

## Separation of X spermatozoa from cauda of epididymis prior to selection of female embryos in local goat

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### Abstract

The objective of this study is the selection of X epididymal spermatozoa of local buck by discontinuous ficoll density gradient and using these spermatozoa for *in vitro* fertilization and identify the ratio of produced female embryos after identification by polymerase chain reaction (PCR). Epididymal spermatozoa were harvested from the cauda epididymis by slicing, and then spermatozoa were estimated and submitted to *in vitro* maturation and capacitation prior to separation of X from Y bearing spermatozoa and use for *in vitro* fertilization. Modified discontinuous ficoll density gradient method used for the separation of X and Y-bearing sperm by using 3 layers of discontinuous ficoll (60, 40, and 20%) or 4 layers of discontinuous ficoll (80, 72, 64, and 50%). The centrifugation applied at 200×g or 300×g. In the protocol of 3 layers of discontinuous ficoll (60, 40, and 20%) and centrifugation at 200×g and 300×g the results showed that the mean of the sperms at the 3<sup>rd</sup> was  $79.014 \pm 3.12$  and  $79.39 \pm 2.11$  respectively and this show the sperms was not completely separated. In the protocol of 4 layers of discontinuous ficoll (80, 72, 64, and 50%) and centrifugation at 200×g showed that the mean of the sperms at the 4<sup>th</sup> layer was  $(42.79 \pm 1.38)$  as compared with the first three layers and with the protocol centrifugation at 300×g. The mean of the sperm lost showed  $(7.066 \pm 0.56)$  and a little percent from centrifugation at 300×g showed  $11.28 \pm 1.55$ . Oocytes collected from ovaries which obtained from the slaughter house then matured oocyte was applied. The maturation rate for grade A and B used for IVF by spermatozoa after ficoll density gradient it was 67.60% and 52.74%, while the fertilization rate for the grade A and B was 70.68% and 78.26% respectively. The sex of embryos was determined by polymerase chain reaction (PCR) using specific primers for detection of SRY gene. The percentage of goat embryos obtained by IVF by sperms selected with ficoll density techniques after centrifugation at 200×g at different stage of development was 70.13%. The percentage of the male embryos was 16.67% while the female embryos was 83.33%. The results showed that the best method for selection of female embryos was by using 4 layers of discontinuous ficoll gradient method (80, 72, 64, and 50 %) with centrifugation at 200×g.

**Key words:** gender, female, ficoll, SRY gene, goat, cauda of epididymis, embryo, spermatozoa.  
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### عزل النطف الأنثوية من ذيل البربخ لغرض اختيار الأجنة الأنثوية في الماعز المحلي

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### الخلاصة

الأهداف الرئيسية لهذه الدراسة هي فصل نطف ذيل البربخ التي تحمل X عن الحاملة Y كروموسوم لذكور الماعز المحلي بواسطة تدرج الكثافة المتقطعة (Ficoll) واستخدام هذه النطف للإخصاب الخارجي لتقدير النسبة المئوية للأجنة الذكور والإناث المنتجة مختبرياً بعد تطبيق التقنية. يتم تحديد جنس الأجنة بواسطة تفاعل سلسلة البلمرة (PCR). تم الحصول على خصى ذكور الماعز المحلي من المجزرة لغرض حصاد النطف من الذيل البربخ بواسطة التشطيب و تم تحديد تركيز النطف، العدد الكلي، الحركة الفردية، النطف الحية والميتة وقد تم تقييمها وتكييفها للإخصاب الخارجي قبل البدء بفصل النطف الحاملة X عن الحاملة Y واستخدامها في إخصاب بويضات مختبرياً. اختبرت طريقة تدرج الكثافة المتقطعة والمحورة لفصل النطف الحاملة X و Y باستخدام ثلاث طبقات من Ficoll المتقطع (60، 40، 20%) أو أربع طبقات من Ficoll متقطع (80، 72، 64، 50%). قوة الطرد المركزي طبقت 200g و 300g. في نظام الثلاث طبقات من Ficoll متقطع (60، 40، 20%) وقوة طرد

مركزي  $g \times 200$  و  $g \times 300$  أظهرت النتائج ان معدل النطف في الطبقة الثالثة كانت  $3.12 \pm 79.014$  و  $2.11 \pm 79.39$  على التوالي وهذا يدل على ان الحيامن لم يتم فصلها بشكل كامل. في نظام الأربع طبقات من Ficoll متقطع (80، 72، 64 و 50%) ويقوة طرد مركزي  $g \times 200$  أظهرت ان معدل الحيامن في الطبقة الرابعة كان ( $1.38 \pm 42.79$ ) مقارنة بالطبقات الثلاث الأولى وأيضا مع الطبقات المستعملة في قوة الطرد المركزي  $g \times 300$ . أما معدل الحيامن المفقودة ظهرت ( $0.56 \pm 7.066$ ) وبنسبة مئوية اقل من قوة الطرد المركزي  $g \times 300$  التي ظهرت ( $1.55 \pm 11.28$ ). تم جمع البويضات من المبايض التي تم الحصول عليها من المجزرة وكانت  $67.60\%$  و  $52.74\%$ . أما معدل الإخصاب الخارجي للبويضات من النوعين A و B بتقنية السباحة للأعلى كانت  $73.08\%$  و  $84.26\%$  على التوالي، في حين بلغ معدل الإخصاب الخارجي للبويضات من النوعين A و B في تقنية الكثافة المتدرجة (Ficoll)  $70.68\%$  و  $78.26\%$  على التوالي. تم تحديد جنس الأجنة عن طريق تقنية سلسلة تفاعل البلمرة (PCR) وباستخدام برايمر خاص للكشف عن جين SRY. بلغت مجموع نسب الأجنة التي تم الحصول عليها بواسطة الإخصاب الخارجي وبواسطة النطف المختارة بطريقة الفيكول وبعد قوة طرد مركزي  $g \times 200$  وفقا لمراحل تطورها ( $77.05\%$ ) و ( $70.13\%$ ) على التوالي. سجلت نسبة الأجنة الذكور الأجنة الذكور ( $16.67\%$ ) بينما سجلت الأجنة الأنثوية ( $83.33\%$ ). في نهاية البحث، أظهرت النتائج إمكانية استعمال النطف من ذيل بريح ذكور الماعز في تقنية فصل نطف ذيل البريح التي تحمل X عن الحاملة Y كروموسوم، وإمكانية اختيار نسب عالية من الأجنة الأنثوية باستخدام اربع طبقات (80، 72، 64، 50%) من تدرج الكثافة المتقطع (Ficoll) ويقوة الطرد المركزي  $g \times 200$ .

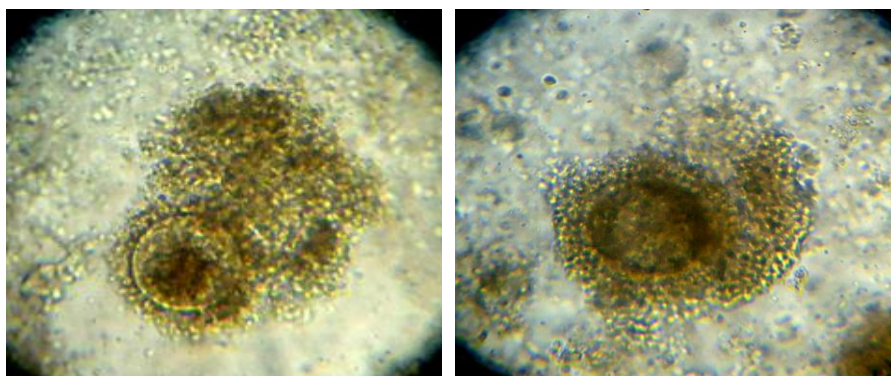
الكلمات المفتاحية: إناث، فيكول، جين SRY، ماعز، ذيل البريح، جنين، نطف.

## Introduction

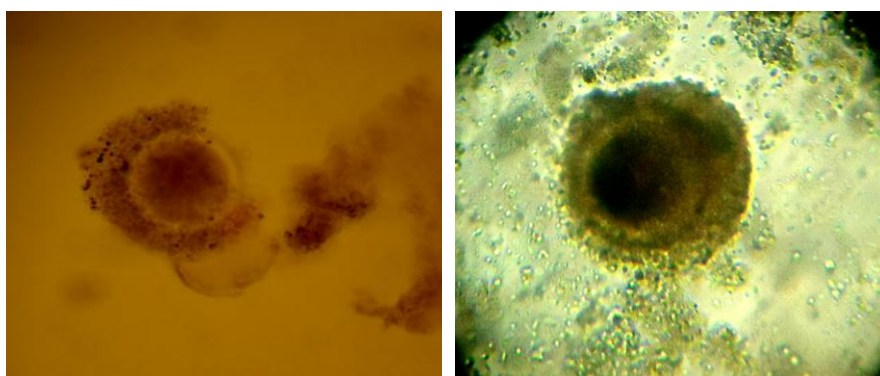
The animal production depends upon adequate increase in the number of productive livestock and a major concern is to increase the efficiency of offspring from them. Among livestock, the goat plays a significant role in socioeconomic development because of its contribution of milk, meat, skin and fur to humans and plant nutrition as farmyard manure, being small in size and having a short gestation period, the goat is a convenient domestic species for recombinant protein secretion in milk by transgenesis. Because the female is preferred for protein production (1). Males of high genetic merit are still required as sires in artificial insemination programs. The current development of state-of-the-art reproductive techniques has made it possible to predetermine the sex, involving the separation of X- from Y- chromosomes-bearing sperms, used in artificial insemination (AI), in vitro fertilization, and embryo transfer (2, 3). However, none of these methods has been able to produce statistically significant separation of fertile sperm populations nor have they been reproducible. Spermatozoa bearing X and Y chromosomes can be separated on the basis of DNA using flow cytometric sperm sorter (4). In recent years, sexing sperm has progressed from research to commercial application for humans and cattle (5). Furthermore, it has become a reliable tool for basic research. The effectiveness of sex preselection in semen has been demonstrated for many species, including endangered species in zoos and aquarium animals (6, 7). To date, it has produced millions of pre-sexed offspring in a wide variety of species including, rabbits, pigs, cattle, sheep, horses, dogs and domestic cats, buffalos, farmed deer and elk, bottlenose dolphins, humans, and non-human primates like gorilla, chimpanzee, baboon, and marmoset. In general, contemporary sperm-sorting procedures result in skewed offspring sex ratios of between 85 and 95% accuracy (8, 9). The aim of this study was the possibility of selection, in vitro, a high percentage of female embryos by application the ficoll density method on spermatozoa collected from the cauda epididymis of local buck.

## Materials and Methods

- **Sperm preparation:** Spermatozoa from cauda epididymis of local bucks were obtained by slicing of cauda in normal saline. Evaluation of samples was applied, lower than 60% of individual motility samples were discarded. Sperm maturation was done by incubation of sperm in 5% CO<sub>2</sub> incubator at 35°C for 6 hrs. Sperm properties were recorded before the separation technique, including volume, concentration (sperm/ml) and motility (%) as well as the forward progressive motility (10, 11, 12). Selected sperms incubated in minimum essential media (MEM) (GIBCO, USA) supplemented with 100 µg/ml of heparin sodium, incubated for 45 minutes at 38 °C (10).
  - **Sperm separation techniques:** By the discontinuous Ficoll density gradient method described by (13) for the separation of X-bearing from Y-bearing was used. By this technique, the sperms samples were diluted 1:1 with Hepe's buffered Hank's solution (Biowest) and layered onto 10 ml centrifuge tube, which contained increasingly dense layers of isotonic Ficoll media as follows:
    - **Protocol 1: (200×g or 300×g), (using 3 layers of Ficoll):** Three layers density-gradient, formed from a top layer of 20% (v/v), medium layer of 40% (v/v) and a lower layer of 60% (v/v), each layer was supplemented with BSA.
    - **Protocol 2: (200×g or 300×g), (using 4 layers of Ficoll):** Four layers density-gradient, formed by a top layer of 50% (v/v), medium layer of 64% (v/v), sub medium layer of 72% (v/v), and a lower layer of 80% (v/v).
- In each of the above protocols, the density gradient was prepared by layering 1 ml of each concentration, and then 1 ml of washed caudal sperms sample suspension was layered over the upper layer, and centrifuged at 200×g or 300×g for 30 minutes. After centrifugation, most of the supernatant were gently removed, 0.25 ml from each layers was collected for sperm count. The layer which was chooses (contains less than 50% of sperms), diluted 1:2 with Hepe's buffered Hank's solution to remove the density gradient medium. Then centrifugation force which was used according to the procedure at 200×g or 300×g for 10 minutes, the supernatant is then removed, and the final pellet was re-suspended in sterile Hepe's buffered Hank's solution. Selected sperm samples was diluted 1:10 with normal saline, then diluted 1:1 with heparin containing (100 µg/ml Heparin salt) and then incubated for 45 minutes at 38°C according to the procedure described by Al-Timimi, (10) prior to be used in IVF.
- **Oocyte collection and classification:** Goat ovaries were obtained from Al-shu'alah abattoir and transported to the laboratory within 2 hours in cold normal saline solution. Each ovary was subjected to 3 washing in normal saline and 2 washings in normal saline and gentamycin. Ovaries were placed in saline solution and were chopped into small pieces with a surgical blade. The cumulus-oocyte complexes (COCs) were transferred from saline solution to MEM, the media with harvested oocytes were transferred to one petri dish (14). Collected oocytes were graded according to (15) as grade A grade B and grade C. Good and fair classified oocytes (Fig. 1 and 2) were selected, washed twice in maturation medium MEM, and incubated at 39 °C, 5% CO<sub>2</sub> and 90% relative humidity for 24-28 hrs. 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 (16).



**Fig. (1) Grade A goat oocytes surrounded by many layers of cumulus cells ( $\times 40$ ).**



**Fig. (2) Grade B goat oocytes with thin or incomplete layers of cumulus cells and uniform cytoplasm ( $\times 40$ ).**

- ***In vitro* fertilization:** Capacitated sperms were diluted to obtain  $1.0 \times 10^6$  sperm/ml in MEM and used for IVF. Only matured oocytes were kept petri dish containing fertilization medium with sperms and incubated at  $39^\circ\text{C}$ , 5%  $\text{CO}_2$  and 90% relative humidity (16). Twenty four to twenty seven hours later, oocytes with 2<sup>nd</sup> polar body were evaluated as fertilized oocyte. The numbers of fertilized oocytes were estimated (16).
- ***In vitro* Culture:** Previously fertilized oocytes were cultured in MEM at  $38.5\text{--}39^\circ\text{C}$ , 5%  $\text{CO}_2$ , and 90% humidity. Embryonic developments were observed every 24 hr., 50% of the media volume was replaced with fresh medium at 24 hr. intervals. According to (17) procedure, fertilized oocytes were evaluated at 24hr interval.
- **Extraction of embryonic DNA:** DNA of embryos was extracted by using (tissue DNA extraction kit Geneaid. USA). The purity and concentration of extracted DNA was applied by using Nano-drop spectrophotometer.

The primers which used to detect *SRY* (Promiga, Germany) prepared according to the information of the company. The primers with their sequences and product size:

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

The PCR master mix reaction was applied by using (GoTaq® Master Mix from INTRON, USA) and according to the producer instructions. We used to analyses the PCR product agarose gel (2%) electrophoresis (Cleaver, Japan), the final 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 detect of difference factors in study parameters. Chi-square test was used for significant comparison between percentage and least significant difference-LSD test (ANOVA) was used for significant differences between means in this study.

### Results

Table (1) briefly revealed the results concerning the separation of the two types of caudal buck spermatozoa by using the three layers of ficoll density (20%, 40% and 60%) technique at 200×g or 300 × g, the percentage of sperms were  $0.960 \pm 0.17$ ,  $0.898 \pm 0.26$ ,  $8.982 \pm 2.37$  and  $79.014 \pm 3.12$  for the upper layer, 20%, 40% and 60% respectively using 200×g, while at 300 × g the percentage were 0.0%,  $2.924 \pm 0.75$ ,  $5.754 \pm 0.51$ ,  $79.39 \pm 2.11$  respectively. There was a significant difference ( $P < 0.05$ ) in the % of spermatozoa in the upper, 20% and 40% layer of the two different centrifugation forces. There were no significant differences between the total percentage of recovered sperms and the lost sperms in the two protocols which were  $89.85 \pm 1.12$  and  $10.15 \pm 1.12$  at 200×g respectively and at 300 × g they were  $88.04 \pm 1.39$  and  $11.95 \pm 1.39$  respectively.

**Table (1) Percentage of sperms after centrifugation at 200×g or 300 × g using three layers of Ficoll density gradient**

Parameters		Centrifugation Force		T-Test
		200×g	300 × g	
Post separation sperm % in different layers (Mean ± SE)	Upper layer	$0.960 \pm 0.17$	$0.00 \pm 0.00$	0.412 *
	20 % layer	$0.898 \pm 0.26$	$2.924 \pm 0.75$	0.762 *
	40% layer	$8.982 \pm 2.37$	$5.754 \pm 0.51$	1.446 *
	60 % layer	$79.014 \pm 3.12$	$79.39 \pm 2.11$	3.972 NS
recovered sperm % (Mean ± SE)		$89.85 \pm 1.12$	$88.04 \pm 1.39$	4.056 NS
Sperms lost % (Mean ± SE)		$10.15 \pm 1.12$	$11.95 \pm 1.39$	2.398 NS

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

- **Four layers of Ficoll:** As shown in table (2), the percentage of sperms in the four layers of ficoll density gradient (50, 64, 72 and 80 layers)% at 200×g were  $10.59 \pm 0.51$ ,  $13.99 \pm 0.63$ ,  $25.00 \pm 0.69$  and  $42.79 \pm 1.38\%$  respectively and the percentage of recovered and lost sperms were  $92.93 \pm 0.56$  and  $7.066 \pm 0.56$  respectively. When centrifugation at 300 ×g was used, the percentage of sperms in the four layers were  $2.690 \pm 0.75$ ,  $3.494 \pm 0.32$ ,  $19.84 \pm 1.91$ ,  $62.47 \pm 2.25$  respectively, and the percentage of recovered and lost sperms were  $88.73 \pm 1.56$  and  $11.28 \pm 1.55$  respectively. Significant differences ( $P < 0.05$ ) were reported between the data of the two protocols ( $P < 0.05$ ).

**Table (2) Percentage of sperms after centrifugation at 200×g or 300×g using 4 layers of ficoll density gradient**

Parameters		Centrifugation Force		T-Test
		200 x g	300 x g	
Post separation sperm % in different layers (Mean ± SE)	Upper layer	$0.552 \pm 0.08$	$0.236 \pm 0.11$	0.194 *
	50 % layer	$10.59 \pm 0.51$	$2.690 \pm 0.75$	2.473 *
	64 % layer	$13.99 \pm 0.63$	$3.494 \pm 0.32$	2.582 *
	72 % layer	$25.00 \pm 0.69$	$19.84 \pm 1.91$	3.041 *
	80 % layer	$42.79 \pm 1.38$	$62.47 \pm 2.25$	6.793 *
Recovered sperm % (Mean ± SE)		$92.93 \pm 0.56$	$88.73 \pm 1.56$	3.911 *
Sperms lost % (Mean ± SE)		$7.066 \pm 0.56$	$11.28 \pm 1.55$	2.477 *

\* ( $P < 0.05$ ).

- **Oocyte collection:** Table (3) showed the mean weight of the 320 ovaries involved in the research with the mean of their weight was  $0.672 \pm 0.06$  gm., the total number of oocyte recovered was 1680 which represent  $5.25 \pm 0.72$  oocyte per ovary. The number of grade A, B and C harvested were 798 (47.65%), 404 (23.94%) and 478 (28.41%) respectively with a significant differences between the three grades ( $P < 0.05$ ).

**Table (3) Weight of local doe ovaries and the number and grades of oocyte recovered**

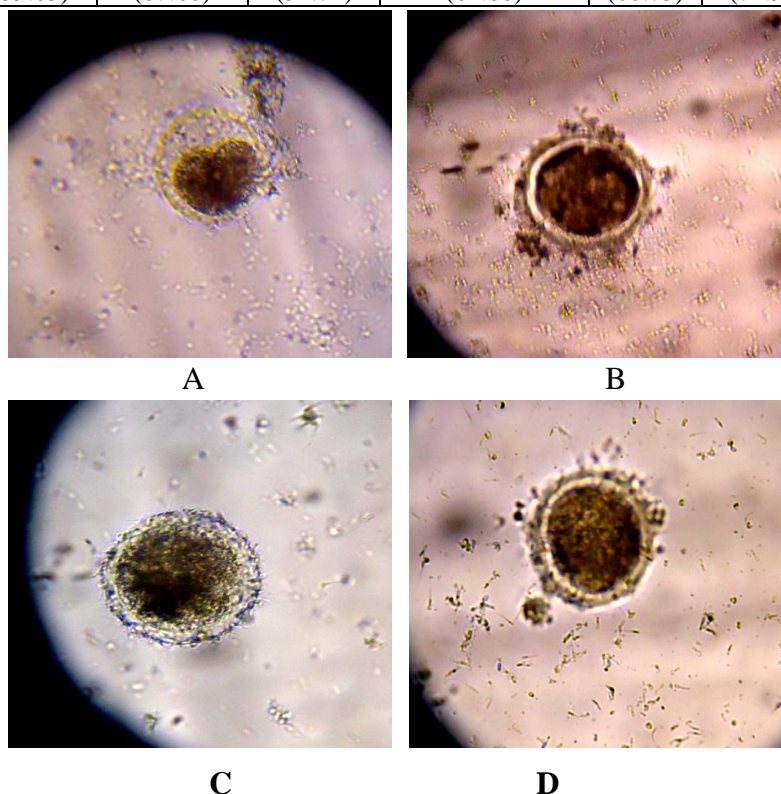
No. of ovaries	Mean weight of ovaries (gm.) (Mean $\pm$ SE)	Total no. of collected Oocytes	Number of oocyte recovered per ovary (Mean $\pm$ SE)	Grades of collected oocytes (Number and %)		
				Grade A	Grade B	Grade C
320	$0.672 \pm 0.06$	1680	$5.25 \pm 0.72$	798 (47.65%)	404 (23.94%)	478 (28.41%)
Chi-Square	---	---	---	8.483 **		

\*\* (P<0.01).

- **In Vitro Maturation and Fertilization:** In the experiment in which IVF was done by using spermatozoa collected from the 4<sup>th</sup> layer of ficoll density (layer of 80%) after centrifugation at  $200 \times g$ , we used 233 of grade A and grade B oocytes, represented as 142/233 (60.94%) grade A and 91/233 (39.05%) grade B oocytes. The maturation rate of grade A oocyte was 67.60% (96/142) while for grade B it was 52.74% (48/91), so the total maturation rate for the 2 types of oocytes was 61.80% (144/233) (Table 4). The fertilization rate was also calculated as 68.75% (66/96) for grade A and 72.91% (35/48) for grade B, the total fertilization rate in this part of the experiment was 70.14% (101/144). The embryos obtained after IVF of mature goat oocyte are shown in (Fig. 3).

**Table (4) Maturation rate of grade A and B oocytes of slaughtered local goats and IVF rate using sperms obtained from the 4th layer of ficoll density (layer of 80%) after centrifugation at  $200 \times g$ .**

Cultured oocytes			Matured oocytes		Total maturation rate No (%)	Fertilized oocytes		Total Fertilized oocytes No and (%)
Total No	Grade A No (%)	Grade B No (%)	Grade A No (%)	Grade B No (%)		A No (%)	B No (%)	
233	142 (60.94)	91 (39.05)	96/142 (67.60)	48/91 (52.74)	144/ 233 (61.80)	66/96 (68.75)	35/48 (72.91)	101/144 (70.14)



**Fig. (3) Different stages of local goat embryos produced in vitro using caudal epididymal spermatozoa; the embryos at 2 cell stage (A), the 4 cell embryos (B), the embryos at 8 cell (C) and embryos at the stage more than 16 cell (D).**

- **Embryonic stages:** When sperms selected from the 4<sup>th</sup> layer of ficoll after centrifugation at 200×g were used for fertilization, 101 of 144 (70.13%) mature oocytes were fertilized and the number of embryos recovered as 2, 4, 8, 16 and >16 cells were 8 (7.92%), 11 (10.89%), 23 (22.77%), 33 (32.67%) and 25 (24.75%) respectively.

**Table (5) The numbers of goat embryos obtained by IVF using sperms selected by 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
Ficoll (4 layers at 200×g)	144	101 (70.13%)	8 (7.92%)	11 (10.89%)	23 (22.77%)	33 (32.67%)	25 (24.75%)
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.

- **PCR amplification of SRY gene:** 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 size 116 bp, the embryos were amplified successfully and when a single band stained by ethidium bromide 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. 4).

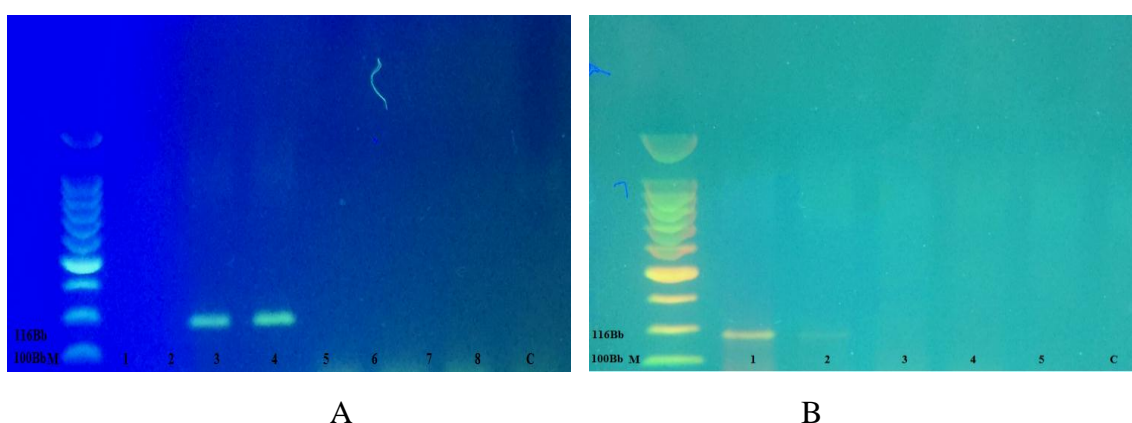


Fig. (4) Electrophoresis pattern of PCR product of SRY gene with 116 bp size in local goat embryo using sperms after ficoll density gradient technique. In (A) the lane's 3-4 appear the male embryos while the lanes 1-2-5-6-7-8 negative indicated the female embryos. In (B) the lanes 3-4-5 show the female embryos, while the lanes 1-2 indicated the male embryos

- **Embryonic sex percentage:** After sexing of embryos by using PCR, table (6) showed the percentage of the male and female caprine embryo *in vitro* fertilization was applied by sperms harvested from the 4<sup>th</sup> layer after ficoll density technique by using centrifugation at 200×g, the number and percentage of the male and female embryo were 11 (16.67%) and 55 (83.33%) respectively from 66 sexed embryos, also there was a significant difference between the two sex (P<0.01).

**Table (6) Number and percentage of male and female goat embryos after detection of their sex by PCR after IVF using sperms selected by ficoll density techniques.**

Technique used	Total no of embryos	No of sexed embryos	No and % of Male embryos	No and % of Female embryos	Chi-Square
Ficoll (4 layers 200×g)	101	66	11 (16.67%)	55 (83.33%)	13.427 **

\*\* (P<0.01).

## Discussion

Assisted reproductive techniques (ARTs) have become the treatment of choice in many cases of male and female infertility, and it is known that successful fertilization requires a sperm with normal integrity and function (18) and this include procedures used for gender selection. In the standard IVF procedures, sperm 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, and penetrate the zona pellucida before fertilization takes place (19). Hence, our study was directed toward application a method having the reliable and low cost and separation of good quality spermatozoa to increase the rates of *in vitro* techniques, so we used Ficoll density gradient for the separation of goat epididymal spermatozoa to use it in *in vitro* fertilization and selection of female embryos of local goat.

- **Harvesting of spermatozoa from cauda epididymis of local buck:** The sperm preparation technique is one which prepares a sample of spermatozoa having good viability, maturity, and normal intact morphology so that the sperms are potent for positive fertilization. Furthermore, the technique should be simple, cost-effective and should not cause any damage to the cell or alteration in the sperm membrane, and nuclear integrity. In addition, the separation material should be nontoxic and isotonic to the sperm, without any impedence to the osmotic pressure. The technique should, remove nonviable, dead, abnormal sperm, epithelial cells, leukocytes, and bacterial contaminants (20). The results of spermatozoa harvested from local buck cauda epididymis were within the normal values of good samples used for *in vitro* fertilization, similar data were obtain by other authors in ovine (12). From our data we can conclude that conditions for the preparation of caudal spermatozoa was perfect, it was in accordance also with results of (21) from semen of black Bengal goat.
- **Ficoll:** In caprine, as in other species, the Y-sperms are lighter than X-sperms due to the lower DNA content of Y-sperms than X-sperms. Heavier spermatozoa (X sperms) should settle down faster than lighter spermatozoa (Y sperms) through media gradient, therefore centrifugation force and time could positively influence X-bearing sperm moving down the gradient. Most of the methods for sexing were based on suggested physical differences between X and Y bearing sperms, such as swimming velocity, density, surface charge, or presence of H-Y antigen (22, 23, 24, 25, 26). The Ficoll density gradient method was found to meet these requirements in the present study, yielding a good quality fraction of normal, viable spermatozoa and could be an effective alternative to most routinely used methods of IVF (20).

When we tried the technique of caprine caudal spermatozoa separation by three layers of discontinues ficoll density gradient at 200× g or 300× g, the result were not satisfactory because most of spermatozoa ( $79.014 \pm 3.12\%$ ) were accumulated in the 60% layer (lower layer), so this procedure was not suitable for separation of spermatozoa when we obtained a mixture of  $79.014 \pm 3.12\%$  (mixture of both types of spermatozoa) with no significant differences between the two procedures (200× g or 300× g). However (27) founded that, when she used the same conditions, better results of separation for ovine semen sperms ( $40.05 \pm 0.81\%$  and  $52.05 \pm 1.55\%$  for 200×g and 300× g respectively). This might be due to the difference in DNA content and chromosomes number between the two species. There was a significant differences ( $P < 0.05$ ) of sperm lost percentage after the application of this procedure which were  $10.15 \pm 1.12$  and  $11.95 \pm 1.39$  for 200× g and 300× g respectively, which were more than those reported by (27).

When we had not obtained favorable results after using three layers of ficoll density gradient, we tried using 4 layers of ficoll with higher concentrations (50, 64, 72 and 80%) at 200×g or 300×g to ameliorate the separation of the two types of spermatozoa. When centrifugation at 300 × g was applied, most of spermatozoa were present in the 80% layer ( $62.47 \pm 2.25\%$ ) which mean that the two types of spermatozoa were not well separated when centrifuged, in the other hand, best result of separation was obtained in 80% layer ( $42.79 \pm 1.38\%$ ) using 200×g centrifugation force with significant differences ( $P < 0.05$ ) between the two forces of centrifugation. So we had chosen spermatozoa from this layer for IVF of goat oocytes. Similar results were obtained by (28) in caprine ( $40 \pm 2.0\%$ ). Our results also were not far from that of (27) when she used similar concentrations for separation of spermatozoa at 200× g centrifugation in the 80% layer ( $44.80 \pm 2.40\%$ ), but our data are not in accordance with her results using 300× g ( $43.80 \pm 1.58\%$ ) and (29) in bovine using four layers (80, 70, 55 and 40% Percoll media) at 300×g for 15 minutes, and in rabbit (30) using two layers (90 and 40% Percoll media) at 250×g for 15 minutes. There was a significant differences ( $P < 0.05$ ) in the percentage of sperm lost between the two centrifugation forces which were  $7.066 \pm 0.56\%$  and  $11.28 \pm 1.55\%$  for 200× g and 300× g respectively, these percentages of lost spermatozoa were higher than those reported in ovine semen (27). We used Ficoll gradient for the separation of X-spermatozoa in this research but not the Percoll gradient which used by some authors because the sperm evaluation parameters after sperm separation by gradient Percoll are not reliable in predicting the outcome of IVF (31), and several techniques which used Ficoll was found to be more efficient method for separation of spermatozoa, Ficoll density gradient yielded higher percentage of live, mature, morphologically normal spermatozoa in an isolated (20)

- **Recovery and maturation of oocytes:** In this study we used 320 local doe ovaries, their weight were ( $0.672 \pm 0.06$  gm) similar to those recorded by (32) who recorded  $0.69 \pm 0.01$  gm. and the mean number of oocyte recovered by slicing was  $5.25 \pm 0.72$  per ovary while, this difference might be due to using ovaries of goat in the season of reproduction in our experiment, and also our laboratory equipment were from trusted sources plus the very good conditions of application of techniques. We reported a high number of grade A oocyte harvested from these ovaries compared with grade B and C oocytes with significant differences ( $P < 0.01$ ), similar observations were recorded by (27) in the ewe.
- **In vitro fertilization:** When spermatozoa from the 4<sup>th</sup> layer of ficoll density gradient were used in fertilization, the percentages were 7.92%, 10.89%, 22.77%, 32.67% and 24.75% of 2, 4, 8, 16 and > 16 cells of embryo respectively and the fertilization rate was 101/144 (70.14%). The fertilization rate was good as compared with other results of (33) who reported a fertilization rate of 45.5% using sperms after swim up in the Bengal black goat, and others authors reported higher percentages reached between 64 and 77% of fertilization (14, 34, 35) who obtained 61.3% of fertilization.
- **Sexing of embryos:** Sex of IVF produced embryos, by spermatozoa selected from the 4<sup>th</sup> layer of ficoll separation technique with an accurate and reliable protocol for PCR-based, it is based on detection of the target sequence which is the SRY genome. The amplification of only the Y-specific target sequence has been done successfully in bovine and caprine embryos sexing studies (36, 37, 38). So the present PCR protocol is reliable, accurate and efficient for sexing of IV produced local goat embryos. All embryos produced *in vitro* were analyzed by PCR. SRY is a gene located in this region and both conserved during evolution and Y chromosome specific in a number of mammals, so it is considered as perfect method for sex determination in mammals (38). The SRY gene on sex chromosome must amplified

by PCR reaction because the success of sex determination is based on the amplifying the genome DNA of the samples (38). The results after determination of the sex of embryos produced by spermatozoa selected from the 4<sup>th</sup> layer of ficoll density gradient which represented by 83.33% female embryos, was in accordance with the results of (27) who recorded 81.80% of female embryos in local sheep using the same concentration of ficoll layer and the same centrifugation force.

It was concluded from this study that, it possible to select a high percentage of X bearing spermatozoa from the cauda epididymis of local buck by application of ficoll density gradient, and successfully used in IVF of doe oocytes prior to obtain a high percentage of female goat embryos.

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