Separation of X-Bearing Ovine Sperm by Centrifugation in Discontinuous Ficoll Density Gradient: Effect on Gender of *In Vitro* Produced Embryos

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Abstract

The aim of this study was the separation of X-bearing ovine sperm by centrifugation in discontinuous "Ficoll" density gradient and their effect on gender of *in vitro* produced embryos. The percentage of male and female ovine embryos produced after IVF which determined by polymerase chain reaction (PCR). The discontinuous Ficoll density gradient (three layer) centrifugation at 200×g (F_{1a}) and 300×g (F_{1b}) showed a significant differences ($P<0.05$) in the sex ratio toward female embryos (63.6 and 54.5%) respectively. While the discontinuous Ficoll density gradient (four layer 80, 60, 40 and 20) centrifugation at 200×g (F_{2a}) and 300×g (F_{2b}) showed a significant differences ($P<0.05$) in the sex ratio toward female embryos (72.7 and 63.6%) respectively. While at four layer (80, 72, 64 and 50) concentration of ficoll the ratio was at 200×g (F_{3a}) and 300×g (F_{3b}) showed a significant differences ($P<0.05$) in the sex ratio toward female embryos (81.8, 72.7)%. From our results we can conclude that the purity of selected X-sperms and the percentage of female embryos obtained by IVF were good when we use $F_{2a,2b}$ and $F_{3a,3b}$ protocols than $F_{1a,1b}$ protocols, and the best result of IVF obtained when we apply high concentration of Ficoll media $F_{3a,3b}$ than $F_{2a,2b}$.

عزل حيامن الاغنام الحاملة للكروموسومات الانثوية بواسطة الطرد المركزي وباستعمال وسط الفيكول وتأثير ذلك على نسب جنس الأجنة المخصبة خارج الجسم

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الخلاصة

الهدف من الدراسة هو عزل الحيامن الحاملة للكروموسوم–X للاغنام بواسطة الطرد المركزي في وسط الفيكول وتاثير ذلك على نسب جنس الاجنة المخصبة خارج الجسم. باستعمال وسط الفيكول (ثلاث طبقات $F_{1a,1b}$) وقوة الطرد المركزي تساوي 200 ×g و300 ×g تم الحصول على فرق معنوي $p<0.05$ في نسبة جنس الجنين 54.5%,63.6 باتجاه جنس الإناث على التوالي. في حين باستعمال وسط الفيكول (أربع طبقات$F_{2a,2b}$) (80,60,40,20%) وقوة طرد مركزي تساوي 200×g و300×g تم الحصول على فرق معنوي $p<0.05$ في نسبة جنس الجنين 63.6%,72.7 باتجاه جنس الإناث على التوالي. في حين باستعمال وسط الفيكول (أربع طبقات $F_{3a,3b}$) (80,72,64,50%) وقوة طرد مركزي تساوي 200×g و 300 ×g تم الحصول على فرق معنوي $p<0.05$ في نسبة جنس الجنين تساوي 81.8%, 72.7% باتجاه جنس الإناث على التوالي. وقد استنتج من البحث ان نسبة الحيامن الحاملة للكروموسوم–X ونسب الاناث للاجنة المخصبة خارجيا كانت جيدة عند استعمال $F_{2a,2b}$ و $F_{3a,3b}$ اكثر من $F_{1a,1b}$ وافضل النتائج تم الحصول عليها باستعمال طريقة $F_{3a,3b}$.

Introduction

Sperm sexing rouses 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. The technique which provides a high accuracy is the flow cytometry that separates, by DNA content, two populations of

sperm (X and Y-bearing) with an accuracy of 90% (1). However this technique has disadvantages, which includes damage to sperm during sexing (2) and altered mRNA expression of embryos (3). Using a simple methodology, the density gradient was capable to separate X and Y-bearing sperm with lower cost and without damages to sperm viability. However, this technique presents accuracy of about 70%. In previous studies using the discontinuous Percoll density gradient, Hossepian De lima *et al.* (4) observed many difficulties that prevent commercial application such as the impossibility of gradient storage, that should be used immediately after semen collection or thawing, and moreover difficulties to prepare (many layers) that can increase the variability and it is time consuming. The primary objective of this study was to facilitate the procedure of preparing and storage continuous Percoll and OptiPrep density gradients for separation of X-bearing sperm and to evaluate their efficacy by PCR of embryos in vitro produced.

Materials and Methods

- **Oocytes collection from slaughterhouse ewe:** Ewes genetalia, only with follicular activity (n= 400 ovaries), were collected from Al-Shu'alah abattoir, transported within one hour in a normal saline in cool box to the Laboratory. The ovaries washed in normal saline and two washings in collecting media (Modified Tyrodes Albumin Lactate Pyruvate Media (mTALP). The follicles were counted and their diameters were measured with an automatic vernier. Oocytes were collected by aspiration from 4-8 mm size follicles. The media with harvested oocytes were transferred to one well out of 24 wells dish after grading to good, fair, and poor (type A, B and C).
- ***In vitro* maturation:** Only good and fair classified oocytes were selected. The oocytes were washed twice in a maturation medium (TALP), incubated in appropriate maturation medium at 39 ^{o}C, 5% CO_2 and 90% relative humidity for 27 hrs. The presence of the first polar body was a good criterion for maturation of oocytes *in vitro* (IVM).
- **Semen collection:** Three local rams of proven fertility used in this experiment, presented in the farm of College of Agriculture, Baghdad University, fresh semen was collected by artificial vagina (AV). Ejaculates from rams were pooled in equal quantities for final volume of 1-1.5 ml in order to minimize the variation between rams. Semen sample were examined under light microscope. The mass and individual motility was assessed, samples which showed less than 60% progressive motility was rejected.
- **Modified discontinuous Ficoll density gradient method:** The discontinuous Ficoll density gradient method described by (5) separates X-bearing and Y-bearing sperm was used. By this technique, the semen sample is diluted 1:1 with Hepe's buffered Hank's solution and layered onto a 15 ml centrifuge tube, which contain increasingly dense layers of isotonic Ficoll media as follow:-
- **Protocol 1 using 3 layers (200×g F_{1a}, 300×g F_{1b}):** 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). Density gradient media are available in commerce ready to use or ready to make the different density layers; the top layer phase (20%) is prepared by adding 2 ml of Ficoll density gradient medium to 8 ml isotonic sterile medium; the medium layer phase (40%) is prepared by adding 4ml of Ficoll density gradient medium to 6 ml isotonic sterile medium; the lower layer phase (60%) is prepared by adding 6 ml of Ficoll density gradient medium to 4 ml of isotonic sterile medium, each layer is supplemented with BSA.

- **Protocol 2 using 4 layers (200×g F_{2a}, 300×g F_{2b}):** Four layers density-gradient, formed by a top layer of 20% (v/v), medium layer of 40% (v/v), sub medium layer of 60% (v/v), and a lower layer of 80% (v/v).
- **Protocol 3 using 4 layers (200×g F_{3a}, 300×g F_{3b}):** 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 is prepared by layering 1 ml of each concentration, and then 1 ml of washed semen is layered over the upper layer and centrifuged at 200×g, 300×g or 400×g for 30 minutes. After the centrifugation, most of the supernatant must be gently removed and the pellet is placed into a new sterile test tube; here, the pellet is well re suspended in 5 ml of Hepe's buffered Hank's solution to remove the density gradient medium. It is centrifuged at 200×g, 300×g or 400×g for 10 minutes, the supernatant is then removed and the final pellet is re suspended in the sterile Hepe's buffered Hank's solution for the IVF.

- **Capacitation of sperms:** Selected sperm samples were diluted 1:10 with TALP, and then diluted 1:1 with heparin chloride containing media (100 µg/ml) and incubated for 45 minutes at 38 ^{o}C according to the procedure of (6).
- ***In vitro* fertilization:** Capacitated sperms suspension were diluted to yield a final concentration of 1.0 $\times 10^{6}$ sperm/ ml in the fertilization medium (TALP), pH 7.4-7.8. Only matured oocytes were kept in group of 5 to 10 oocytes in one well of 24 wells petridish containing fertilization medium with sperms and incubated at 39 ^{o}C, 5% CO_2 and 90% relative humidity for 24-27 hr (7).
- **Evaluation of fertilized oocytes:** 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.
- ***In vitro* Culture:** Cultures of previously fertilized oocytes (zygotes) were performed. Embryos were cultured in (TALP) at 38.5-39 ^{o}C, 5% CO_2, and 90% humidity. Embryonic developments were observed every 24 hrs and 50% of the media volume was replaced with fresh one at 24 hrs intervals. According to (8) procedure, ova that did not show cleavages were removed from the wells at the time of each change of medium. Proportions of fertilized oocytes reached 4 cells stage were recorded, and then extraction of DNA from embryos was applied.
- **DNA extraction from Embryos:** The cultured embryos were washed twice in culture medium and three times in KCl medium with 2 g/L bovine serum albumin. DNA was isolated from the embryos by the single step method described by (9) in a 1×PCR buffer (10 mM Tris HCl, pH 8.3, 50 mM KCl, 1.5 mM MgCl2 and 0.01% gelatin) containing Proteinase-K (150 µg/ml) and incubated for 1 hr at 37°C. Then Proteinase-K was inactivated by incubating at 99°C for 10 min. The tubes were kept frozen at -20°C until sexing was carried out by PCR.
- **Polymerase Chain Reaction (PCR) Technique:** Identification of DNA was performed in 20µl reactions containing approximately 5ng of template DNA isolated from embryo, 5 pmole primer(Promiga, Germany), and 250 µM each: dNTP (dATP, dCTP, dGTP, and dTTP), 1U Taq DNA polymerase, 10 mM Tris-HCL (pH 9.0), 30mM KCL and 1.5 mM $MgCL_2$. (Bio Neer, Kory).

The PCR amplification was carried out using a "MJ research thermal cycler" with the following amplification procedure: An initial denaturation for 5 min at 95°C was followed by 40 cycles of denaturation (60 sec at 94°C), primer annealing (for 60 sec at 56°C) and strand synthesis (for 120 sec at 72°C), and in the last cycle, the samples were held at 72°C for an additional 5 min. The amplification was confirmed by agarose gel electrophoresis (1%), stained with 2.5 µl ethidium bromide and visualized under UV light (260-280nm).

- **Restriction enzyme analysis:** PCR products (15 µl each) were subjected to digestion at 37°C for 3 h with 20 units of *Sac I* (Promiga, Germany). The Restriction fragment length polymorphism (RFLP) was then analyzed using 2.5% agarose gel electrophoresis and visualized under UV light (260-280nm).
- **Statistical Analysis:** Student t-test and Chi-square test were used for analysis of data according to (10).

Results and Discussion

- **Oocyte collection, maturation and fertilization:** From of 400 active ovaries collected from the slaughter house, the number oocytes collected from large follicles were 1020, the number of oocytes recovered from the right ovary were 490, while, those collected from the left ovary were 530 table (4-7). There was a significant difference in the numbers of oocytes ($P<0.05$) of the right and left ovaries. Similar observations have been revealed by several investigators (11, 12 and 13).
- **Grading of oocytes:** Visual assessment of morphological features is the most important vehicle for selection of oocytes before maturation during oocytes recovery. Our results showed that a high recovery rate was obtained of good oocyte (Grade A) 42.35% (432/1020), fair oocyte (Grade B) 37.54% (383/1020) followed by and poor oocyte (Grade C) 17.84% (182/1020). There was a significant difference ($P<0.05$) between the 3 different grades. Embryo development is influenced by events occurring during oocyte maturation, so for successful IVM, oocytes must undergo nuclear and cytoplasmic maturation. Only grades A and B oocytes (815/1020), 79.9% of recovered oocytes were cultured. Maturation rate was 86.38% (704/815). Similar observations had been reported by other workers (14, 15 and 7). It is obvious that oocyte quality is essential for embryonic development (16 and 7). The ability to identify good quality oocytes prior to *in vitro* culture is as important consideration for IVP of embryo system. The in vitro fertilization rate observed was 21.8% (132/604) of matured oocytes which Show the presence of the 2^{nd} polar body of fertilized oocyte. The percentage of fertilization was low as compared with the results reported by (17, 18 and 19). The fertilization rate obtained from slaughter house samples could be affected by several factors plays a role in successful IVF such as, cultural media, semen preparation with capacitating agents, season, and size of follicle and oocyte collection techniques.
- **Identification of sex of the embryo by PCR:** PCR technique has been applied for the identification of sex of embryos with WBC obtained from male and female sheep as follow: By choosing universal primers from sequences that are highly conserved in the X and Y chromosomes, sex-specific sequences were successfully amplified in embryonic lysates. The embryos subjected to PCR with "universal primer" showed uniform banding patterns (447 bp) irrespective of sex. This technique has recently been used as a reliable technique for the quantification of X and Y sperm cells in semen samples, especially to validate techniques for sexing sperm (20, 21 and 22).
- **Using sperms selected by modified discontinuous Ficoll density gradient technique (F_{1a}, F_{1b}, F_{2a}, F_{2b}, F_{3a}, and F_{3b}):** The discontinuous Ficoll density gradient (three layer) centrifugation at 200×g (F_{1a}) and 300×g (F_{1b}) showed a significant differences ($P<0.05$) in the sex ratio toward female embryos (63.6 and 54.5%) respectively. The discontinuous Ficoll density gradient (four layer 80, 60,40 and 20) centrifugation at 200×g (F_{2a}) and 300×g (F_{2b}) showed a significant differences ($P <0.05$) in the sex ratio toward female embryos (72.7 and 63.6%) respectively. While at four layer (80, 72, 64 and 50) concentration of ficoll was

(F_{3a}=81.8%, F_{3b}=72.7%) as shown in Table (1) (Fig. 1-3). These data show that discontinuous Ficoll gradients can be explored to separate sperm cell populations of different weights, as it can increase the proportion of X-bearing sperms. Results of sexing by discontinuous Percoll gradient made with 12 layers were described in bovines (4 and 23) ranged from 55.7 to 74.3% of sexual deviation for females; while using 2 layers by (24 and25) founded the results range from 55.2% to 63.41%. In humans, using the same continuous Percoll density gradient made by 12 layers, there was a variation to ward female 94% and 55% (26 and 27). In pigs, using 2 layers of discontinuous Percoll density gradient, there was a variation among 90.7% (28). But inflexible using continuous Percoll density gradient made by 1 layer in bovine by (29 and 30) found 63% and 62% respectively. In bovine, one centrifugation on 12-layer discontinuous Percoll gradient produced a female sex ratio deviation that ranged from 55.7 to 74.3% (4 and 23). Separation of X from Y-bearing sperm by means of discontinuous Percoll, Sephadex or Ficoll density gradient, an 82% X-bearing sperm population recorded in the bottom fraction of an 8layers. Increasing the number of step of the gradient to 12 improved the purity of X-bearing sperm to 94% (5). The sedimentation velocity of human X-bearing sperm was revealed as being faster than that of Y-bearing sperm (5). In spite that (23) believed that separation occurs as a result of X- and Y-bearing sperms density difference. However, when a gradient is centrifuged, the effect of sperm motility is minimized and their mass difference effect is maximized, it makes the heavier sperms reach the bottom faster. Another factor might affect the speed of sperm penetrating through Ficoll gradient, heavier sperms should settle down faster than lighter sperms; therefore centrifugation time could positively influence X-bearing sperms moving down the gradient. The shorter the centrifugation time, the less time the Y-bearing sperm (lighter ones) would have to reach the bottom towards higher ficoll concentration. The use of larger volume gradients in order to make sperm penetration more difficult could be an alternative. Smaller volumes (1 to 4 ml) may not be enough to promote separation. Larger volumes (>7 ml), however, and higher Percoll concentrations (close to 90%) would turn the swimming down of lighter sperm more difficult (25). From our results we can conclude that the purity of selected X-sperms and the percentage of female embryos obtained by IVF were good when we use $F_{2a, 2b}$ and $F_{3a, 3b}$ protocols than $F_{1a, 1b}$ protocols, and the best result of IVF obtained when we apply high concentration of Ficoll media $F_{3a, 3b}$ than $F_{2a, 2b}$. The only difference showing lowest rate of female embryos (63.60 and 54.50%) was observed in three layers of Ficoll gradient ($F_{1a, 1b}$). This might be due to low concentrations, low number of layers of Ficoll media and speed of centrifugation used. Also, when we compare the rate of female embryos produced from IVF by sperms isolated by four layer of Ficoll media ($F_{2a,b}$ $F_{3a , 3b}$), the a moderate results obtained in F_{2b} (63.6%) while the best results were with F_{3a} separation protocol (81.8%), results shown in table (1).

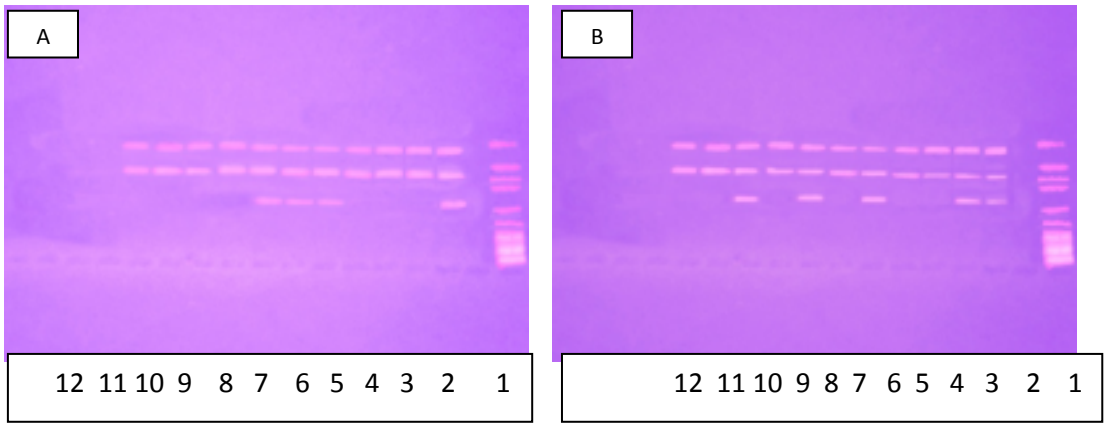

Fig. (1) Restriction patterns from Sac 1 digested P1-5EZ and P2-3EZ- PCR products of sheep embryos.

Lane1: 100 bp ladder (marker).

a- Sexed embryos F_{1a}: Male lanes 2, 6, 7 and 8 only. Female lanes 3, 4, 5, 6, 9, 10, 11 and 12.

b- Sexed embryos F_{1b}: Male lanes 2, 3, 6, 8 and 10 only. Female lanes 4, 5, 7, 9, 11 and 12.

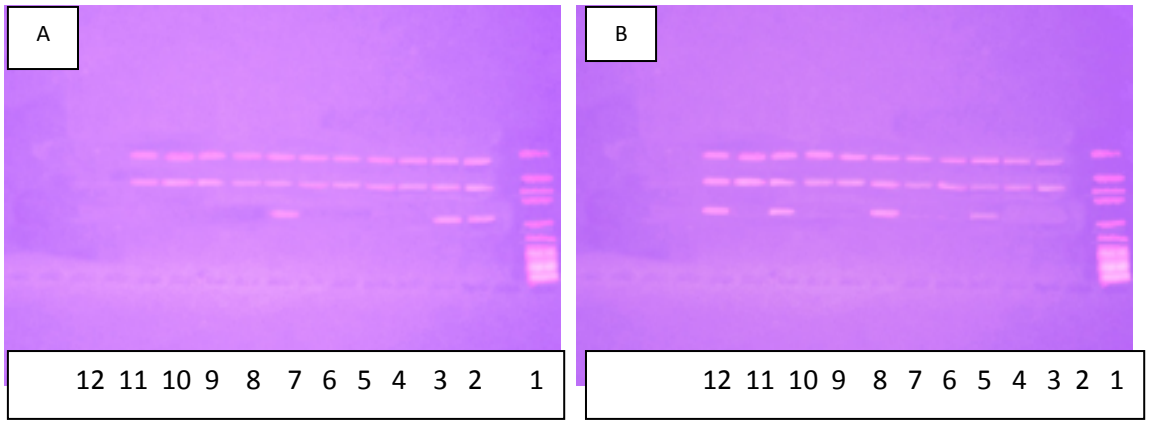

Fig. (2) Restriction patterns from Sac 1 digested P1-5EZ and P2-3EZ- PCR products of sheep embryos.

Lane1: 100 bp ladder (marker).

a- Sexed embryos F_{2a}: Male lanes 2, 3 and 8 only. Female lanes 4, 5, 6, 7, 9, 10, 11 and 12.

b- Sexed embryos F_{2b}: Male lanes 4, 7, 10 and 12 only. Female lanes 2, 3, 5, 6, 8, 9, 10 and 11.

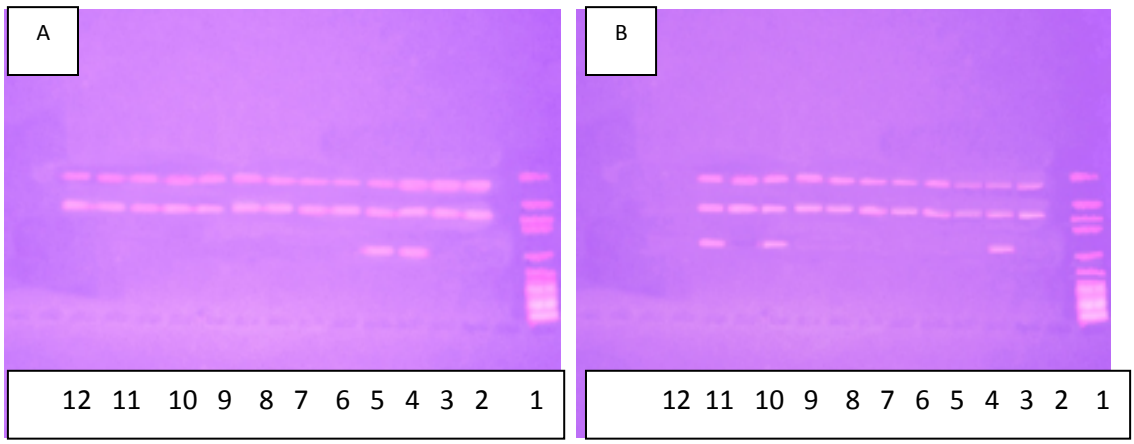

Fig. (3) Restriction patterns from Sac 1 digested P1-5EZ and P2-3EZ- PCR products of sheep embryos.

Lane1: 100 bp ladder (marker).

a- Sexed embryos F_{3a}: Male lanes 4, and 5 only. Female lanes 2, 3, 6, 7, 8, 9, 10, 11 and 12.

b- Sexed embryos F_{3b}: Male lanes 3, 10 and 12 only. Female lanes 2, 4, 5, 6, 7, 8, 9, 10 and 11.

Table (1) Percentage of Male and Female Embryos obtained after IVF using Sperms separated by centrifugation in discontinuous Ficoll density gradient

Treatment	No. of embryos	Female		Male	
		No	%	No	%
$F1_a$	11	7	(63.60)	4	(36.40)
$F1_b$	11	6	(54.50)	5	(45.50)
$F2_a$	11	8	(72.70)	3	(27.30)
$F2_b$	11	7	(63.60)	4	(36.40)
$F3_a$	11	9	(81.80)	2	(18.20)
$F3_b$	11	8	(72.70)	3	(27.30)

(F1a) = Ficoll 3 layers, 200×g, (F1b) = Ficoll 3 layers, 300×g, (F2a) = Ficoll 4 layers, 200×g, (F2b) = Ficoll 4 layers, 300×g, (F3a) = Ficoll 4 layers, 200×g, (F3b) = Ficoll 4 layers, 300×g.

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