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In vitro embryo production from early antral follicles of goats fed with a whole full-fat linseed based diet

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Research Article

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Summary

The present study aimed to use an *in vitro* follicle culture (IVFC) biotechnique as a tool to evaluate the influence of whole flaxseed as a feed supplementation in the diet on the *in vitro* development of caprine early antral follicles (EAFs) and further embryo production. In total, 18 adult goats were homogeneously allocated into two diet groups: Control and Flaxseed. EAFs from both experimental groups (300–400 µm) were isolated and cultured *in vitro* for 18 days. After IVFC, recovered cumulus–oocyte complexes were submitted to *in vitro* maturation, and subsequently to IVF and *in vitro* embryo culture. The endpoints evaluated were follicular growth and morphology, oocyte recovery rate and diameter, sperm penetration, pronuclei formation, embryo development, and estradiol production. The addition of the whole flaxseed in the diet did not affect ($P > 0.05$) follicular growth and diameter. A higher ($P < 0.05$) percentage of oocytes ≥ 110 µm was recovered from the flaxseed treatment. However, the sperm penetration rate was higher ($P < 0.05$) in the control treatment when compared with the flaxseed treatment, but no differences were found regarding the rate of fertilization nor cleaved embryos. In conclusion, dietary flaxseed increased the recovery rate of fully grown oocytes, but it did negatively affect the sperm penetration rate, even though there was no further effect on the cleavage rate.

Introduction

The developing *in vitro* follicle culture (IVFC) biotechnique aims to mimic ovarian folliculogenesis *in vivo*. This biotechnique could be used to maximize the number of potentially fertilizable oocytes for assisted reproductive technologies in human, and for *in vitro* embryo production in livestock (Figueiredo *et al*., 2011). Also, IVFC may serve as an *in vitro* model for toxicological research (Stefansdottir *et al*., 2014), limiting the number of experimental animals needed.

It has been shown that IVFC efficacy may be influenced by many variables, such as base medium composition and supplementation (Ferreira *et al*., 2016), the animal model, and the follicular category (Cadenas *et al*., 2017). Nonetheless, the vast majority of the studies have been performed using ovaries from a slaughterhouse. As a result, the ovaries used in IVFC are from animals of different ages, breeds, and physiological status (Silva *et al*., 2014). Under these conditions, it is impossible to determine, for instance, the effect of nutrition, another essential variable on *in vitro* follicle development.

In vivo, the association between nutrition and reproduction has been widely described, and it is accepted that a diet with a deficiency, excess, or imbalance in energy, such as proteins, vitamins, and minerals may compromise reproductive efficiency (Mostafa *et al*., 2020). In this sense, dietary fatty acids may positively affect reproduction in ruminants (Mattos *et al*., 2000). However, the relationship between the lipid content in the diet, specifically polyunsaturated fatty acids (PUFAs), and oocyte quality is still controversial (Fouladi-Nashta *et al*., 2007; Fernandes *et al*., 2014), although the presence of PUFAs in the follicular fluid has been positively correlated with oocyte competence (Matoba *et al*., 2014). Nevertheless, despite the importance of PUFAs for reproduction, to the best of our knowledge, there has been no information about the effect of a diet rich in PUFAs on *in vitro* follicle development.

Among the different dietary supplementation protocols, several lipid sources have been tested to promote an increase in the energy density of the diet and positively influence reproduction. Flaxseed has been highlighted as an important source of lipids and its use in animal feed

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(*c.* 10% of dry matter) is mainly due to the high concentration of PUFA in its composition and the potential effects of this lipidemic profile on animal reproduction (Bernacchia *et al.*, 2014). Recently, it was demonstrated that supplementation with flaxseed in goats was able to increase the rate of *in vivo* follicular growth in goats (Alves *et al.*, 2019), as well as change the follicular fluid environment, the expression of oocyte mitochondrial genes and *in vitro* embryo development (Alves *et al.*, 2021).

Therefore, this study aimed to use the *in vitro* follicle culture (IVFC) biotechnique as a tool to evaluate the influence of whole flaxseed as a feed supplementation in the diet on the *in vitro* development of caprine early antral follicles (EAFs) and further embryo production. The endpoints evaluated were follicular daily growth and diameter, estradiol production, oocyte recovery rate and diameter, sperm penetration, pronuclei formation, and embryo development.

Materials and methods

Reagents

Unless mentioned otherwise, all chemicals and reagents used in the current study were purchased from Sigma-Aldrich Chemical Co. (St. Louis, Mo., USA).

Animals and experimental design

All procedures in this study were approved by the Ethics Committee in Animal Experimentation of the Ceará State University, Brazil (no. 3047564/2017, CEUA-UECE).

In total, 18 Anglo-Nubian crossbred, non-lactating adults, between second and third birth, were selected for cyclicity.

Goats were allocated to two diet groups: control and flaxseed, according to homogeneity in body weight, (33.4 ± 3.6 kg; overall mean ± standard deviation (SD)), body condition scores (2.7 ± 0.3, from 1 to 5), and ages (45.5 ± 7.25 months). In the control treatment ($n = 9$), the diet consisted of elephant grass hay (*Pennisetum purpureum*) and commercial concentrate with ground corn grain, soybean meal, wheat bran, and mineral mixture. In the flaxseed treatment ($n = 9$), it was added at 30% to the concentrate dry matter basis of whole full-fat linseed. The total lipids of the diets were 2.8 % and 8.5% on a dry matter basis, respectively, for the control and flaxseed groups, and were isonitrogenous (13% crude protein to dry matter basis). The composition of the experimental diets and their chemical composition are presented in Table 1.

Animals from each group, homogeneously segregated for use in three replicates were kept in collective stalls (Fernandes *et al.*, 2014), receiving mineral salt and water *ad libitum*. In total, seven, six, and five animals were used in the first, second, and third replicates, respectively. In all the experimental groups, diets were provided twice a day (07:00 h and 15:00 h) for 30 days, up to follicle recovery. All animals received the diets to satisfy their energy requirements for maintenance and breeding according to the National Research Council (NRC, 2007) for adult non-dairy does.

Table 1. Ingredient composition of the concentrate-based diets

Parameters	Diet	
	Control	Flaxseed
Ingredients, %		
Elephant grass hay	40.0	40.0
Ground corn grain	34.5	20.3
Soybean meal	12.5	4.0
Wheat bran	10.0	14.8
Mineral mixture	3.0	3.0
Whole full-fat linseed	–	17.0
Chemical fraction, g.kg DM $^{-1}$		
Dry matter	892.1	878.5
Crude protein	133.1	133.9
Ether extract	28.2	84.6
Ash	82.0	84.4
Neutral detergent fibre	704.1	703.1
Acid detergent fibre	322.6	345.6

Collection of ovaries, isolation, selection and culture of early antral follicles

After 30 days of feeding, the animals were randomly selected for slaughter. The goats' reproductive tracts were collected immediately post mortem. Ovaries from both groups were collected and transported to the laboratory as previously described (Chaves *et al.*, 2008). For the IVFC and oocyte *in vitro* maturation (IVM) we used the methodology previously described by our group (Cadenas *et al.*, 2017). Briefly, in the laboratory, EAFs (300–400 µm) from each dietary treatment, i.e. control and flaxseed were isolated and individually cultured *in vitro* in 100-µl drops of α-MEM (pH 7.2–7.4), supplemented with 3 mg/ml bovine serum albumin (BSA), 10 ng/ml insulin, 5.5 µg/ml transferrin, 5 ng/ml selenium, 2 mM glutamine, 2 mM hypoxanthine, 50 µg/ml ascorbic acid, and 50 ng/ml growth hormone (GH) for 18 days at 38.5°C and 5% CO_2 in air under mineral oil. The fresh medium was prepared and pre-equilibrated overnight before use. The medium was replaced partially (60 µl) every other day.

Morphological evaluation of follicle development: follicle survival, daily growth, and levels of estradiol

The classification of normal or degenerated follicles was performed every 6 days based on their morphological aspects, and those showing morphological signs of degeneration, such as darkness of the oocytes and surrounding cumulus cells, or misshapen oocytes, were classified as degenerated. The average of two measurements (height and length) of the follicle was used as a measure of follicle diameter. The daily growth rate was calculated based on the diameter changes over the culture period (18 days).

At the end of the culture period, levels of estradiol were measured in spent medium using a competitive immunoassay commercial kit (enzyme linked fluorescence assay VIDAS, Biomerieux, Marcy l'Etoile, France). The analytical sensitivity of the E2 was 9 pg/ml (range, 9–3000 pg/ml) and the intra-assay coefficient of variation was 5%.

In vitro maturation (IVM) and fertilization (IVF)

All follicles were mechanically opened and only those oocytes ≥ 110 µm in diameter (zona not included) were submitted to IVM in groups of 10 cumulus–oocyte complexes (COCs)/100-µl drop) for 30 h at 38.5°C and 5% CO_2 in air under mineral oil. The IVM medium consisted of tissue culture medium 199 supplemented with 1 µg/ml 17β-estradiol, 5 µg/ml luteinizing hormone, 0.5

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Table 2. Percentages of morphologically normal, extruded and degenerated follicles, daily growth, and estradiol production at the end of *in vitro* culture (day 18)*

Treatments	MIF (%)	Ext (%)	Deg (%)	Daily growth (µm) (mean ± SEM)	E2 (ng/ml) (mean ± SEM)
Control ($n = 100$)	78.0 (78/100)	8.0 (8/100)	14.0 (14/100)	22.57 ± 1.6	36.37 ± 13.1
Flaxseed ($n = 76$)	85.5 (65/76)	6.6 (5/76)	7.9 (6/76)	23.77 ± 1.4	53.69 ± 37.7

*There was no difference between control and flaxseed treatments
Abbreviations: Deg, degenerated follicles; E2, estradiol; Ext, extruded follicles; MIF, morphologically intact follicles.

µg/ml rFSH (bovine), 10 ng/ml epidermal growth factor, 1 mg/ml BSA, 1 mM pyruvate, 50 ng/ml, insulin-like growth factor 1, and 100 mM cysteamine.

After the IVM, all COCs were washed and transferred in groups of 10 into 100-µl drops of fertilization medium under mineral oil. The fertilization medium consisted of IVF-TALP (Parrish *et al.*, 1986) supplemented with 30 µg/ml heparin (Calbiochem 375095), 15 µM hypotaurine, and 5 µg/ml gentamicin. The IVF-TALP medium was pre-equilibrated for at least 2 h before use. Refrigerated semen diluted in extender from two fertile bucks were pooled and motile sperm were selected by the swim-up procedure (Fukui *et al.*, 2000) in Sperm-TALP medium (Papa *et al.*, 2015). Viable sperm were diluted in the appropriate volume of fertilization medium to achieve a final concentration of 2×10^6 sperm/ml. Spermatozoa and COCs were co-incubated for 18 h at 38.5°C in a humidified atmosphere of 5% CO_2.

In vitro *embryo culture, transfer and pregnancy diagnosis*

After the IVF, all presumptive zygotes were washed and transferred to microdrops of embryo culture medium (G1™, Vitrolife, Gothenburg, Sweden) (10 zygotes: 10 µl medium) under mineral oil, and incubated for 2 days at 38.5°C in a humidified atmosphere of 5% CO_2 and 5% O_2. On day 3 post-IVF, all cleaved embryos were surgically transferred into the oviduct of three synchronized recipient goats, as previously reported by Sá *et al.*, (2020). Pregnancy diagnosis was performed on day 36 after transfer by transrectal ultrasonography.

Statistical analysis

Statistical analysis was carried out using Sigma Plot 11 (Systat Software Inc., USA). Comparison of means (follicle and oocyte diameters, estradiol and daily growth rate) between treatments were analyzed using *t*-test. One-way repeated measures analysis of variance (Holm–Sidak post-hoc test) was performed to compare the effect of treatment among days of culture. When appropriate, chi-squared or Fisher's exact tests were used to evaluate the percentage variables (*in vitro* fertilization parameters, intact, degenerated, and extruded follicles) between treatments. Data are presented as mean [± standard error of the mean (SEM)] and percentage, and the statistical significance was defined as $P < 0.05$ (two-sided).

Results

Follicle morphology and growth after in vitro culture

The results of follicle morphology, growth rate, and estradiol production are summarized in Table 2.

There was no influence of the administration of the whole flaxseed in the diet on follicle morphology, growth rate, and estradiol production. Both treatments, control, and flaxseed, increased ($P < 0.05$) follicle diameter from day 0 (368.12 ±7.8 and 376.28 ± 8.1 µm, respectively) to day 18 (779.70 ± 25.6 and 783.71 ± 25.7 µm, respectively). Also, follicle daily growth rate was lower ($P < 0.05$) in the first 6 days of culture than from day 6 onwards (10.36 ± 1.1 and 10.90 ± 1.1 vs. 34.76 ± 2.2 and 34.35 ± 2.0 µm/day, respectively).

Oocyte parameters, evaluation of in vitro *embryo development, transfer, and pregnancy diagnosis*

The results of oocyte diameter, percentage of oocytes ≥ 110 µm, sperm penetration, male pronucleus formation, two pronuclei formation, and cleaved embryos after the IVFC of caprine EAFs are summarized in Table 3.

After the IVFC, the recovery rate of oocytes ≥ 110 µm was higher ($P < 0.05$) in the flaxseed treatment when compared with control group. However, oocytes from goats fed with flaxseed based diet showed lower ($P < 0.05$) overall sperm penetration with no further effect on the cleavage rate.

Regardless of the diet, most of the fertilized oocytes showed only one pronucleus (MPN) and only five embryos were produced on day 3 post-IVF, three at the 4-cell to 6-cell stage, and two at the 2-cell to 3-cell stage. All five embryos were surgically transferred into the oviduct of three recipient goats. At 36 days after the embryo transfer, none of the recipient showed signs of estrous behaviour, however no pregnancy was detected after ultrasonography examination.

Discussion

It is well established that lipid supplementation in the diet of ruminants affects ovarian activity (Robinson *et al.*, 2002). In this context, different lipid sources have been tested in animal feed, as the composition of fatty acids seems to have a crucial role in specific reproductive processes, including the follicular fluid environment (Alves *et al.*, 2019), follicular development (Childs *et al.*, 2008), hormonal production (Robinson *et al.*, 2002), oocyte quality (Fernandes *et al.*, 2014) and embryo development (Leroy *et al.*, 2005). Our study aimed to evaluate for the first time the efficiency of the use of whole flaxseed, an oilseed rich in polyunsaturated fatty acids, in the diet of goats, on the *in vitro* development of isolated EAFs and subsequent embryo production. The current results showed that there was no influence of the administration of the whole flaxseed in the diet on follicle morphology, growth rate, and estradiol production. However, a higher recovery rate of oocytes ≥ 110 µm was observed in the flaxseed treatment, with lower overall sperm penetration in the oocytes. Despite this, no effect of the diet was observed on the embryo cleavage rate.

Recently, several studies have highlighted the composition of flaxseed and its potential for animal feed as a lipid source, especially in the possible effects on reproduction (Alves *et al.*, 2019,

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Table 3. Oocyte diameter, percentage of oocytes ≥ 110 µm, sperm penetration, male pronucleus formation, two pronuclei formation, and cleaved embryos after the IVFC of caprine EAFs

Treatments	Oocyte diameter (mean ± SEM)	Oocytes ≥ 110 µm (%)	Oocyte fertilization (%)*			Cleaved (%)†
			Sperm penetration	MPN*	2PN*	
Control (n = 100)	120.1 ± 1.0	62.0 (62/100) ^{A}	40.3 (25/62) ^{B}	76.0 (19/25)	24.0 (6/25)	12.0 (3/25)
Flaxseed (n = 76)	119.0 ± 0.9	76.3 (58/76) ^{B}	20.7 (12/58) ^{A}	75.0 (9/12)	25.0 (3/12)	16.7 (2/12)

*Only oocytes ≥ 110 µm were submitted to IVM, IVF and IVC.
†Values calculated out of the overall fertilized oocytes.
^{A,B}Within a column ($P < 0.05$).
Abbreviations: 2PN, two pronuclei; EAFs, early antral follicles; IVFC, *in vitro* follicle culture; MPN, male pronucleus.

2021). To date, studies have been limited to the effects on follicular dynamics, oocyte quality, embryo development, metabolic parameters in plasma and follicular fluid, and oxidative stress, with no study evaluating the possible effects on *in vitro* follicular development. In the present study, the use of whole flaxseed in the diet of goats did not significantly affect follicle morphology and growth after *in vitro* culture and estradiol production. Similar results were shown *in vivo* where dietary PUFAs did not affect either follicular dynamics or steroid production in bovine (Childs *et al*., 2008). Also, PUFAs had no effect on granulosa cell proliferation and steroidogenesis during *in vitro* culture in ovine (Wonnacott *et al*., 2010) and bovine (Lammoglia *et al*., 1997). Conversely, several authors have shown that increasing dietary PUFAs increased the size of preovulatory follicles and the total number of follicles (Beam and Butler, 1997).

Both treatments, control and flaxseed, increased ($P < 0.05$) follicle diameter from day 0 to the end of the culture period, and also follicle daily growth rate was lower ($P < 0.05$) in the first 6 days of culture than from day 6 onwards. These growth patterns (first third of culture vs. second and last third) are in agreement with previous published papers under the same culture conditions (Cadenas *et al*., 2018). However, compared with a previous study performed by our group, the end points: percentage of morphologically normal follicles (~80 vs. 65%), follicle diameter (~780 vs. ~550 µm) and growth rates (~23 vs. ~11 µm/day) showed higher figures when using ovaries from controlled well nourished animals (present study) compared with ovaries from slaughterhouse (Cadenas *et al*., 2017). It is well known that animal nutritional status (Abecia *et al*., 2006) and age (Ottolenghi *et al*., 2004) can profoundly affect *in vivo* follicle development. Therefore, the use of a homogenous group of animals (same body weight, body condition score, and age) could contribute to provide a high quality starting material for the IVFC.

There are vast reports in the literature about how the composition of follicular fluid affects follicular development, as well as the quality of the oocyte and embryo (Sinclair *et al*., 2008). Moreover, there is strong evidence that maternal metabolic condition and diet are one of the factors that most influence the follicular microenvironment (Valckx and Leroy, 2015). Fat supplementation with unsaturated fatty acids, for example, is considered one of the main strategies for modulating the lipidic composition of follicular fluid (Zachut *et al*., 2008). According to Wathes *et al.* (2007), the amount and proportion of different unsaturated fatty acids in the reproductive tissues reflect the animal's dietary consumption, and these unsaturated fatty acids influence reproductive processes through a variety of mechanisms, including the provision of precursors for the synthesis of prostaglandins, as well as the modulation of the expression patterns of many key enzymes involved in the metabolism of steroids. Using a diet contained an amount of lipids similar to that of present study, Fernandes *et al.* (2014) observed a higher concentration of total cholesterol in the follicular fluid of goats compared with the control group. Also, previous studies have shown that a diet rich in lipids, with flaxseed as a lipid source, was able to change the lipid profile of the plasma, as well as affect the expression of genes related to the accumulation of lipids in the granulosa cells (Alves *et al*., 2019) and the concentration of glutathione peroxidase in the follicular fluid (Alves *et al*., 2021).

The effect of lipid supplementation, including a PUFA-rich diet, on oocyte quality has been widely studied in ruminants. There is a consensus that the effects are mediated by changes in follicular fluid fatty acids composition (Childs *et al*., 2008; Fouladi-Nashta *et al*., 2007). However, according to Santos *et al.* (2008), changes in the composition of oocyte fatty acids are relatively small, suggesting a possible mechanism of selective uptake of PUFAs. After the IVFC, the recovery rate of oocytes ≥ 110 µm was higher ($P < 0.05$) in the flaxseed treatment. The beneficial effect of the whole flaxseed in the diet on oocyte growth may be due to the fact that, among other important functions, fatty acids are stored within the oocyte and cumulus cells, providing a potent source of energy via β-oxidation (Dunning *et al*., 2014). However, oocytes from goats fed with flaxseed based diet showed lower ($P < 0.05$) overall sperm penetration. This result is consistent with other studies that associated elevated fatty acid concentration in diet with low oocyte competence (O'Callaghan *et al*., 2000; Wakefield *et al*., 2008). Conversely, Cardoso *et al.* (2019) feeding cows with flaxseed for a long period found no differences in oocyte viability. In general, the relationship between fatty acids and oocyte quality is still controversial, as some studies showed positive effects (Fouladi-Nashta *et al*., 2007; Matoba *et al*., 2014), while in others no effect was observed (Fernandes *et al*., 2014). Percentages of normal fertilization (2PN) and cleaved embryos on day 3 post-IVF were not affected by the presence of the whole flaxseed in the diet. Alves *et al.* (2021), feeding goats with a similar amount of flaxseed for the same period, also found no difference in the cleavage rate and proportion of blastocysts compared with the control group. Similar results were described in caprine when another important source of PUFAs, cashew walnuts, was added to the diet (Fernandes *et al*., 2014). The low embryo production when using *in vitro* grown caprine oocytes has been described before (Saraiva *et al*., 2010; Silva *et al*., 2014). However, those previous studies showed low oocyte nuclear maturation as well, unlike our system, which was able to produce a considerable amount of metaphase II oocytes (Cadenas *et al*., 2017). In this current study, 40% of the *in vitro* grown oocytes were fertilized, even though most of the fertilized oocytes formed only MPN. To our knowledge, this is the highest

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fertilization rate described in this species so far for *in vitro* grown oocytes. These findings indicated that the oocytes were penetrated by the spermatozoa and were able to contribute toward MPN formation. However, the second meiotic resumption did not occur appropriately, preventing normal female pronuclei formation and subsequent cleavage. Taken together, these facts suggest that oocyte developmental competence (oocyte cytoplasmic maturation) should be improved.

In conclusion, except for the increase in the percentage of fully grown oocytes, in general, dietary flaxseed did not affect goat *in vitro* folliculogenesis. However, it did negatively affect the sperm penetration rate, even though with no further effect on the cleavage rate. Also, our current IVFC system was able to produce a considerable amount of meiotically matured oocytes that were able to be fertilized. Nevertheless, the low percentage of oocytes showing normal fertilization and embryo production highlights the need for further research to improve oocyte cytoplasmic maturation.

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Ethical standards. All procedures in this study were approved by the Ethics Committee in Animal Experimentation of the Ceará State University, Brazil (no. 3047564/2017, CEUA-UECE).

Conflicts of interest. The authors declare no conflicts of interest.

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