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Effect of season on intrafollicular fatty acid concentrations and embryo production after *in vitro* fertilization and parthenogenic activation of prepubertal goat oocytes

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ABSTRACT

In this study we have assessed the concentration of 13 fatty acids (FAs) in follicular fluid (FF) and tested the blastocyst development of prepubertal goat oocytes after *in vitro* fertilization (IVF) and parthenogenic activation (PA) during spring, summer, autumn and winter. Ovaries were collected from a local slaughterhouse and transported to the laboratory during the four seasons. Samples of FF were recovered from follicles and FAs were analyzed by gas chromatography. Oocytes were *in vitro* matured in TCM-199 with hormones, epidermal growth factor and 10% of fetal bovine serum for 24 h. After maturation, oocytes were inseminated with fresh semen capacitated with heparin or parthenogenically activated with ionomycin and 6-dimetylaminopurine. Zygotes from both groups were cultured in SOF medium for 8 days. The results showed that the polyunsaturated FA (PUFA) concentration of n3 was significantly reduced in autumn compared to winter (46.17 μM and 116.45 μM, respectively) and the n6 to n3 (n6:n3) PUFA ratio was significantly higher in autumn than in winter (11.17 μM *vs* 4.23 μM, respectively). Blastocyst production was significantly higher ($P < 0.05$) in winter than autumn after IVF (16.8% and 5.5%, respectively) and showed a statistical tendency ($P < 0.1$) after PA (22.7% and 11.5%, respectively). In conclusion, in ovaries of prepubertal goats we have observed higher concentrations of n3 PUFAs and lower n6:n3 PUFA ratio in the follicular fluid and oocytes with higher competence to develop up to blastocyst in winter compared to autumn. Moreover, spring, which is a non-breeding season in goat, have not affected *in vitro* blastocyst production from suckling kid oocytes.

1. Introduction

Goats, in the northern hemisphere, start breeding season in response to the shortening day-length. In our geographical conditions, the annual breeding season in Spanish Mediterranean goats begin in late summer (September) and ends in spring of the following year (March) (Gómez-Brunet et al., 2010). The *in vitro* embryo production (IVEP) allows to obtain viable embryos avoiding the negative effect of the day-light. The IVEP involves three mains steps: oocyte *in vitro* maturation (IVM), oocyte *in vitro* fertilization (IVF) with capacitated sperm and embryo *in vitro* culture (IVC) up to blastocyst stage to be transferred to recipient females or cryopreserved for future use. Consequently, the blastocyst yield obtained by IVEP programs should not be affected by the season. However, most laboratories experience periodic reductions in embryo yield throughout the year. In cat, a seasonal species, it has been observed that oocytes recovered during non-breeding season present a significantly reduced *in vitro* maturation and embryo development (Spindler and Wildt, 1999). Seasonal variations on the number of IVEP results in small ruminants have also been described. In sheep, Mara et al. (2013) observed a significantly higher *in vitro* blastocyst yield during the breeding season compared to the anoestrous season (36 *vs* 30%, respectively). However, in adult goats de Souza-Fabjan et al. (2014), obtained higher oocyte cleavage and blastocyst development from oocytes recovered during the non-breeding season. It is well known the crucial impact of oocyte quality on the IVEP success assessed by the percentage of blastocyst reached.

Oocyte quality presents a close and positive relationship with the follicular size from which the oocyte comes. Because of the close and physical relation between oocyte and follicular fluid (FF) the composition of this fluid can be determinant in oocyte competence (Bender et al., 2010; Dumesic et al., 2015; Leroy et al., 2012). Fatty acids are an important compound of the FF because they are a considerable source

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of energy for the growing and developing oocytes (Fouladi-Nashta et al., 2007; McKeegan and Sturmey, 2012). However, some studies have showed that elevated concentrations of fatty acids could be toxic and impair oocyte development. Lipotoxicity could affect oocyte competence by inducing endoplasmic reticulum stress and altering cell metabolism (Alves et al., 2015; Latham, 2015). Thus, fatty acid profile appears to have a predictive value of oocyte competence (O'Gorman et al., 2013). In ruminant, the most prevalent FAs on follicular fluid are linoleic, oleic and palmitic acids (Sinclair et al., 2008). These FAs are also the most prevalent in oocyte composition (Dunning et al., 2014). Different FAs have been used as biomarker of oocyte quality. Thus, total saturated fatty acids (SFAs) and palmitic acid were significantly higher in FF of poor quality oocytes (Matoba et al., 2014). On the other hand, polyunsaturated fatty acids (PUFAs), including omega-6 (n6) and omega-3 (n3) essential FAs, affect oocyte quality and its ability to develop until blastocyst stage (Wathes et al., 2013). In addition, reduced n6:n3 PUFA ratios in FF are associated with good oocyte quality (Dumesic et al., 2015).

Regarding these facts, the aim of this study was to assess 13 fatty acid concentrations in follicular fluid of prepubertal goat follicles during the four seasons. Moreover, we analyzed the blastocyst development after IVF and parthenogenic activation during the four seasons in order to determine if the FAs could influence the developmental capacity of prepubertal oocytes.

2. Materials and methods

2.1. Chemicals and reagents

All the chemicals were purchased from Sigma-Aldrich Chemicals Co (St. Louis, USA) unless otherwise specified.

2.2. Oocyte and follicular fluid collection

All experiments were performed during the four seasons: spring (March 21 to June 20; 18.8 °C main temperature), summer (June 21 to September 20; 23.1 °C main temperature), autumn (September 21 to December 20; 13.9 °C main temperature) and winter (December 21 to March 20; 10.3 °C main temperature).

Ovaries from prepubertal goats were collected from a local slaughterhouse (41° 29′ 50.766″ N latitude and 1° 57′ 31.682″ E longitude) and transported in Phosphate Buffered Saline (PBS) at 35–37 °C. Commercial goat meat in Spain comes from suckling kids younger than 45 days old, with a live weight from 7 to 10 Kg. Once in the laboratory, the ovaries were washed three times in PBS at 37 °C. Cumulus oocyte complexes (COCs) were recovered by slicing the surface of the ovary in TCM-199 medium containing HEPES, sodium bicarbonate and antibiotic-antimycotic (AB). COCs with homogeneous cytoplasm and three or more compact cumulus cells layers were selected for *in vitro* maturation.

Follicular fluid was recovered by aspiration of follicles larger than 2 mm. The FF was centrifuged at 500 g for 20 min. The supernatant was immediately recovered and frozen in eppendorf at −80 °C. Follicular fluid was recovered three times per season taking into account to recover it at the midpoint of each season.

2.3. Fatty acid analysis of follicular fluid

Fatty acids were analyzed by chromatography using Sukhija and Palmquist (1988) protocol with some modifications. Briefly, 200 µL of sample (100 µL FF + 100 µL PBS) with 450 µL toluene, 50 µL of nonadecanoic acid (C:19, 767 µg/mL in toluene) as internal standard, and 1 mL methanolic HCl (5%) was vortexed for 60 s and then warmed in a water bath for 1 h at 70 °C. Subsequently 1.25 mL K_2CO_3 (12%) and 500 µL toluene were added, vortexed for 30 s and centrifuged for 5 min at 1000 G. Finally the supernatant (organic matter) was recovered and dried with Na_2SO_4. The extracted samples were maintained at -20 °C until gas chromatographic analysis, on an Agilent 6890, with a chromatographic column Agilent DB23 60 m × 0.32 mm × 0.25 µm. For each sample, 2 µL were injected using pulsed split-less mode, with oven initial and final temperatures of 140 and 250 °C during 93 min. Fatty acids were identified and quantified comparing the retention time of samples in the column with the ones of commercial standards Supelco 37 FAME Mix and Supelco cis-11-Vaccenic Methyl ester. A total of thirteen fatty acids were analyzed. Specifically, five saturated fatty acids (SFAs): myristic (C14:0), pentadecanoic (C15:0), palmitic (C16:0), margaric (C17:0) and stearic (C18:0) acids; three monounsaturated fatty acids (MUFAs): palmitoleic (C16:1), oleic (C18:1n9c) and vaccenic (C18:1n11c) acids; three n3 polyunsaturated fatty acids (n3 PUFAs): linolenic (C18:3n3; ALA), eicosapentaenoic (C22:5n3; EPA) and docosahexaenoic (C22:6n3; DHA) acids; and two n6 polyunsaturated fatty acids (n6 PUFAs): linoleic (C18:2n6c; LA) and arachidonic (C20:4n6) acids. Three samples of follicular fluid were analyzed in each season.

2.4. Oocyte in vitro maturation

Groups of 30–35 COCs were washed in *in vitro* maturation (IVM) medium and cultured in 100 µL drop for 24 h at 38.5 °C, in 5% CO_2 humidified air atmosphere. IVM medium was consisted of TCM-199 supplemented with 5 µg/mL LH, 10 µg/mL FSH, 1 µg/mL 17β-E_2, 10 ng/mL epidermal growth factor, 0.2 mM sodium pyruvate, 2 mM L-glutamine, 100 µM cysteamine, 1% AB and 10% of fetal bovine serum (FBS).

2.5. In vitro fertilization

Fresh semen was obtained by artificial vagina from three bucks with proven fertility. Only ejaculates with good mass motility were used (David et al., 2015). The motile sperm fraction was selected by centrifugation (20 min at 300 ×*g*) in a density gradient (Nidacon®International AB). The pellet was re-suspended in washing medium and subsequently capacitated in modified Defined Medium (mDM (Younis et al., 1991)) containing heparin (final concentration 50 µg/mL) during 30 min at 38.5 °C in a 5% CO_2 humidified air atmosphere.

A total of 2633 *in vitro* matured oocytes were co-incubated during 20 h with a final concentration of 4×10^6 sperm/mL in fertilization medium (modified Tyrode's medium, TALP (Parrish et al., 1986)) supplemented with 1 µg/mL hypotaurine and 0.3 mg/mL glutathione.

2.5.1. Pronuclear (PN) assessment of zygotes

To assess the nuclear stage of IVF zygotes, a sample of zygotes (17 h post insemination) were completely denuded and fixed in ethanol:acetic acid (3:1) and stained with 1% of orcein in 45% acetic acid. Zygotes were categorized as normally fertilized if one female and male pronuclei with a sperm tail were visible (2 P N), polyspermic if three or more pronuclei (3 P N) were observed, and non-fertilized if no pronuclei or Metaphase II spindle was observed. A total of 461 oocytes were *in vitro* fertilized and after 17 h post insemination were evaluated for nuclear staining.

2.6. In vitro embryo culture

After 20 h of IVF, 2172 presumptive zygotes were completely denuded by gently pipetting and cultured in 2 µL/zygote of synthetic oviductal fluid (SOF) (Holm et al., 1999) supplemented with 10% (v/v) of fetal bovine serum and incubated at 38.5 °C, 5% CO_2 and 5% O_2 in a humidified atmosphere during 8 days. Embryo cleavage was assessed 48 h post fertilization. Embryo culture media was changed at day 5.