Selection of male goat embryos *in vitro* by using swim-up of epididymal spermatozoa

Sura Shakir Hammood Al-Dulaimi and Ihsan H. S. Al-Timimi
Department of Surgery and Obstetrics, College of Veterinary Medicine, University of Baghdad, Iraq.
E-mail: vdr.ihsan@gmail.com
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Summary

The main objectives of this study is the separation of X from Y bearing epididymal spermatozoa of local buck by swim-up, and the use of this spermatozoa for *in vitro* fertilization to determine the percentage of produced male and female embryos. The sex of produced embryo was identified by polymerase chain reaction. Testis of the local buck were obtained from Al-Shu'alah abattoir and the epididymal spermatozoa were harvested from the cauda by and submitted to *in vitro* maturation prior to separation of X from Y bearing spermatozoa and prior to their use for *in vitro* fertilization. For the separation of epididymal spermatozoa, swim-up technique was used with centrifugation at 200×g or 300×g. The centrifugation at 200×g showed that 41.84±1.39 % of spermatozoa were detected in the supernatant while the precipitate contained 50.69±0.71 and the mean of the sperm lost was 7.65±0.93. After centrifugation, spermatozoa in the supernatant were used for *in vitro* fertilization of matured oocytes. The sex of *in vitro* produced goat embryos was determined by polymerase chain reaction using specific primers to detect of SRY gene. The percentage of total goat embryos obtained after *in vitro* fertilization by sperms selected using swim-up at centrifugation force of 200×g recorded 79.66 % male embryos while female embryos recorded only 20.33 %. At the end, the results showed the ability of selection male embryos in caprine by application of swim-up technique on epididymal spermatozoa with centrifugation at 200×g.

Keywords: Swim up, Male, Gender, Goat, Cauda, Epididymis, Embryo.

Introduction

Pre implantation sex determination is a necessary tool to control the sex ratio in goats (1). After a dramatic development of cellular biology, a lot of research efforts have been moved towards the implementation of embryo-technologies involving multiple ovulation and embryo transfer (MOET), *in vitro* production (IVP) of embryos, cloning and transgenesis to transfer a targeted number of embryos (2). Among all, IVP of embryos has become a routine method of producing embryos from abattoir derived ovaries with minimal cost, although, goat oocytes can be recovered in relatively large numbers from abattoir ovaries, the oocytes frequently have reduced development potential when compared to *in vivo* matured or immature oocytes collected after gonadotropin treatment (3). Sperm sexing raises great interest due to extensive application in animal production as well as medicine, and new separation techniques which present both better accuracy and low costs are necessary. A technique which provides accuracy is the flow cytometry that separates by DNA content, two populations of sperm (X and Y-bearing) with an accuracy of 90% (4). However this technique has disadvantages such as equipment costs, damage to sperm during sexing (5) and altered mRNA expression of embryos (6). Several investigators have attempted to separate X- and Y spermatozoa using various techniques based on principles of differing mass and motility, swimming patterns, surface changes, volumetric differences, centrifugal counter-current distribution and immunologically relevant properties (7 and 8). Preselecting of offspring by altering the sex ratio has been a priority for centuries. In the livestock industry, predetermining the sex of animals has been a main goal of producers for generations because of its financial advantage (8). The goal of this research is the separation of X- from Y-bearing epididymal spermatozoa of local buck by swim-up which with low cost and can be applied easily in small laboratory with minimum laboratory equipments.

Materials and Methods

Gonads of local goat were obtained from Al-Shu'alah abattoir and transported to the laboratory within 1-2 hours in cold normal saline solution. Sperms from cauda epididymis of local bucks were harvested by slicing of cauda by scalpel using the normal saline. Incubation of sperm sample in 5% CO_2 incubator at 35˚C for 6 hrs for sperm maturation, sperms properties were recorded before and after commencement of swim up technique, including volume, concentration (sperm/ ml) and the forward progression motility (9-11). Selected sperms were suspended in minimum essential media (MEM) (GIBCO, USA) contained 100 µg/ ml heparin sodium and then incubated for 45 minutes at 38˚C according to the procedure described by (9).

Swim-up separation technique (200×g, 300×g): The procedure of the modified swim-up technique followed in this study is basically the same as that described by (12), Ham's F10 (Euro-Lone, Italy) was used as an alternative media to evaluate the efficiency of the swim-up technique in separating X and Y chromosome bearing sperms. Sperms sample transferred to 10 ml centrifuge tube. Sperms were washed twice using 0.5 ml Ham's F-10 medium then centrifuged at 300×g or 200×g for 10 min. Supernatant removed and overly sperm pellets with 0.5 ml of Ham's F-10 medium in each tube. The tubes were put in the incubator, inclined at an angle around 45° and incubated at 37°C and 5% CO_2 for 60 minutes. By inclining the tubes at 45 to improve the capability of the sperms to swim out of the sample and reach the medium. Following incubation, the first 0.25 ml was discarded and the final 0.25 ml was used for *in vitro* fertilization after sperm evaluation (12).

Goat ovaries were subjected to three washing in normal saline and two washings in normal saline and gentamycin to avoid contamination and were chopped into small pieces in normal saline with a surgical blade. The cumulus-oocyte complexes (COCs) were selected from the saline solution to MEM. The media with harvested oocytes were transferred to one Petri dish (11 and 13). Collected oocytes were examined and graded according to (14) as grade A, grade B and grade C. Only grade A, and grade B oocytes were selected, washed twice in MEM, and incubated in appropriate maturation medium at 39˚C, 5% CO_2 and 90% relative humidity for 24-28 hrs, the numbers of matured oocytes were calculated (15). Capacitated sperms suspension was diluted to yield a concentration of 1.0×10^6 sperm/ml in MEM. Only matured oocytes were kept in group in of 10 in petri dish containing fertilization medium with sperms and incubated at 39˚C, 5% CO_2 and 90% relative humidity for 24-27 hrs (15). Twenty four to twenty seven hrs after fertilization, oocytes having 2nd polar body or oocyte with sperm head in the cytoplasm were evaluated as fertilized oocyte. The numbers of fertilized oocytes were counted (15).Cultures of previously fertilized oocytes were performed; embryos were cultured in MEM at 38.5-39˚C, 5% CO_2, and 90% humidity. Embryonic developments were observed every 24 hrs, 50% of the media volume was replaced with fresh medium at 24 hrs intervals according to (16) procedure, and then extraction of DNA from embryos was applied.

DNA extraction from embryos: The primers (Promega, Germany) were used to detect *SRY* gene, they prepared according to the information of the producer. The primers with their sequences and product size are: The forward primer sequence was: ATGAATAGAACGGTGCAATCG (OD-260: 12.9, Microgram: 382, Pico moles: 58704). The reverse primer sequence was: GAAGAGGTTTTCCCAAAGGC (OD-260:11.7, Microgram: 363, Pico moles: 58842).

DNA of embryos was extracted by using tissue DNA extraction kit (Geneaid. USA) then DNA sample was stored at -20˚C until use. The purity and concentration of extracted DNA was measured using Nano-drop spectrophotometer (ActGene, USA). Polymerase chain reaction master mix reaction was prepared by using (GoTaq® Master Mix from INTRON, USA), these master mix were done according to the producer instructions. It is a very important step to complete PCR assay, which is used to analyses the PCR product by agarose gel (2%) electrophoresis (Cleaver, Japan). Finally PCR products (bands) were visualized using a UV trans-

illuminator and photographed by digital camera.

The Statistical Analysis System- SAS (2012) program was used to effect of difference factors in study parameters. Chi-square test was used to significant compare between percentage and least significant difference –LSD test (ANOVA) was used to significant compare between means in this study.

Results and Discussion

The total number of collected sperms was $3.085\pm0.17\times10^9$ from each cauda of local buck and the mean of number of spermatozoa was $154.54\pm8.82\times10^6$/ ml and the percentage of motility was 87.12±0.62. The live sperms recorded as 89.50±1.89% while dead sperms represented 5.93±0.30 and 6.92±0.64% of spermatozoa diagnosed as abnormal.

It is known that successful fertilization requires a sperm with normal integrity and function (17) and this include procedures used for gender selection. In the standard IVF procedures, perm function is essential for normal fertilization that has to take place *in vitro*: Sperm must be able to bind to zona pellucida, undergo the acrosome reaction, penetrate the zona pellucida, and fuse with the oolemma before fertilization takes place (18). Hence, our study was directed toward application an ideal method having the reliable and low cost for caudal sperm preparation and the separation of good-quality fraction of spermatozoa, this is required to increase the success rates of *in vitro* techniques, so it used swim up methods for the separation of goat epididymal Spermatozoa prior to *in vitro* fertilization and gender selection in local goat.

The study of (19) suggests that the separation material should be nontoxic and isotonic to the sperm, without any impedance to the osmotic pressure. The technique should, in addition, remove nonviable, dead, abnormal sperm, epithelial cells, leukocytes, and bacterial contaminants. The results obtained after harvesting spermatozoa from cauda were within the normal values of good samples used for IVF, similar data were obtain by other authors in ovine (11), who reported 85% alive sperm, 14% dead sperm and 90% of motility. From the above data it can said that the perfect conditions for the preparation of caudal spermatozoa. Our results were in accordance also with those of (20) which recorded for semen of black Bengal goat, which were 89.64±5.1 % of live sperms and 80.83±3.5 % of fresh sperm motility, while other authors like (21) who reported lower individual motility which not exceed 76.66±0.33 %.

When centrifugation at 300×g was applied, the mean number of sperms after washing, the mean of the percentage of sperm in the upper and lower parts of the tube and total sperm recovered were 90.48±1.26, 40.61±1.16, 49.99±1.26 and 90.60±0.71% respectively. At centrifugation rate of 200×g, the mean of number of sperms after washing, the mean of the percentage of sperm in the upper and lower parts of the tube and total sperm recovered were 93.39±0.74, 41.84±1.39, 50.69±0.71 and 92.53±0.93 respectively. No Significant differences were found between the two groups (Table, 1).

The mean of the sperms lost through the procedure was 9.39±0.71 % using centrifugation at 300×g and it was 7.65±0.93% using centrifugation at 200×g and there was a significant difference between these two groups (P<0.05) (Table, 1).

Table, 1: Number of local buck sperms ($\times10^6$/0.25 ml) used in swim-up technique before and after centrifugation at 200×g or 300×g.

Parameters		Centrifugation Force 200×g (Mean±SE)	300×g (Mean±SE)	T-Test
Sperm no after washing $\times10^6$		93.39 ± 0.74	90.48 ± 1.26	4.831 NS
Post swim up sperm %	Sperms in the upper part of the tube	41.84 ± 1.39	40.61 ± 1.16	2.984 NS
	Sperms in the lower part of the tube	50.69 ± 0.71	49.99 ± 1.26	2.719 NS
Total recovered sperms %		92.53 ± 0.93	90.60 ± 0.71	4.227 NS
Lost sperms %		7.65 ± 0.93	9.39 ± 0.71	1.052 *

* (P<0.05), NS: Non-Significant.

Table (2) shows the results of IVF by buck sperms of the upper layer prepared by swim up technique after centrifugation at 200×g, the total number of doe oocytes used was 218 divided as 131 (60.91%) grade A and 87

(39.90%) grade B oocytes, the total number of maturated oocyte was 122 (55.96%) from which 78/131 of (59.54%) grade A matured oocytes and 44/87 (50.57%) of grade B mature oocytes, while the total number of fertilized oocytes was 94/122, the fertilization rate of grade A oocytes was 73.08% (57/78) and the fertilization rate of grade B oocytes was 37/44 (84.09 %). When it followed, the method used by many authors for selection of Y bearing spermatozoa which depends on the difference between the movement of X and Y bearing spermatozoa (22 and 23). The swim up technique is the standard method used for patients with normozoospermia and female sub fertility (24). In an attempt to improve the sperm yield in oligospermic males the "swim-up" can be performed directly from the liquefied semen avoiding the centrifugation and multiple washing steps (24). The methodology of this conventional swim-up is based on the active movement of spermatozoa from the prewashed cell pellet into an overlaying medium. A yield of very high percentage (>90%) of motile morphologically normal spermatozoa can be obtained with the help of this technique (25). Our data revealed that the recovery of spermatozoa, when swim up technique was used, using centrifugation force of 200×g or 300×g which were not high, differ from the result of (26) which was obtained in application of this technique on fresh ovine semen, while other authors like (19) founded a very low percentage of recovery in human (30%). In our study, centrifugation at 200×g was chosen because sperm lost after separation of spermatozoa were relatively low; 7.65±0.93% and 9.39 ±0.71% for 200×g and 300×g respectively with a significant difference (P<0.05) between them. This percentage of lost spermatozoa was lower than the data reported by (27) who used the swim up protocol for fresh semen of bull.

Table, 2: Maturation rate of grade A and B oocytes of slaughtered local goats and IVF rate using sperms obtained by swim-up technique.

Cultured oocytes			Matured oocytes		Total maturation rate No and %	Fertilized oocytes		Total Fertilized oocytes No and %
Total oocyte no	Grade A No and %	Grade B No and %	Grade A No and %	Grade B No and %		A No and %	B No and %	
218	131 (60.91%)	87 (39.90%)	78/131 (59.54 %)	44/87 (50.57 %)	122/218 (55.96 %)	57/78 (73.08%)	37/44 (84.09%)	94/122 (77.04%)

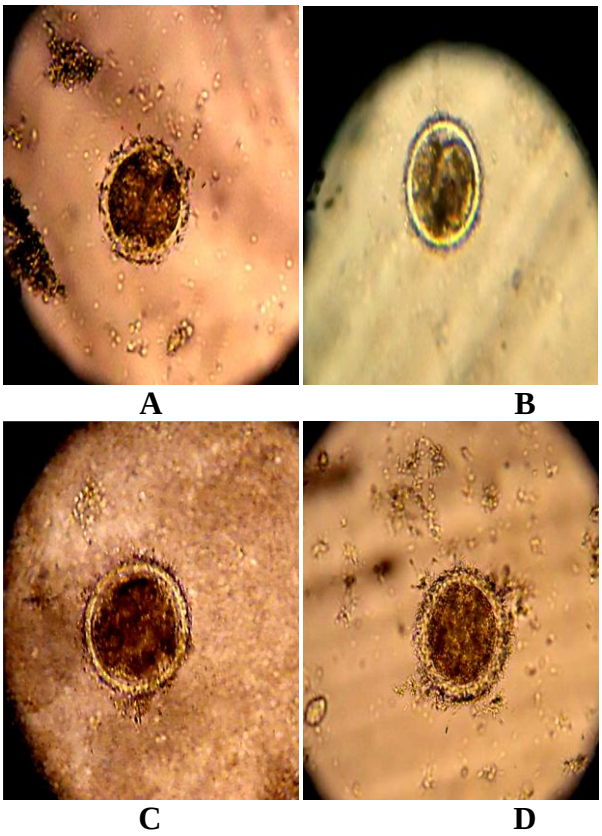
After IVF by spermatozoa obtained from the upper layer of swim up procedure, 77.05% were fertilized from which were 5.31, 15.95, 22.34, 27.65 and 28.72% of 2, 4, 8, 16 and >16 cells of embryo, respectively. The fertilization rate was good when compared with results of other authors, (15) had a fertilization rate reach to 45.5% when use sperms after swim up technique in the Bengal black goat while (28) who reported 41.91% of fertilization using fresh semen and oocytes collected by slicing in the same media that it used (MEM), (21) also reported 42.56 % using fresh semen in caprine, while others authors reported high percentages; 64.92% and 70-77% of fertilization (13 and 29). While (27) obtained only 21.9% of fertilization rate in ovine and (11), obtained not 26% also in ovine, however (30) reported 61.3% of cleaved embryo in ovine. After swim up technique, from 122 IV matured oocytes 94 (77.04%) oocytes were fertilized and the embryonic stage of 2, 4, 8, 16 and >16 cells embryos were 5 (5.31%), 15 (15.95%), 21 (22.34%), 26 (27.65%) and 27 (28.72%) respectively. Figure (1) shows different stages of *in vitro* produced local goat embryos after fertilization by sperms obtained from cauda of local bucks and after swim up technique (Table, 3).

In the present research, the primers for SRY gene of caprine were used for amplification genomic DNA of *in vitro* produced embryos of local goat, and the PCR products were separated on 2% agarose gels. The result showed that amplification fragment of size 116 bp, the embryos were amplified successfully and when a single band stained by ethidium bromide or red gel was obtained the embryos recorded as male embryos while the result which showed the absence of such bands the produced embryos recorded as female goat embryos (Fig. 2).

Table, 3: Number of goat embryos obtained by IVF using sperms selected by swim-up or ficoll density techniques after centrifugation at 200×g and according to their stage of development.

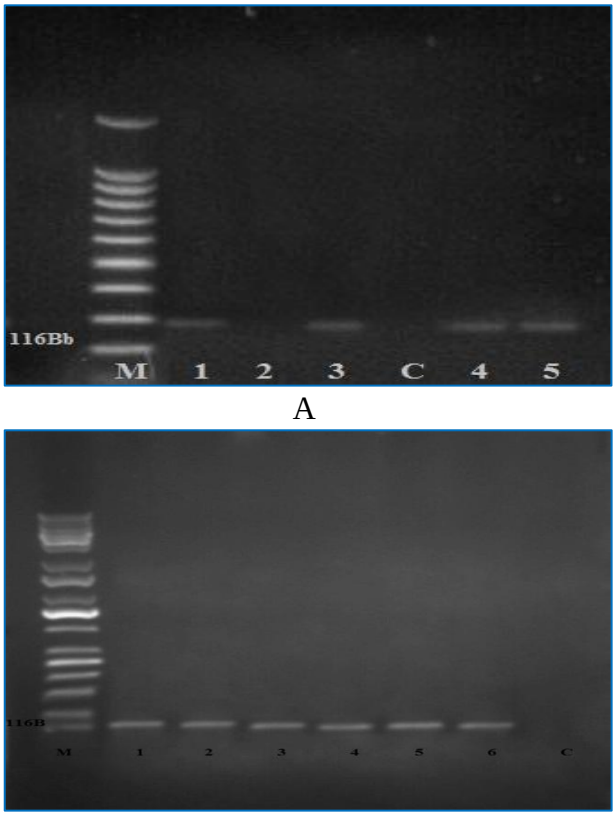
Technique used	No of matured ova used	No of fertilized ova	Embryonic stage				
			2 cells	4 cells	8 cells	16 cells	>16 cells
Swim-up (200×g)	122	94 (77.05 %)	5 (5.31 %)	15 (15.95 %)	21 (22.34 %)	26 (27.65 %)	27 (28.72 %)
Chi-Square	---	4.06 *	0.882 NS	1.892 NS	0.007 NS	1.953 NS	1.426 NS

* (P<0.05), NS: Non-Significant.



Figure, 1: Different stages of local goat embryos produced in vitro using caudal spermatozoa; 2 cell stage (A), 4 cell stage (B), 8 cell stage (C) and 16 cell stage (D), (× 40).

The determination of the sex of IVF produced local goat embryos by caudal spermatozoa, selected from the upper layer of swim up technique, gave 79.66% male embryos. The Y-bearing chromosome is faster than the X-bearing chromosome because X sperm has more DNA than Y sperm which results in different migration velocity (31). Based on this affirmation, some authors described that supernatant of swim-up procedure contained more Y sperm (32 and 33), it used accurate and reliable protocol for PCR-based caprine embryos sexing, it based on detection of the presence of target sequence, i.e., SRY gene. The amplification of only the Y-specific target sequence was sufficient to determine the gender as has been done successfully in bovine and caprine embryos sexing studies (34-36).



Figure, 2: Electrophoresis pattern of PCR product of SRY gene with 116 bp size of *in vitro* produced local goat embryos, PCR amplification product in: A- columns 1, 3, 4, and 5 indicated the male embryos while the in column 2 indicated the female embryo. In B- all column indicated the male embryo. Amplified DNA stained by red gel in the 2% agarose gel.

After sexing of embryos by using PCR, (Table, 4) shows the percentage of male and female caprine embryo after IVF by spermatozoa from the upper layer after swim up technique at 200×g, the result revealed that 47 (79.66%) from 59 sexed caprine embryos were male, while female embryos represented only 12 (20.33%) with a significant difference between the two detected sex (P<0.01).

Table, 4: Number and percentage of male and female local goat embryos after detection of their sex by PCR and obtained after IVF using caudal sperms selected by swim-up.

Technique used	Total no of embryos	No of sexed embryos	No and % of Male embryos	No and % of Female embryos	Chi-Square
Swim-up (200×g)	94	59	47 (79.66%)	12 (20.33%)	11.536 **
** (P<0.01).					

The percentage of male embryos after swim up technique considered high compared with the results of Marco-Jiménez and Vicente, (2004) using swim up technique in ovine, but our data was not far from that of (27) who obtained 81.80 % of male ovine embryos after swim up procedure. From the above data, it can conclude that it is possible to harvest Y bearing spermatozoa from cauda of epididymis of local buck by swim up technique, and use these sperms successfully for IVF to obtain a high percentage of male embryos.

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اختيار الأجنة الذكرية للماعز مختبرياً باستعمال طريقة السباحة للأعلى لنطف البربخ

سرى شاكر حمود و إحسان حمودي التميمي
فرع الجراحة والتوليد، كلية الطب البيطري، جامعة بغداد، العراق.
E-mail: vdr.ihsan@gmail.com

الخلاصة

الأهداف الرئيسة لهذه الدراسة هو فصل النطف التي تحمل X عن الحاملة Y كروموسوم للحيوانات المنوية من ذيل البربخ لذكور الماعز المحلي بواسطة تقنية السباحة للأعلى واستعمال هذه النطف للإخصاب الخارجي لتحديد النسبة المئوية للأجنة الذكور والإناث المنتجة مختبرياً. حُدد جنس الجنين المنتج بواسطة تفاعل سلسلة البلمرة. حُصل على خصى ذكور الماعز المحلي من مجزرة الشعلة وحُصل على النطف من ذيل البربخ وكُيفَت في المختبر قبل البدء بفصل النطف الحاملة X عن الحاملة Y تحمل واستعمالها للإخصاب في المختبر. لأجل اختيار نطف ذيل البربخ الحاملة Y كروموسوم استعملت تقنية السباحة للأعلى بواسطة استعمال قوة طرد مركزي تعادل 200×g و 300×g. أظهرت قوة الطرد المركزي عند 200×g أن 41.84 ± 1.39٪ من النطف التي كشف عنها في الطافي بينما احتوى الراسب على 50.69±0.71 % وكان معدل الحيوانات المنوية المفقودة 7.65±0.93. بعد الطرد المركزي، استعملت الحيوانات المنوية في الجزء الطافي للإخصاب الخارجي للبويضات الناضجة. حُدد جنس أجنة الماعز المنتجة في المختبرعن طريق تفاعل سلسلة البلمرة باستعمال برايمر خاص للكشف عن جين SRY. بلغت النسبة المئوية لأجنة الماعز الكلية التي حُصل عليها بعد الإخصاب الخارجي من النطف المختارة باستعمال تقنية السباحة للأعلى وبقوة الطرد المركزي 200×g سجلت 79.66٪ من الأجنة الذكور بينما سجلت الأجنة الإناث فقط 20.33٪. أخيراً، أظهرت النتائج قدرة اختيارالأجنة الذكور في الماعز عن طريق تطبيق تقنية السباحة للأعلى على النطف من ذيل البربخ وبقوة الطرد المركزي 200×g.

الكلمات المفتاحية: السباحة للأعلى، ذكر، الجنس، الماعز، ذيل، البربخ، جنين.