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Research Article

Effects of Various Levels of Luteinizing Hormone and Caprine Follicular Fluid on In Vitro Embryo Production of Shami Goat

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Abstract
In the current study, the hypothesis of the effects of luteinizing hormone (LH) and follicular fluid (FF) derived from follicles of varying size on in vitro embryo production of the Shami goat breed were tested. The caprine follicular fluid (cFF) was obtained from healthy female's ovaries by aspiration method and classified into two main classes (follicles with a diameter of ≤ 2mm and ≥3mm). The resulting cFF was added to the culture medium TCM-199 through six Treatments (A, B and C with a source of follicle size of ≤ 2mm; D, E and F with a source of size of ≥3mm). LH was added only to four of the previous Treatments with the levels of 50 µg ml^{-1} (B and E) and100 µg ml^{-1} (C and F). Results of the study showed that the oocytes incubated in Treatment F achieved a clear superiority (p=0.001) in the rates of maturation (87.0%), fertilization (80.0%) and cleavage (82.3%). The oocytes incubated in the same Treatment (F) continued to outperform (p= 0.006) by achieving the best rates across cleavage stages at 2-16 cell (16%; the lower value of arrest) and blastocyst (42%). Significant differences (P=0.03) were observed among the rates of Type 1embryos (the highest rate: 45.3%; Treatment F) and Type 3 embryos (the highest rate: 45.1%; Treatment A). No significant differences were observed in the rates of morula and Type 2 embryos. It is advised to add 15% of the cFF derived from follicles with a diameter of ≥3mm and 100 µg of LH ml^{-1} in the maturation media to obtain higher rates of maturation and cleavage of goat oocytes.

Keywords
Follicular fluid, goat, luteinizing hormone, oocytes

INTRODUCTION

Shami goat is one of the five best international goat breeds that characterized by the production of milk and the ability to give birth twice a year in appropriate environment. The breed raising goes back to the original and local habitat in Syria, from about 70 years it was exported to Cyprus to raise the level of the Cypriot goats in terms of production efficiency of meat and dairy. Due to the economic importance of goat breeds, there has been a great deal of research related to raising the productivity. In the field of assisted reproductive techniques (ART), many studies have been carried out concerned with increasing the yield of in vitro-produced embryos of this breed with the aim of producing individuals who are productively superior and have a high genetic potential.

FF is an important natural biological media due to the richness in steroid hormones in addition to nutrients and growth factors (Tripathi et al., 2015). FF plays the primary regulating role in the growth and development of oocyte during the folliculogenesis stage until the oocyte acquires the developmental competence in preparation for fertilization and subsequent divisions. LH is a glycoprotein hormone that is secreted with follicle-stimulating hormone (FSH) by the anterior pituitary gonadotrophs (Nedresky et al., 2020). LH plays the main role in folliculogenesis and oocyte maturation and the timing of exogenous LH activity administration in controlled ovarian stimulation (Filicori et al., 1986).

This study aimed to compare the combined levels of LH and source of cFF to determine which treatment that are capable of maturation, fertilization and cleavage of Shami goat oocytes. In addition, this study aimed to study the embryo quality.

MATERIAL and METHODS

Animals and ovaries collection

The ovaries were collected from the local slaughterhouses in the city of Aleppo. The ovaries were selected from healthy females whose ages ranged between 2-4 years; during the breeding season. The ovaries were kept in saline solution (9%) in a specially designed thermos at 39 °C. The ovaries were transferred directly to the biotechnology laboratory at the University of Aleppo - Faculty of Agriculture within a period not exceeding 1 hour during each collection process.

Oocytes collection

The oocytes were obtained by the slicing method, where many incisions were made in several directions on the entire surface of the ovary by a special blade in several directions. After words, the ovaries were washed with TCM-199 culture medium supplemented with heparin at 9 °C. The resulting solution was received in Petri dishes. The oocytes (cumulus oophorus complexes (COCs) were examined under a microscope at 300x magnification and collected by the micropipette. Healthy oocytes surrounded by more than three layers of cumulus cells were selected for further work.

cFF collection

According to the size of the follicle, the cFF was aspirated by a 5 ml syringe connected to a needle. The total cFF was received in test tube and centrifuged for 10 minutes and kept at a temperature of 12 °C for further use.

Experimental design

According to the source of cFF, the size (diameter) of the follicle was assigned in two basic classes: ≤ 2 mm, and ≥ 3 mm. The levels of the LH and the volume of the cFF added to the culture medium TCM-199 were determined according to six Treatments as shown in Table 1. The two groups A and D were considered as control groups to determine the effect of the cFF source on the studied traits. The experiment follows the one-factor experimental design (levels of LH and source of cFF on a set of traits (IVM, IVF, cleavage and embryo quality).

Table 1. Levels of the LH and the volume of the cFF added to the culture medium TCM-199

Levels and volumes	A	B	C	D	E	F
LH (µg /ml)	-	50	100	-	50	100
cFF: ≤ 2 mm (ml)	15	15	15	-	-	-
cFF: ≥ 3 mm (ml)	-	-	-	15	15	15

In vitro maturation (IVM)

The oocytes were subjected to IVM as described previously by Dos Santos-Neto et al. (2020) with some modifications. Briefly, COCs were washed three times in TCM HEPES and twice in TCM-199 supplemented with 0.25 mmol l^{-1} sodium pyruvate, 2 ng ml^{-1} epidermal growth factor (EGF), 5 ng ml^{-1} follicle stimulating hormone (FSH) and penicillin/streptomycin (200 U ml^{-1} penicillin, 200 µg ml^{-1} streptomycin). Each Treatment was added to the previous formula of maturation (TCM-199, Earle's salt with L- glutamine and sodium bicarbonate; Invitrogen, USA) so that the final volume became 100 ml. Oocytes were matured in groups of 5 per 50 µl droplets in culture dishes under mineral oil. Incubation lasted for 27h at 39 ºC and 5% CO_2 in air with 100% humidity.

In vitro fertilization (IVF)

Following maturation, oocytes were transferred to TCM-199 maturation medium supplemented with 300 IU ml^{-1} hyaluronidase. Next, a gentle pipetting was done to remove surrounding cumulus cells of oocytes and washed three times in Tyrode's albumin lactate pyruvate (TALP). Straws of frozen semen of proven Shami bucks were used in IVF. The sperms have undergone a capacitation process. Briefly, the semen was washed in TALP medium and centrifuged twice in a Percoll gradient (2 ml at 45% over 2 ml at 90%) for 25 min at 700 X× g at room temperature. The viable spermatozoa at the bottom were collected, washed in TALP and subjected again to centrifugation at 200 X× g for 18 minutes at room temperature. The resulting aliquots of spermatozoa were diluted to give a concentration of 5 X 10^6 spermatozoon/ml. Afterword, the final concentration (2 X 10^6 spermatozoon ml^{-1}) was obtained by adding 250 µl of the previous suspension in TALP medium to each fertilization well. COCs were incubated for 24 h in 5% CO in humidified air at 39 °C (De Oliveira Bezerra et al., 2019; with some modifications). After the incubation period, the fertilized oocytes (zygotes) were investigated by the emergence of the two pronuclei. A microscope with a magnification of 300X marginations was used for this purpose.

In vitro culture (IVC)

IVC took place according to Vajta et al. (1999) with some modifications. Very briefly, the presumptive zygotes were washed three times in 100 µl modified synthetic oviduct fluid (SOF) HEPES medium before being transferred to the SOF culture media droplets (in groups of 1-3 zygotes μl^{-1} medium). Within 48 hours of culture up to the eighth day, embryos were investigated in different stages (2-16 cell, morula and blastocyst; Figure 1). Embryo culture took place under mineral oil in a humidified atmosphere of 5% CO_2 and 5% O_2 and 90% N_2 at 39 ºC.

Figure 1. Stages of Shami goat early embryos. A: 2-4 cell stage, B: morula, C: blastocyst

Determination of embryo quality

Embryo quality was determined into three main classes based on Wintner et al. (2017); (Figure 2) with some modification as follows:

-Type 1: cells are of equal size; no fragmentation is seen.

-Type 2: cells are of equal size; minor fragmentation only.

-Type 3: cells are of equal or unequal size; fragmentation is moderate to heavy.

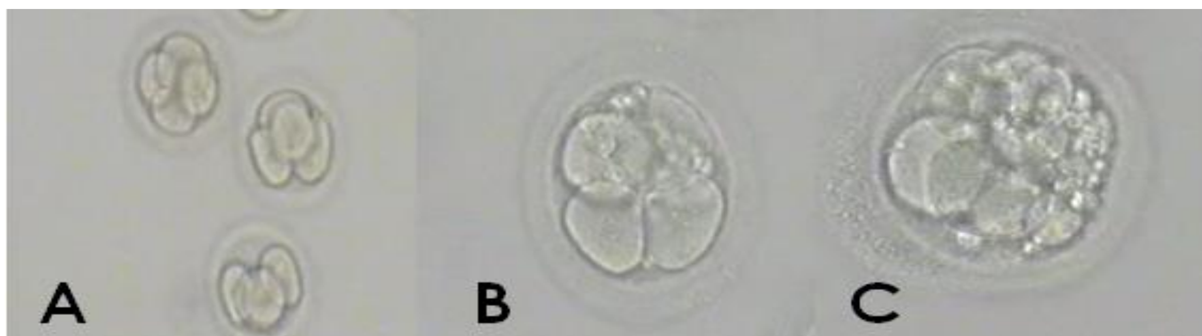

Figure 2. Types of Shami goat early embryos. A: Type 1, B: Type 2, C: Type 3

Statistical analysis

All values were expressed as percentage. Differences between parameters were evaluated by Pearson Chi-square of contingency tables. All statistical analyses were conducted using SAS Institute Inc. (2017) statistical package. The difference between rates of different traits was evaluated by using Fisher exact test.

Chemicals and reagents

All chemicals, reagents and media constituents were purchased from Sigma-Aldrich Chemicals, USA.

RESULTS

IVM, IVF and cleavage

The results of IVM, IVF and cleavage for different Treatments are presented in Table 2. The rates of oocytes at M-II stage differed significantly (p=0.0001) among the six treatments. The oocytes matured in Treatment F achieved the highest rate (87.0 %), while the lowest rate was in Treatment A (53.1%). Similarly, the rates of IVF (zygote) in the six groups differed significantly (p=0.0001). The oocytes matured in Treatment F achieved the highest rate (80.0 %). In cleavage stage, the oocytes incubated in treatment F continued to outperform the rest of the other groups (82.3%; p=0.001). Despite the significant superiority, the difference did not exceed 19.3 % between oocytes of Treatments F and A. On the other hand, the differences between the rates for the three traits between the two control groups (A and D Treatments; without LH supplementation) were not proven, despite the superiority of the oocytes in group D (55.1%; M-II, 52.6%; zygotes, 63.7%; cleavage) over that of group A (53.1%; M-II, 50.0%; zygotes, 63.0%; cleavage).

Table 2. Rates of M-II, IVF and cleavage of Shami goat oocytes under the influence of LH and cFF levels

cFF source	LH level	Incubated oocytes	M-II- oocytes		Zygotes		Cleaved oocytes	
		NO	NO	%	NO	%	NO	%
A: ≤ 2 mm	-	305	162	53.1[a]	81	50.0[a]	51	63.0[a]
B: ≤ 2 mm	50 µg /ml	320	186	58.1[a]	95	51.1[a]	62	65.3[a]
C: ≤ 2 mm	100 µg /ml	335	208	62.1[a]	110	52.9[a]	72	65.5[a]
D: ≥3 mm	-	352	194	55.1[a]	102	52.6[a]	65	63.7[a]
E: ≥3 mm	50 µg /ml	341	263	77.1[b]	165	62.7[a]	119	72.1[b]
F: ≥3 mm	100 µg /ml	316	275	87.0[b]	220	80.0[b]	181	82.3[b]
p			0.0001		0.0001		0.001	

Values with different superscript (a and b) within the same column are significantly different at the assigned probability for each column

Embryo stage

It is evident from the data of Table 3 that the arrest rates of 2-16 cell embryos stage of oocytes incubated in Treatment A were the highest (45.1%; p=0.0001) compared to the rest of the groups, while the oocytes incubated in Treatment F showed the lowest rate of arrest of 2-16 cell stage embryos (16.0%). Upon the morula stage, an increase in the rates was observed for the oocytes of the two Treatments E (43.7%) and F (42%) compared to the rest of the groups without significant difference. Down to blastocyst stage, the oocytes incubated in the two Treatments F and E achieved the highest rates (42.0% and 38.7% respectively; p=0.006). The lowest rates were at the oocytes in the two Treatments A and B (21.6% and 24.2% respectively). Also, a difference in the rates of blastocyst was observed between the oocytes of the control A and D Treatments, the difference was 4.6%.

Table 3. Rates of 2-16 cell, morula and blastocyst stages of Shami goat cleaved oocytes under the influence of LH and cFF levels

cFF source	LH level	Cleaved oocytes	Embryonic stage status 2-16 cell		Morula		Blastocyst	
		NO	NO	%	NO	%	NO	%
A: ≤ 2 mm	-	51	23	45.1[a]	17	33.3	11	21.6[a]
B: ≤ 2 mm	50 µg /ml	62	25	40.3[a]	22	35.5	15	24.2[a]
C: ≤ 2 mm	100 µg /ml	72	29	40.3[a]	25	34.7	18	25.0[a]
D: ≥3 mm	-	65	22	33.8[a]	26	40.0	17	26.2[a]
E: ≥3 mm	50 µg /ml	119	21	17.6[b]	52	43.7	46	38.7[a]
F: ≥3 mm	100 µg /ml	181	29	16.0[b]	76	42.0	76	42.0[b]
p			0.0001		NS		0.006	

Values with different superscript (a and b) within the same column are significantly different at the assigned probability for each column

Embryo quality

Data in Table 4 indicate that the embryos resulting from B Treatment achieved the highest rates in the Type 1 embryos (45.3%; p=0.03) followed by the E Treatment oocytes (38.7 %). Regarding Type 3 embryos, the rate of embryos in Treatment F oocytes decreased to a value of 23.2% (p=0.03), while the rate increased for Treatment A oocytes (45.1%). In Type 2 embryos, no significant difference was observed in the rates. In general, the difference between the highest (B Treatment; 40.3%) and lowest value (A Treatment; 25.5) was 14.8%.

Table 4. Rates of Type 1, Type 2 and Type 3 of Shami goat early embryos under the influence of LH and cFF levels

cFF source	LH level	Cleaved oocytes	Type 1		Type 2		Type 3	
		NO	NO	%	NO	%	NO	%
A: ≤ 2 mm	-	51	15	29.4[a]	13	25.5	23	45.1[a]
B: ≤ 2 mm	50 µg /ml	62	17	27.4[a]	25	40.3	20	32.3[b]
C: ≤ 2 mm	100 µg /ml	72	22	30.6[a]	22	30.6	28	38.9[ab]
D: ≥3 mm	-	65	19	29.2[a]	25	38.5	21	32.3[b]
E: ≥3 mm	50 µg /ml	119	46	38.7[b]	34	28.6	39	32.8[b]
F: ≥3 mm	100 µg /ml	181	82	45.3[c]	57	31.5	42	23.2[c]
p			0.03		NS		0.03	

Values with different superscript (a, b and c) within the same column are significantly different at the assigned probability for each column

DISCUSSION

In general, the two main mechanisms of LH and FF are that the first breaks the dormant phase of the oocytes, which are often arrested in the germinal vehicle phase (GV), while the FF is the normal environment for the development of oocytes in the follicle (*in vivo*).

In the current study, it became evident that the oocytes incubated with a mixture of 100 µg /ml LH and 15 ml cFF of ≥3 mm follicle size source (Treatment F) were the superior in IVM, IVF, cleavage and Type 1 embryo rates compared to the rest of the groups (Tables 2, 3 and 4). The current results can be traced back to a set of factors and scenarios under which both the LH and the FF work. During the process of nuclear maturation, LH surge releases oocytes from meiotic prophase arrest. Thus, the oocytes will be induced to resume the meiosis and complete of the first meiotic division (Mehlmann, 2005). More clearly, inside the follicle, when LH signals start to progress, the G protein will be activated by binding the mural granulosa cell LH receptor (LHR). This will result in the activation of the cyclic adenosine mono phosphate (cAMP). The final target in this scenario are the EGF network, CNP/NPR2 system and gap junctions (Conti et al., 2012; Jaffe and Egbert, 2017).

Another mechanism by which LH may enhance the oocytes IVM is through modifying the nutritional environment to increase the energy available for the oocyte to support subsequent development in fertilization and cleavage (Harper and Brackett, 1993). The net metabolism effect of LH exposure during maturation period could be shown as increased glycolysis combined with increased mitochondrial glucose oxidations and increased tricarboxylic acid (TCA) cycle activity within cumulus cell-enclosed oocytes (Zuelke and Brackett, 1992). In the context of ART, most studies are concerned with the main role of LH in the stage of maturation and the effect on the developmental component of oocytes only. By comparing the results of our study with some studies, in a previous study of the author (Mardenli, 2020), two levels of LH were added to maturation media (TCM-99), the IVM maturation rates of sheep oocytes were 57.69% (50 µg/ml LH) and 78.43 (100 µg/ml LH) respectively. In a study conduct by Dinopoulou et al. (2016), the rates of maturation, fertilization and cleavage under the effect of LH (1.5 IU/ml) were 47.5%, 33.8% and 45.5% respectively. By adding the LH (0.05 mg/ml) combined with cysteamine, Silva et al. (2010) obtained cleavage rates ranged between 84% and 90% and blastocyst rates ranged between 28% and 40% respectively. At a concentration of 0.1 UI/ml r-LH, Accardo et al. (2004) obtained rates of 60.7%, 55.2 and 27.0 for IVM, cleavage and blastocyst respectively.

On the other hand, the biochemical constituents of the FF surrounding the oocytes in a period of growth are the factors that determine the quality and the level of

subsequent developments to initiate subsequent IVF stages (Leroy et al., 2004). Moreover, in the very complex follicle wave scenario, the follicles that are eligible for follow-up in development processes are determined according to a system governed by the secretion of FSH according to specific levels of estradiol, insulin-like growth factors (IGFs), and inhibin/activin peptides (Ginther et al., 2001).

Also, our current results can be attributed to the fundamental relationship through which the timing of LH action and the content of FF are determined with temporal follicle development. Although FF is rich in all the elements and compounds that support the developmental competence of oocytes to complete their nuclear and cytoplasmic maturation, FF at the same time contains compounds that inhibit maturation (inhibin A and inhibin b) and this is related to the dynamic follicular wave scenario, according to which the dominant follicle is chosen (Wen et al., 2006). In relation to FF, LH is necessary in the selection of the dominant follicle. This dominance was more clear in cattle by an increasing dependence of the follicle on LH, mainly at the signaling and transcription levels (Fayad et al., 2004; Mihm et al., 2006). Some studies advise against introducing FF into the maturation medium at rates exceeding 50% due to the high arrest rates of the oocytes during IVM.

The addition of FF up to 60% to the maturation medium led to the inhibition of the cytoplasmic and nuclear development of the oocytes due to coagulating the cumulus cell mass by fibrin-like substance in FF. The lower-dose (10%) stimulated both the maturation and developmental competence of the oocytes (Kim et a1., 1993). Using porcine follicular fluid (pFF) derived from small follicles (2 to 5 mm) led to IVM rates similar to the rates of *in vivo* matured oocytes (Naito et a1., 1988; Naito et a1., 1989). The results of our current study were relatively higher than those of Dell'Aquila et al. (1997) who used an equine FF aspirated from follicles with diameters of <3 mm (68.5%, 8% and 11.5% for IVM, IVF and cleavage respectively). In the study of Ikeda et al. (1999), the rates of nuclear maturation, cleavage and blastocyst of oocytes matured in bovine FF (aspirated from small follicles (2–5 mm in diameter)) were 70%, 65% and 23% respectively .The previous results support the hypothesis of positive support of the FF derived from relatively large follicles, which explains the superiority of the control group D over the other control group A in our study for most of the studied traits although this superiority was relatively simple. Addition of 0.023 U / ml LH to TCM-199 media resulted in IVM rate of value 95.68% (Barakat et al., 2012). In literature, the study of factors affecting the quality of embryos is not clearly studied, and there is still some ambiguity surrounding the mechanism of influence. The results of our study (Table 4) suggested the prolonged effect of FF source and LH on quality. Logically, the developmental competence of the oocytes develops with the advancement of the oocytes and the follicle by growth and the increase in the volume of the FF, which provides the oocytes with all the components that this ability acquires (Da Broi et al., 2018). In general, many studies have indicated that the quality of the embryo is affected by a set of factors such as the nutritional status of the female (Ashworth et al., 2009; Chundekkad et al., 2020), the reproductive status of females (Twigg-Flesner et al., 2014), the dominant follicle and dynamic follicular wave (Webb and Campbell, 2007), the characteristics of the semen (Kusumaningrum et al., 2015) and the vital characteristics of the sperm used in fertilization (Ervandi et al., 2013; Chapuis et al., 2017).

CONCLUSION

It is concluded from the results of the current study that the maturation of goat oocytes in a combination containing 100 µg /ml LH and 15% cFF of ≥3 mm follicle size

improves the rates of maturation, cleavage and the quality of the resulting embryos.

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