicular cells, it may be used as a guide for the formulation of specific IVP medium to enhance *in vitro* process efficiency.

In conclusion, follicular dimensions and phase of the ovarian cycle influenced FF composition; in turn this could affect oocyte quality. The concentrations of fatty acids that have been proposed as marker of oocyte quality seem to suggest that oocytes collected from large follicle during the luteal phase could be less competent, although this aspect has to be confirmed.

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