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Original Article

Effect of Epididymal Spermatozoa of Local Iraqi Goat on *in vitro* Fertilization and Evolution of Embryos

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Abstract

In vitro fertilization (IVF) has been considered one of the greatest improvements in livestock science and production. Epididymal spermatozoon is a valuable gamete source obtained from slaughtered animals, and it has great importance in livestock IVF. This study aimed to create caprine embryos in Baghdad using local Iraqi goats. Ovaries were obtained from 50 female local goats at the Al-shu'alah abattoir. The ovaries were transported to the laboratory in physiological saline solution at 38°C. After oocyte recovery from the mature antral follicle, the oocytes with grades A or B were used for in vitro maturation (IVM). Moreover, 10 testicles were obtained from slaughtered adult goats, and the sperm was recovered from the caudal epididymis. All the stage-specific culture mediums were incubated at 39°C with 5% CO_2 and 90% relative humidity. In this study, embryonic development on a 24-h basis was evaluated and *in vitro* culture media was replaced 50% of the volume every day for nine days with fresh media until an enlarged or hatched blastocyst appeared. The recorded data showed that the mean size of the large follicles was 6.23±1.34 mm, while the small follicles were in the mean size of 3.10±0.62. On the other hand, the results showed a significant increase in the oocyte recovery rate from the large size follicle (81.22%), compared to the small size follicle (62.67%). The recorded data showed that the total number of retrieved oocytes with grades A and B was 290 (79.5%). The recorded data showed that 50.1% of these oocyte cohorts had reached metaphase II (145/290). Furthermore, 45.45% of the recovered oocytes by aspiration from large follicles were developed to the blastocyst stage, compared to 25% of recovered oocytes from the small follicles reaching the same developmental stage (blastocyst). On the other hand, the recorded data showed that the oocyte recovery method had a great influence on the oocyte competence regardless of the follicle size. The results revealed that the number of recovered oocytes by aspiration was greater than slicing. In conclusion, the results approved that the slicing method could be considered a safe and more efficient method in oocyte recovery from the slaughtered animals, compared to the aspiration method. On the other hand, according to the findings, the epididymal spermatozoa from local Iraqi goats are a reliable source of oocytes for IVF and *in vitro* conversion.

Keywords: Dead, Embryo Production, Epididymis, *In Vitro*, Local Iraqi Goat

1. Introduction

To create an *in vitro* embryo, there are three steps involved. First, oocytes recovered from large follicles are matured in *in vitro* maturation (IVM) medium. Next, secondary oocytes are fertilized with fresh or epididymal spermatozoa. Finally, the presumptive embryos are cultured for up to a week until blastocysts formation completely passed, and the produced embryos got ready to be transferred or cryopreserved (1, 2). Obtainment of the caudal epididymal spermatozoa after significant damage to animals or from dead animals is crucial in animal specimen propagation and conservation with high genetic values (1).

It is possible to produce *in vitro* embryos at a reasonable cost by using the testicles and ovaries obtained from slaughtered or dead animals (3). A valuable source of oocytes for *in vitro* fertilization (IVF) has long been thought to be abattoir offal (Ovaries). Oocytes from abattoir ovaries can be harvested via a variety of methods, including aspiration, slicing, and piercing, to name a few (4-6). Assisted reproductive technologies (ART), including multiple ovulation, embryo transfers, and *in vitro* production (IVP) of embryos, are being used to improve animal genetics rapidly (7). Research on stem cells and genetic improvement has benefited from the use of ART (8). As a result, it is possible to learn more about fundamental biological processes, such as oocyte maturation, embryo implantation, and metabolic pathways that regulate early embryo development by studying the vast numbers of IVP embryos produced by an animal (7).

Applying the superovulation and subsequent ovum pick-up procedure in the livestock for the purpose of *in vitro* embryo production can only lead to the recovery of a limited number of mature oocytes. Therefore, slaughtered house materials are considered the most abundant and inexpensive sources of oocytes and spermatozoa for *in vitro* embryo production (IVEP) (1). In the last 50 years, slaughterhouse material has been considered an invaluable source for IVEP purposes. However, in this way, there are several factors affecting the IVEP yield. Therefore, the optimization of *in vitro* techniques could improve the IVEP efficiency (6, 9). This study was designed to use epididymal spermatozoa obtained from slaughtered local goats to perform IVF and IVP.

2. Materials and Methods

2.1. Chemicals and Biological Materials

All the chemicals were purchased from Sigma Aldrich (the USA) unless otherwise indicated. The corresponding media used for IVM, IVF, and IVP were incubated at 38.5˚C-39˚C under 5% CO_2 with 95% humidity for 2 h before starting each step. Goats' female reproductive organs (n=100) were collected from local goats breed of a local abattoir in Al-shu'alah, Iraq. The reproductive organs were placed in an insulated container with physiological saline solution (0.9% NaCl) supplemented by penicillin/streptomycin (50 µg/mL), at 38.5˚C-39˚C. The goats' reproductive organs were transported to the laboratory at the earliest time following ovaries dissection from the slaughtered animals. All the superfluous tissue and bursa were removed from the ovaries' surface. Each ovary was subjected to three washing in Dulbecco's phosphate-buffered saline (DPBS) and two washing in oocyte harvesting medium at 38.5˚C-39˚C (DPBS+4 mg/mL, bovine serum albumin [BSA]+50 IU/mL penicillin) prior to processing (6).

2.2. Oocyte Recovery and Grading

The oocytes have been collected under the Biosafety Cabinet Class II by either aspiration or slicing according to the procedures previously described by Wani, Wani (5).

2.2.1. Follicle Aspiration

The aspiration technique was conducted following the brief description below:

2-8 mm follicles were punctured and the containing oocytes were recovered using an 18-gauge needle and a sterile 3 mL disposable syringe containing 2 mL of collection media.

2.2.2. Follicle Slicing

After holding the ovaries with forceps, they were transferred to an autoclavable petri dish with 10 mL of collection media, and then the ovarian surface was incised by a sterile scalpel blade. A 24-well petri dish was viewed under a dissecting microscope before the oocytes were transferred using a sterile pasture pipette. The oocytes in the petri dish wells were counted after examining them using an inverted microscope (5).

2.2.3. Oocytes grading

The recovered oocytes were analyzed under the stereomicroscope and divided into three classes as the previously grading method described by Wani, Wani (5). Grade A oocytes have a homogeneous cytoplasm and several compact layers of cumulus cells. Grade B

oocytes have a homogeneous cytoplasm and 3-4 layers of cumulus cells. Grade C or inferior oocytes have a small number of cumulus cell layers (1-2 layers) and cytoplasm with nonhomogeneous and fragmented cytoplasm.

2.3. *In Vitro* Maturation of Oocytes

In a 50-µL droplet of maturation medium consisting of tissue culture medium (TCM199) with Earle's salts and bicarbonate supplemented with 10% heat-treated fetal calf serum, 0.5 pg/mL follicle-stimulating hormone and 5ug/mL gentamicin 20 COCs were added. To prevent unwanted evaporation of culture droplets, the petri dish was filled with mineral oil until covering the surface of maturation droplets. The maturation condition was as follows: 38.5˚C, 5% CO_2, and 95% air atmosphere with maximum humidity (10).

2.4. Sperm Preparation and *in Vitro* Fertilization

The IVF procedure was conducted according to the method previously published by Davachi, Shahneh (11) with some modifications. In total, 10 testicles were obtained from the slaughtered adult goats, and the sperm was recovered from the caudal epididymis. The recovered spermatozoa were diluted in TCM 199 with Earle's salts (Gibco, Grand Island, NY, USA) (pH 7.8). Thereafter, this suspension was centrifuged at 500× g for 1 min. After centrifugation, the supernatant was carefully discarded, and the spermatozoa pellet was pre-incubated for 60 min at 38.5˚C prior to fertilization in a 1000 µl of capacitation medium.

A 90 µl drop of IVF medium which contained *in vitro* matured COCs (n=10-20) was mixed with 10 µl of pre-incubated spermatozoa. The final concentration of spermatozoa in the fertilization droplets was 1×10^6. The IVF medium included the following compositions: 12 mM KCL, 25 mM $NaHCO_3$, 90 mM NaCl, 0.5 mM NaH_2PO_4, 0.5 mM $MgSO_4$, 10 mM sodium lactate, 3 mg/mL bovine serum albumin (BSA; fatty acid free), 50 µg/mL gentamicin, and 5 mM caffeine. Following a 12-h co-incubation of oocytes with spermatozoa, by performing mechanical forces (gentle pipetting), the cumulus investment and the attached spermatozoa were dissociated from the inseminated oocytes, and then these fertilized oocytes were transferred to the culture medium. Presumptive zygotes were cultured in KSOMaa. All cleaved embryos were transferred into fresh culture medium 72 h after insemination. The cleaved embryos were cultured for 7 days to evaluate their ability to develop into morula and blastocyst stages. An examination of the Morulae and Blastocysts' morphology was carried out following Kharche, Goel (12). Morulae were defined as embryos that appeared under an inverted microscope as a compacted mass of cells (about 16 cells). Blastocysts were defined as embryos with a blastocoel. Hatched blastocysts are embryos that have a blastocoel and are either emerging from the zona pellucida or do not have a zona pellucida.

2.5. Embryo Evaluation and Staining

For the assessment of the resulted embryos after 7 days *in vitro* culture (IVC), all the developed embryos to the morulae and blastocysts stage were removed from the IVC medium and washed twice in PBS-polyvinylpyrrolidone (PVP) (1 mg/mL). Subsequently, the embryos were fixed in a 100 µL drop of paraformaldehyde solution (4% [w/v] in PBS, pH 7.4) at room temperature. At the final step, the embryos were removed from the fixation drop and washed three times in PBS-PVP. Following that, all the embryos were stained for 15 min in 1 µg/mL Hoechst33342; after which all the stained embryos were evaluated under a fluorescent microscope with a UV filter (11).

2.6. Statistical Analysis

Data were expressed as mean±SEM. After IVF, the proportion of oocytes reaching each stage of meiosis, fertilized oocytes, and embryos were subjected to arc-sin transformation before the analysis of variance (ANOVA).

3. Results and Discussion

The recorded data showed that the mean size of the large follicles was 6.23±1.34 mm, while the small follicles were in the mean size of 3.10±0.62. On the

other hand, the results showed a significant increase in the oocyte recovery rate from the large size follicle (81.22 %), compared to the small size follicle (62.67%) ($P \leq 0.05$) (Table 1). It is obviously noticeable that the recovered oocytes from large follicles have better quality, compared to the oocytes recovered from small follicles (Table 1). Grade A oocytes obtained from the large follicles (35.82%) was significantly higher than the recovered oocytes from small size follicle (22.9%). In the same line, the recovered oocytes with grade B were higher in the large follicle group (54.01%), compared to the recovered oocytes from the small follicles (46.62%) (Table1). Although the inferior oocytes (grade C) were significantly lower in the group of recovered oocytes from the large follicle (10.16%), compared to the small follicle group (31.45%), these results are in line with the findings of previously published studies (13, 14). This might be the case because larger follicles tend to have more fully developed oocytes, compared to the smaller ones.

On the other hand, age, breeding season, diet, and some other biological and environmental factors can affect the follicular size and oocyte quality (2, 15, 16). These data revealed that grade B oocytes were the most frequent oocytes recovered from both cohorts of large and small follicles (Table 1), which was occurred due to the oocyte recovery methods. Previously several research teams have experienced the same observation (13, 17). The comparison of the large and small follicles has revealed that the large follicles produce more competent oocytes for IVM (13, 17). Other researchers discovered that the size of the follicle influenced the quality of oocytes. According to the studies, more statistically ova with various granulosa cells are produced in cows with follicles more prominent than 6mm in diameter. *In vitro*, a large number of blastocysts were generated from these eggs. According to theory, more prominent follicles might promote the function and structure of cumulus-oocyte complexes (COCs) and the embryo created by growth factors (18, 19).

Before IVC, the ability to select high-quality oocytes is critical for the IVP of the embryos. As previously mentioned by Davachi, Kohram (10), the COC quality is of great importance in the IVM rate and the consequent success of IVF and IVC.

The recorded data showed that the total number of retrieved oocytes with grades A and B was 290 (79.5%) (Table 1). Accordingly, 50.1% of these oocyte cohorts had reached metaphase II (145/290) (Table 2). These findings are consistent with previously published results and similar maturation rates for large and small follicles (58.33% and 38.52%, respectively) (12, 20).

Table 1: Recovery of oocytes from follicles of different size and their grade in slaughtered goats

Follicle size	No. of follicles	Mean size ±SE	No. of oocyte recovered	Recovery rate (%)	Grading of oocytes No. (%)		
					Grade A	Grade B	Grade C
Large follicle (5-8 mm)	229	6.23±1.34[a]	187	81.22[a]	67 (35.82 %)	101 (54.01%)	19 (10.16%)
Small follicle (< 5 mm)	283	3.10±0.62[b]	178	62.67[b]	39 (22.9%)	83 (46.62%)	56 (31.45%)

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

Table 2. 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%
Large follicle (5-8 mm)	168	98	58.33%
Small follicle (< 5 mm)	122	47	38.52%

According to the table 3 the results showed that 45.45% of the recovered oocytes by aspiration from large follicles were developed to the blastocyst stage, compared to 25% of the recovered oocytes from the small follicles reaching the same developmental stage (blastocyst). On the other hand, the recorded data show that the oocyte recovery method has a great influence on the oocyte competence regardless of the follicle size. The results revealed that the number of recovered oocytes by aspiration is greater than that by slicing. However, the results of presumptive embryo development showed that the obtained oocytes via slicing were more competent for embryo production, compared to the aspiration. It may due to the adverse effects of aspiration pressure damages induced on the COCs structures. These findings were in line with the previously published results Davachi, Shahneh (9), (11). Previously conducted studies have all reported similar results (4, 21, 22). All of them mentioned that the culture media, semen preparation methods, the season, follicle size, and collecting techniques all play key roles in the success of IVF.

Table 3. Blastocysts production rate from total numbers of recovered oocytes of slaughtered local goats

Follicle size	Total No. of oocyte recovered	No. of culture embryos	Blastocyst production rate%
Large follicle (5-8 mm)	220 (by aspiration)	100	45.45%[a]
Small follicle (< 5 mm)	160 (by aspiration)	40	25%[b]
Large follicle (5-8 mm)	190 (by slicing)	110	57.89%[a]
Small follicle (< 5 mm)	120 (by slicing)	50	41.66%[b]

Similar to the findings of a study conducted by Zeinoaldinin (6) who has mentioned that the oocyte recovery method has played a key role in the oocyte recovery rates, cleavage, and blastocysts development, our findings in the current study approved their results. It is well documented that by the use of the aspiration technique for oocyte recovery from slaughtered or dead animals, only the visible follicles were used for oocyte retrieving, while it seemed that by slicing, several nonvisible follicles embedded deep within the cortex were also ruptured and the embedded oocyte released from follicles.

In conclusion, the results approved that the slicing method could be considered a safe and more efficient method in oocyte recovery from the slaughtered animals, compared to the aspiration method. On the other hand, the results reveal that the epididymal spermatozoa from local Iraqi goats are a reliable source of oocytes for IVF and IVC.

Authors' Contribution

Study concept and design: S. S. H.
Acquisition of data: S. S. H.
Analysis and interpretation of data: S. S. H.
Drafting of the manuscript: M. A. J.
Critical revision of the manuscript for important intellectual content: M. A. J.
Statistical analysis: M. A. J.
Administrative, technical, and material support: S. S. H.

Ethics

All study protocols have been approved by the ethics committee of the Al-Farabi University College, Baghdad, Iraq.

Conflict of Interest

The authors declare that they have no conflict of interest.

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