In vitro embryo production from oocyte recovered from live and dead Iraqi black goat: A preliminary study

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Abstract
This study was carried out to produce caprine embryo in vitro from Iraqi black local goats at the College of Veterinary Medicine, Al-Fallujah University. Two different source's for oocyte's collection have been used. Oocytes collected from both sources were counted and graded. Only A and B quality oocytes were selected and incubated in an appropriate maturation medium. Fresh semen was collected from two bucks of proven fertility by electroejaculator. Fertilized oocytes were cultured in the different culture media (TCM. MEM and TALP) and incubated at 39 ^{0}C, 5% CO_2 and 90% relative humidity. Embryonic developments were monitored every 24 hrs and 50% of the culture volume was replaced with fresh medium every day for nine days until expanded or hatched blastocyst were observed.

The results revealed that 1. The number of follicles observed in superovulated animals was 114 follicles with a mean of 3.6 follicles per ovary. There was a significant difference (P<0.05) between the right and left ovaries in the mean number of follicles. 2. The total number of small follicles (2-4 mm) was 33.3% while large follicles (5- 8 mm) was 66.6% in superovulated does. There was a significant difference (P<0.05) in the mean size of small (3.36 mm) and large follicles (7.21 mm). The total numbers of small follicles observed was 55.3% and the large follicles was 44.6%. 3. Oocyte recovery rate was 71.9% and 74.06% in superovulated and slaughtered animals. In slaughtered animals aspiration method showed a recovery rate of 76.93% with a mean of 2.28 oocytes per ovary, while slicing showed a recovery of 69.93% with a mean of 1.51 oocyte/ ovary and 4. There was no significant difference in blastocyst production rate in different culture media between superovulated and slaughtered animals. Thus, it can be concluded that abattoir is a good source for obtaining oocytes for IVM, IVF and IVC from Iraqi Black local goat.

Keywords: *In vitro* Embryo production live, Dead, Black Iraqi goat.

Introduction

In vitro embryo production (IVP) is a reproductive biotechnology that has great potential for speeding up genetic improvement in ruminant, but it is also an important research tool for animal embryology.[14] IVP is another technique that may prove useful for obtaining large numbers of embryos for transfer.[60] *In vitro* culture techniques have allowed the study of mammalian embryo under controlled environmental conditions. IVP in small ruminants provides an excellent source of low-cost embryos for basic research on developmental biology and physiology and for commercial application of emerging biotechnologies such as nuclear transfer and transgenesis.[11] *In vitro* embryo production technique provides large scale, low cost economical production of early and late stage embryos required for gene integration and cloning, expected to make efficient utilization of high number of ova left in ovaries.

There is no available work about IV MFC in goats in Iraq except in sheep and cattle. The objective of the study is to establish a reliable IV MFC system for improved *in vitro* production of matured and blastocyst from goats oocytes obtained at slaughter and from living animals.

Material and Methods

Oocytes Collection: Oocytes were collected from two different sources: a-from living does.b-from slaughter house.

Collection of oocytes from superovulated does: A total of 16 black local goats, aged between 2-3 years with 1-2 parturition were present in the Animal farm of College of Veterinary Medicine/ Al-Falloujah University/ Falloujah. The animals were fed green grasses and concentrated diet and supplied with water *ad. lb*. Synchronization of oestrus were carried out during breeding season (May, Jun, July) by insertion of intravaginal sponges.[16]

Surgical operation and oocyte collection: Laparotomy was performed to superovulated does in standing position[36] at left sublumber fossa, under local anesthesia using 2 % Lidocaine Hydrochoride; 10- 12 cm long incision was made. The ovaries were exteriorized and each ovary held with two fingers. The follicles were counted and their diameters were measured with automatic vernier and the follicles were aspirated with 3 ml syringe containing collection media and then transported to a sterile Petri dish containing collecting media. Then the incision was closed with lock stich using catgut suture material. Intraperitoneal antibiotic (Oxytetracycline 200 mg/ kg/ B. W.). The skin was sutured with horizontal matress intrupted using silk no. 1. local (Tincture Iodine) and systemic i.m. injection of oxytetracycline was applied for 7 days until the removal of

sutural materials. The animals showed recovery without complications.

Oocytes Collection from slaughter house dose: Female genital (599) systems were collected from Al-Falloujah abattoir. The materials were transported within one hour in a normal saline at 33-35 ^{0}C in cool box, to the Theriogenology Lab., Department of Surgery and Obstetrics, College of Veterinary Medicine, Al-Falloujah University, Falloujah. The ovaries were removed and subjected to three washing in normal saline and two washings in collecting media (TCM-199, MEM and TALP).[57] The follicles were counted and their diameters were taken with an automatic Vernier.

The oocytes collected by Aspiration: Two to eight mm size follicles were aspirated using a 18 gauge needle attached with a sterile 3 ml disposable syringe containing 2 ml of the collecting medium.[56] The media with harvested oocytes were transferred to one well out of 24 wells dish. Ovaries were placed in a sterile Petri dish containing 10 ml of collecting medium, held with the forceps and the ovarian surface was incised with a scalpel blade. The Petri dish was examined under dissecting microscope and the oocytes transferred with a sterile pasteur pipette to one well out of 24 wells Petri dish. Then in both techniques the Petri dish was kept undisturbed for five minutes allowing the oocytes to settle down then examined under inverted microscope and then the total numbers of collected oocytes were counted. Grading of oocytes was done according to Wani et al.[56]

***In vitro* Maturation:** Only good and fair quality oocytes were selected. The oocytes were washed twice in a maturation medium either TCM-199 or MEM or TALP. They were incubated in appropriate maturation medium at 39 ^{0}C temp, 5% CO_2 and 90% relative humidity for 27 hrs. The incubated Petri dish was examined under inverted microscope. The presence of the first polar body was a good criteria for maturation of oocytes *in vitro* (IVM). The numbers of matured oocytes were calculated.

Semen collection and preparation: Fresh semen was collected from two bucks of proven fertility by electroejaculator and transported within 5 minutes to the Theriogenology Lab at 30-35^{0}C. Semen samples were examined under light microscope to evaluate semen quality. Ejaculates from two bucks were pooled in equal quantities for final volume of 1 ml, in order to minimize the variation between bucks. The semen samples were warmed in a water bath at 35 ^{0}C. Dilution of semen samples was made. 1: 10 with TALP or MEM or TCM-199 solutions. This suspension was diluted 1: 1 with heparin containing media (100 µg/ mL Heparin salt) and then incubated for 45 minutes at 38^{0}C.

***In vitro* Fertilization:** Capacitated sperms suspension was diluted to yield a concentration of 1.0×10^6 sperm/ mL in the fertilization medium (m MEM, m TCM-199 and Fert. TALP) with pH adjusted between 7.4-7.8. Only matured oocytes were kept in groups of 5 to 10 oocytes in one well of 24 wells Petri dish containing fertilization medium with spermatozoa and incubated at 39^{0}C, 5% CO_2, 90% relative humidity for 27 hrs.[27]

Evaluation of oocytes: At 24 h after insemination, oocytes were evaluated as fertilized oocyte having 2nd polar body or oocyte with sperm head in the cytoplasm. The numbers of fertilized oocytes were counted.

***In vitro* Culture:** Culture of previously fertilized oocytes (zygotes) was performed. Embryos were cultured in different cultural media (MEM, TCM-199 and TALP) and incubated at 38.5-39 ^{0}C, 5% CO_2, 90% humidity. Embryonic development was observed every 24 hrs and 50% of the culture volume was replaced with fresh medium at 24 hrs intervals. According to Keskintepe et al[24] procedure, ova that did not show the next cleavage ago, were removed from the wells containing developing embryos at the time of each change of medium. Proportions of fertilized oocytes reaching 2 to 4 cells stage were recorded at 48 hrs morula observed at 120 hrs, blastocyst at 168 hrs and expanded blastocysts at 216 hrs after fertilization.

Evaluation of Morulae and Blastocysts: Morphological evaluation of Morulae and Blastocysts was done according to Koeman et al[29] Ova appearing as a compacted mass of cells (≥16 cells) at 40 x magnification under inverted microscope was classified as morulae. Embryo exhibiting the presence of a blastocoel was classified as blastocysets. Embryo containing a blastocoel and either emerging from the zona pellucida or lacking a zona pellucida was classified as hatched blastocysts.

Staining: All oocytes and stages cleavage of embryo, zygotes and blastocyst were fixed with methanol- acetic acid and stained with 5% giemsa for 8-10min.

Statistical Analysis: Student t-test and Chi-square test were used for analysis of data according to Schefler.[51]

Results and Discussion

Follicles Numbers

In superovulated living Does: The numbers of follicles observed in 32 ovaries were 114 with a mean of 3.6 ± 0.08 follicles per ovary. The numbers of follicles presented at the right ovary were 50 with a mean of 3.12 ± 0.682 follicles per ovary. While, those presented at the left ovary were 64 with a mean of 4.00 ± 0.00 follicles per ovary (table 1). There was a significant ($P<0.05$) difference between the right and left ovaries in the numbers of follicles. The ovulatory response obtained agrees with the results reported by other researchers.[4,5,15,16] This might be attributed to the type and preparation of gonadotrophin used.[18,40,43] It has been found that eCG have a long half-life.

However. It is disadvantageous as it has been reported to lead to the production of a large number of ovarian follicles

which fail to ovulate. These follicles often remain stimulated after ovulation (cystic ovaries), maintaining high blood oestrogen concentration for an extended period of time.[33,35] These unovulatory follicles are associated with lower quality embryos recovered following ovulation. The use of eCG in goats has also been reported to lead to a high incidence of premature luteal regression.[7,9]

Slaughter house ovaries: The number of follicles observed in 1198 ovaries was 3085, with a mean of 2.57 follicles per ovary. The number of follicles presented at the right ovary was 1234, with a mean of 2.06 follicles per ovary. There was a significant difference ($P<0.05$) between the right and left ovaries in the numbers of follicles. Similar observations have been revealed by several investigators.[15,17] The lower number as compared with superovulated animals of follicles observed might be due to nutritional deficiency, age, genetic factor and/ or the lower fertile animals that were slaughtered.[27] In comparison between superovulated and slaughtered animals, there was a significant difference between them ($P<0.05$) in the number of follicles/ovary. A high number of follicles observed in superovulated animals, might be due to the effect of hormonal stimulation of the ovaries by eCG which enhances more follicles formation.[32]

Size of follicles:

In superovulated goats: The number of small follicles (2- 4 mm) was 38 (33.33%) with a mean of 3.36 ± 0.09 mm while, the number of large follicles (5- 8 mm) was 76 (66.66%) with a mean of 7.21 ± 0.11 mm (Table 4). There was a significant difference ($P<0.05$) in mean size of follicles between small and large follicles. Similar observations have been reported.[17,19,27] This might be attributed to superovulated animals subjected to treatment with gonadotropins that increased the size of follicles even in abnormal conditions such as cystic ovaries.[32]

In slaughter house ovaries: The total number of small follicles (2- 4 mm) observed was 1708 (55.33%), with a mean size of 3.08 ± 0.56, while the number of large follicles (5- 8 mm) observed was 1377 (44.66%), with a mean size of 6.79 ± 1.26. There was a significant difference in mean size ($P<0.05$) between small and large follicles. The follicular size is affected by several factors including reproductive status of the animals, breeding season, age, hormonal stimulation and even nutritional status of the animals.[17] In comparison between follicular size in superovulated animals and those collected from slaughter house, the superovulated follicles are significantly higher ($P<0.05$) from that of slaughter house size. This might be attributed to the hormonal stimulation of the ovaries with eCG.[5,32]

Oocytes Recovery:

Superovulated Does: The total numbers of oocytes collected by aspiration only were 82 oocytes, with a recovery rate of 71.9% (82/114) (table 4). This result agreed with Baldassarre and Karatzas.[6] These results might be affected by several factors; superovulation regime, collection method, season, age of the animals, nutritional status and even breed of the animals.[13,27,41]

From slaughtered animals: The total number of recovered oocytes was 2285 with a recovery rate of 74.06% (2285/3085) (table 4-2). Similar results have been obtained by several authors.[20,52] It has been reported that the cost of oocyte recovery from superovulated animal is high due to unpredictable results and low oocytes quantity. Therefore, slaughter house ovaries are attractive alternative source for oocyte recovery as they are less expensive and most abundant source of immature oocytes for large scale production of Caprine embryos because the goats are the main meat source in many countries.[27]

Grading of oocytes: Visual assessment of morphological features remains the most important vehicle for selection of oocytes before maturation during oocytes recovery.

Superovulated animals: Good oocytes (Grade A) showed a recovery rate of 34.1% (28/ 82), while fair (Grade B) and poor (Grade C) showed a recovery rate of 42.6% (35/ 82) and 23.1% (19/ 82) respectively (table 1). Similar trends have been reported by many investigators.[25,27] There was a significant difference ($P<0.05$) between different grade. The higher recovery of Grades (A) and (B) may depend upon the response to the hormonal regime applied.[13]

Slaughtered animals: The results in table 3 showed that a high recovery rate was obtained of fair oocyte (Grade B) 53.4% (1220/ 2285) followed by good oocyte (Grade A) 31.4% (719/ 2285) and poor oocyte (Grade C) 15.4% (346/ 2285). There was a significant difference ($P<0.05$) between different grades.Similar results have been reported by Rahman et al[46] in goats and Wani et al[57] in sheep. Concerning the method of recovery there was no significant difference in Grade (A) between different method of recovery. While, there was a significant ($P<0.05$) difference between Grades (B) and (C) in different methods of oocytes collection.

The observation of low quality or grade of oocyte recovered might be due to the fact that low fertile animals will be slaughtered. High quality oocytes (Grade A and B) were obtained by aspiration as compared with the slicing method. Similar observations have been made by Sogorescu et al[52] in sheep and goats. The results disagreed with the findings of Pawshe et al[42] who claimed that goat ovaries are relatively smaller in size therefore aspiration of follicular oocytes is difficult.

Also, it has been reported that oocytes recovery is significant of higher quality in puncture and slicing method than by aspiration method.[12,57] Therefore, aspiration was the most common technique for obtaining good quality and quantity of oocytes. As a result, number of COCs was denuded from cumulus cells due to repeated washing and ultimately resulted in lower number of normal COCs when compared

to aspiration at the final observation.[27] The lower number and quality of oocytes obtained by slicing in this study, might be due to different slicing techniques.

The slicing technique consisting in chopping the ovary into small pieces with a surgical blade used in this work, while others used blades to incise the follicle on the ovarian surface. So that, numbers of oocytes were retained in the ovary without recovery or damaged due to injury during chopping. In comparison between superovulated animals and slaughtered animals in respect to oocytes grading, higher good quality oocytes were recovered from slaughtered animals (84.9%) as compared with superovulated animals (76.5%).

There was significant difference (P<0.05) in oocyte quality between superovulated animals and slaughtered animals. This might be attributed to adverse effect of hormonal treatments used for synchronization and superovulation of does.[45]

Effect of Follicular size on Recovery rate:

Superovulated animals: The results showed in table 1 revealed high recovery rate of 78.9% (60/76) from large follicles (5- 8 mm diameter) as compared with that from small follicles 57.8% (22/38). There was a significant difference (P<0.05) in recovery rate between small and large follicles. Similar observations have been reported Cognie et al.[11] Several studies have established a relationship between follicle size and oocyte competence. The competence increases as the follicle enlarge.[27,30] Recovery of more culturable oocytes is higher in large size follicles than small ones. This might be due to more maturity that large follicles may be exposed to better hormone stimuli and growth factors.

Slaughtered animals: The number of recovered oocytes from large follicles was 1176 out of 1377 follicles with a recovery rate of 87.9%, while the number of oocytes recovered from small follicles was 1109 out of 1708 follicles with a recovery rate of 64.9% (Table 4-2). There was a significant difference (P<0.05) in recovery rate from large and small follicles. Similar observations have been established by Kharche et al[27] in goats, Cognie et al[11] in sheep and Lequarre et al[33] in cattle. Also, the results agree with the findings of Majeed et al.[36] This result might be due to the fact that ovarian follicle is a balanced physiological unit whose structure or function depends on extracellular factors such as gonadotropins and complex system of intrafollicular relationship.[17]

Good quality oocytes 87.9% (1030/1176) were recovered from large follicles, while those recovered from small follicles were 64.9% (776/1109) (table 4-2). There was a significant difference (P<0.05) in quality of oocytes recovered from small and large follicles. Similar observations have been reported by many investigators.[27,55,57] It has been reported that large size follicles releases good quality oocytes that have the ability to be matured *in vitro* in comparison to small follicles.[13,27]

***In vitro* maturation of oocytes:** Embryo development is influenced by events occurring during oocyte maturation. For successful IVM, Oocytes must undergo nuclear and cytoplasmic maturation.

Superovulated animals: Good quality oocytes were 34.1% (28/82) and fair ones were 42.6% (35/82) which were only used for IVM (table 4-1). Maturation rate was 73.01% (46/63) (table 4-4). Similar results have been obtained by several workers.[13,53] The results obtained may be attributed to several factors including hormonal treatments regime used, culture media, collection techniques and follicular size from which oocytes were recovered.[27]

Slaughtered animals: Only grades A and B oocytes (1806/2285), 79.03% of recovered oocytes were cultured. Maturation rate was 40.1% (725/1806) (table 4-5). Similar observations have been reported by other workers.[27,47,55] In comparison to maturation rate between superovulated animals and slaughtered one, there was a high significant difference (P<0.05) between them, with high maturation rate in superovulated animals. These findings might be due to hormonal treatments regime of synchronization and superovulation applied to the goats.[4,5,16,32] It is obvious that oocyte quality is essential for embryonic development before and after genome activation, it is a fundamental key for subsequent embryo development.[27]

The ability to identify good quality oocytes prior to *in vitro* culture is important consideration for IVP of embryo system. The presence of healthy cumulus cells surrounding the oocytes is mandatory to facilitate the transport of nutrients and signals into and out of oocytes,[8] as energy substrates and to mediate the effects of hormones on COCs.[47,62] The results are also in agreement with the observation of Rajikin et al.[48]

***In vitro* Fertilization rate:**

IVF for matured oocytes recovered from superovulated animals: The results show that fertilization rate was 60.8% (28/46) from matured oocytes (table 6). Similar results have been reported by several investigators.[21,27,61] High percentage of fertilization rate might be attributed to many factors such as fertilization media with its supplements, semen preparation and capacitating agents.[27]

IVF for slaughtered animals oocytes: The fertilization rate observed was 58.06% (421/725) of matured oocytes fertilized in fertilization media.Similar findings have been reported by Sogorescu et al,[52] Khatun et al[28] and Hoque et al.[19] The fertilization rate obtained from slaughter house samples could be attributed to the culture system and techniques for IVF applied. There are several factors playing a role in successful *in vitro* fertilization like cultural media, semen preparation with capacitating agents, season, follicle size and oocyte collection techniques.

Fertilization rate of matured oocytes recovered from slaughtered animals: Fertilization rate was 62.01% (222/358) of matured oocyte in TCM-199 supplemented with EGS, hormones and BSA while fertilization rate was 53.54% (83/155) in mMEM media and was 54.71% (116/212) in mTALP (table 4-13). There was a significant difference (P<0.05) between fertilization rate in mTCM-199 and fertilization rate in mMEM and mTALP. While there was no significant difference between fertilization rate in mMEM and mTALP. Several studies showed similar results.[19,49] It has been reported that the media employed for IVF must be capable of providing sperm motility, capacitation, the union of two gametes and the start of embryo development.

However, the needs and the metabolic activity of the male and female are not the same. It is necessary to use different media for sperm preparation and another for fertilization.[27] The results disagree with Keskintep et al[23] who reported high fertilization and cleavage rates using modified define medium for sperm capacitation and TALP medium for oocyte fertilization. It is also has been observed that addition of oestrus goat serum or from fatty acid, bovine serum albumin was used successfully in the capacitation and fertilization media.[27]

Higher cleavage rate in medium supplemented with EGS might be due to the fact that EGS is likely to provide physiological active substances such as energy substrates, amino acids, vitamins and hormones. The addition of serum to the capacitation media supported cholesterol efflux thought to be a key event in capacitation. Capacitation of goat sperm can be obtained when heat- inactivated estrus sheep or goat serum was present in the medium and also results in high cleavage rates.[22] There was no significant difference in fertilization rate between oocytes recovered from superovulated goats or that recovered from slaughtered animals.

***In vitro* culture of zygotes (Blastocyst production rate %):**

Blastocysts production in superovulated animals: The total numbers of blastocyst produced was 57.1% (16/28) which constitutes 19.5% from the total number of oocyte recovered from superovulated goat (table 5). Similar results have been observed by many investigators.[11,61] These results might be attributed to the procedures applied, culture conditions which consisted of culture medium and atmospheric conditions under, 39 ^{0}C, 5% CO_2 in air atmosphere with high relative humidity.[60] In general, high rates of loss with up to 80% of *in vitro* matured and inseminated oocytes failing to progress to the blastocysts stage; and most importantly exposure to sub optimal environments at the preimplantation stage of development may disrupt the normal developmental program.

Blastocysts production in slaughtered animals: The proportion of blastocysts obtained from fertilized oocytes was 51.78% (218/421) which consisted of 9.65% (218/2285) from total number of oocytes recovered (table 5). Similar results have been reported by many workers.[23,34,61] There was a significant difference (P<0.05) in blastocyst production between oocytes recovered from live animals and those recovered from slaughtered animals (table 4). This might be attributed to good quality oocytes recovered from live animals as compared with those recovered from slaughtered animals.[10,27] It has been reported that although culture conditions can influence the kinetics of early development.[31,44] It is likely that main factors controlling this parameter are intrinsic to the oocyte or the sperm or both.[31,58]

Table 1
Effect of follicular size on recovery and grading of oocytes from superovulated animals

Follicle size	No. of follicles	Mean size ±SE	No. of oocyte recovered	Recovery rate (%)	Grading of oocytes		
					Grade A	Grade B	Grade C
Small follicle (2-4 mm)	38	3.36 ± 0.09^b	22	57.8%^{b}	6 (27.2%)^a	9 (40.9%)^b	7 (31.8%)^c
Large follicle (5-8 mm)	76	7.21 ± 0.11^a	60	78.9%^{a}	22 (36.6%)^b	26 (43.3%)^a	12 (20%)^c

Mean values in the same column with different superscripts differ significantly (P<0.05).

Table 2
Recovery and selection of oocytes from follicles of different size and their grade in slaughtered animals

Follicle size	No. of follicles	Mean size ±SE	No. of oocyte recovered	Recovery rate (%)	Grading of oocytes		
					Grade A	Grade B	Grade C
Small follicle (2-4 mm)	1708	3.08 ^{b} ± 0.56	1109	64.9% ^{b}	258 (23.2%) ^{b}	518 (46.7%) ^{b}	333 (30.02%)^a
Large follicle (5-8 mm)	1377	6.79 ^{a} ± 1.26	1176	87.9% ^{a}	412 (35.03%) ^{a}	618 (52.5%)^a	146 (12.4%)^b

Mean values in the same column with different superscripts differ significantly (P<0.05).

Table 3
Effect of follicular size on maturation rate of superovulated animals in Black local goats

Follicle size	No. of cultured oocytes	No. of mature oocytes	Maturation rate percentage %
Small follicle (2-4 mm)	15	8	53.33%[b]
Large follicle (5-8 mm)	48	38	79.16%[a]

Mean values in the same column with different superscripts differ significantly (P<0.05).

Table 4
Effect of follicular size on *in vitro* maturation rate of oocytes in Black local goats of slaughtered animals

Follicle size	No. of cultured oocytes	No. of mature oocytes	Maturation rate %
Small follicle (2-4 mm)	776	241	31.05%[b]
Large follicle (5-8 mm)	1030	484	46.99%[a]

Mean values in the same column with different superscripts differ significantly (P<0.05).

Table 5
Blastocysts production rate % from superovulated and slaughtered animals from total numbers of recovered oocytes

Animals status	Total No. of oocyte recovered	No. of fertilized oocytes cultured	Blastocyst production rate %
Superovulated	82	28	16 (19.5%)[a]
Slaughtered	2285	421	218 (9.65%)[b]

Mean values in the same column with different superscripts differ significantly (P<0.05).

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