Separation of Y-chromosome Bearing Ram's Sperms using an Albumin Gradient Technique and Identification of Embryos by PCR

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Abstract

Several advantages have been suggested for producing sexed sperms including using fewer and genetically superior female animals for replacement.Four hundred active ovaries collected from the slaughter house of Al-shu'alah, the number and type of oocytes, ratios of maturation and fertilization shown that there was a significant difference in the numbers of oocytes ($P<0.05$) between right and left ovaries. 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. grades A and B oocytes, (815/1020) 79.9% of recovered oocytes were cultured. Maturation rate was 86.38% (704/815).Y- Bearing sperms separation applied by using procedure of the modified albumin technique; either one (8%) or two layers (8 and 16%) of BSA ($M_{1,}$ and M_2) at 200, 300 or 400 xg, then used for *in vitro* fertilization.The *in vitro* fertilization rate observed was 21.8% (132/604) of matured oocytes 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. Bovine serum albumin sexed sperms result in more percentage of male embryos by using one layer of BSA (8%) at 200 × g (M_{1a}) and 300× g (M_{1b}) which were 72.7% and 54.5% respectively, and shows a deviation ($p<0.05$) from the 50% expected percentage for male and female embryos. While using two layer of BSA (16% and 8% BSA) at the 200 × g (M_{2a}) and at 300× g (M_{2b}) were 81.8% and 63.6% respectively. When we compare the rate of male embryos produced from IVF by sperms isolated by two layers of BSA ($M_{2a,}$ and M_{2b}), moderate results obtained with M_{2b} (63.6%) while the best results were with M_{2a} separation protocol (81.8%).

Introduction

Sexing of ovine sperms might affect both biological and economical effectiveness. Several advantages have been suggested for producing sexed sperms including using fewer and genetically superior dairy females for heifer replacement (1), to provide a wider chance for crossbreeding dairy females, fewer numbers of them required for progeny testing (2), and to facilitate endangered species conservation (1).A great concern and debate on sex-selection has resulted when discontinuous albumin gradient technique was used to enrich the Y-chromosome bearing sperms in human (3). Since then, many new techniques have been developed to separate spermatozoa, such as modified swim-up procedure to enrich Y-chromosome bearing sperms(4), Sephadex column technique and 12-step Percoll gradients to enrich X-bearing sperms (5and6) and free-flow electrophoresis (7and8). However, the validation of the enrichment of X- and Y-chromosome is controversial. It has been suggested that, flow cytometry to separate the sperms showed a promising commercial potential to sex sperms (9,10and11). Unfortunately, this technique requires appropriate skills and expertise as well as it is not easily accessible.The objective of this study was to evaluate the effectiveness of an albumin gradient technique in separating the ram sperms carrying Y- chromosomes.

Materials and Methods

Oocytes collection from slaughterhouse ewe:-

Female genitalia (n= 400) of local ewes were collected from Al-shu'alah abattoir. The reproductive history of the animals was unknown. Genetalia were transported within one hour in a normal saline in cool box to the Laboratory. The ovaries were removed from the surrounding tissue and over lying bursa washing in normal saline and two washings in collecting media (Modified Tyrodes Albumin Lactate Pyruvate Media (m TALP).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, 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 temp, 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 1-1.5ml 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 procedures of the albumin separation method (M_{1a}, M_{1b}, M_{2a}, and M_{2b}):-

The procedure of the modified albumin technique followed in this study is basically the same as the one described by the Beernink *et al.* (1993) (12) with minor modifications. The Modified albumins separating X and Y chromosome bearing sperms technique involves the following steps:-

Pooled semen was diluted 1:1 with Hepe's Buffer Hank's Solution and centrifuged for 10 minutes at 200×g. The supernatant was removed and the pellet was re suspended with 0.5 ml Hepe's Buffer Hank's Solution (concentration 3.22-3.97 $\times 10^9$ sperm/sample). In this procedure, we used the following protocols:-

Protocol 1 (200×g-M_{1a}, 300×g-M_{1b}), (using one layer of BSA):-

Test tubes (six tubes) containing 0.5ml of washed semen sample was layered over 1 ml of 8% bovine serum albumin (BSA) (Merk, Germany), incubated for 1hr at room temperature. At the end of the incubation period, the upper layers discarded, the lower layers of each column were pooled and diluted with Hepe's Buffer Hank's Solution 1:1, centrifuged for 10 min at 200×g, 300×g or 400×g, the supernatant was discarded, and each pellet was re suspended in 0.25-0.5 ml of Hepe's Buffer Hank's Solution, then sperms analysis was applied.

Protocol 2 (200×g M_{2a}, 300×g M_{2b}), (using 2 layers of BSA):-

Test tubes (six tubes) containing 0.5ml of washed sperms was layered over 2 layers of BSA; 1 ml of 8% BSA over 0.5 ml of 16% BSA, incubated at room temperature. After 60min, the sperm suspension and upper half of the 8% BSA layer were removed. After further 30min, the remained 0.5 ml of the 8% BSA layer and upper part of the 16% BSA layer were removed. The remainder of the 16% BSA layers was pooled in a test tube, diluted with Hepe's Buffer Hank's Solution 1:1, centrifuged for 10min. at (200×g, 300×g or 400×g). The supernatant was removed and the final sperm pellet was re suspended in 0.25-0.5 ml

of Hepe's Buffer Hank's Solution prior to sperm analysis.

Capacitating of sperms:-

Selected sperm samples were dilution 1:10 with TALP, 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 Palamo *et al*.,(13).

***In vitro* fertilization:-**

Capacitated sperms suspension were diluted to yield a final concentration of 1.0 x10^{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 27 hr (14).

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 Keskintepe *et al*., (15) procedure, ova that did not show cleavage 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 Taneja *et al.* (16) 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.

Polymers 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).

Results and Discussion

Oocytes collection, maturation and fertilization:-

Four hundred of active ovaries collected from the slaughter house, the number and type of oocytes, ratios of

maturation and fertilization were recorded. There was a significant difference in the numbers of oocytes ($P<0.05$) of right and left ovaries. Similar observations have been revealed by several investigators (17, 18, and 19).

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 oocytes (Grade A) 42.35% (432/1020), fair oocytes (Grade B) 37.54% (383/1020) followed by and poor oocytes (Grade C) 17.84% (182/1020). There was a significant difference ($P<0.05$) between the 3 different grades.Same observations have been reported by Wani *et al.*, (20) in sheep and by Sogorescu *et al.* (21) in sheep and goats. The ability to identify good quality oocytes prior to *in vitro* culture is as important consideration for IVP of embryo system.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). Our results were similar observations which had been reported by other workers (22, 23, and 24). It is obvious that oocyte quality is essential for embryonic development. The ability to identify good quality oocytes prior to *in vitro* culture is as important consideration for IVP of embryo system (24 and 25).The *in vitro* fertilization rate observed was 21.8% (132/604) of matured oocytes. The percentage of fertilization was low compared with the results reported by Sogorescu *et al.* (21), Khatun *et al.* (26) and by Hoque *et al.* (27). The fertilization rate obtained from slaughter house samples could be affected by several factors playing a role in successful IVF like cultural media, semen preparation with capacitating agents, season, follicle size 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 (Figure 1). 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 (28, 29, and 30).

Using sperms selected by modified bovine serum albumin gradient technique (M_{1a}, M_{1b}, M_{2a}, and M_{2b}):-

Bovine serum albumin sexed sperms result in more percentage of male embryos by using one layer of BSA (8%) at 200 × g (M_{1a}) and 300× g (M_{1b}) which were 72.7% and 54.5% respectively, and shows a deviation ($p<0.05$) from the 50% expected percentage for male and female embryos. While using two layer of BSA (16% and 8% BSA) at the 200 × g (M_{2a}) and at 300× g (M_{2b}) were 81.8% and 63.6% respectively.Corson *et al.*, (1984) (31) using the albumin separation technique, reported that 28 males were born out of 35 conceptions (80%). Also male-birth ratio of 73.5% using a three-layer separation was reported by Beernik and Ericsson, (1982) (32), the two-step, two-layer separation technique was reported to produce a male birth ratio of 73%.Ericsson *et al.*, (3) reported separation of X- from Y-bearing sperm by placing a solution of sperm on a layer of BSA. Apparently, Y-bearing sperm moved more quickly into the albumin. The researches hypothesize that Y-bearing sperm swim faster in BSA than X- bearing sperm.

From our results we can conclude that the purity of selected Y-sperms and the percentage of male embryos obtained by IVF were good when we use $M_{2a, 2b}$ than $M_{1a, 1b}$ protocols.The only difference showing lowest rate of male embryos (54.5 %) was observed in one layer of BSA (M_{1b}). This might be due to low concentrations of BSA, low number of layer and speed of centrifugation used.Also, when we compare the rate of male embryos produced from IVF by sperms isolated by two layers of BSA ($M_{2a,}$ and M_{2b}), the a moderate results obtained with M_{2b} (63.6%) while the best results were with M_{2a} separation protocol(81.8%), results shown in table (1).

Table1:- Percentage of male and female embryos obtained after separation by albumin gradient technique compared with the expected percentage of 50% of each gender and between treatments.

Treatment	No of embryos	Male		Female	
		No	%	No	%
M1a	11	8	(72.70)	3	(27.20)
M1b	11	6	(54.50)	5	(45.40)
M2a	11	9	(81.80)	2	(18.10)
M2b	11	7	(63.60)	4	(36.30)

M1a:-BSA one layer 200xg, M1b:-BSA one layer 300xg, M2a:-BSA two layer 200xg, M2b:-BSA two layer 300xg.

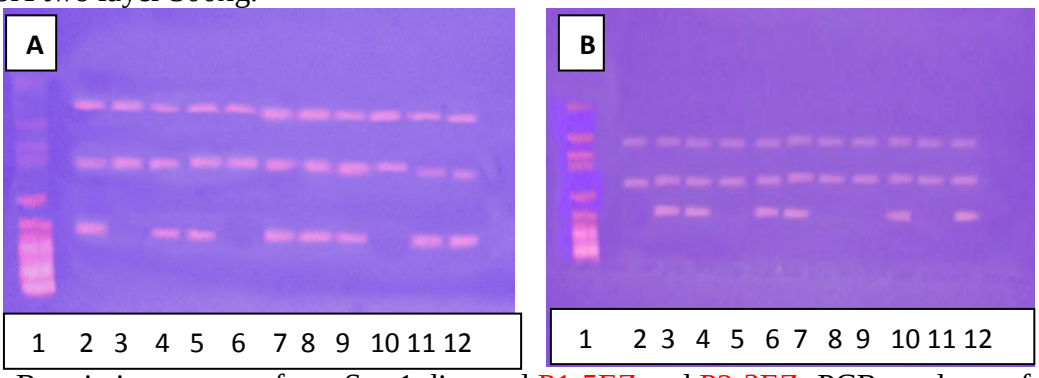

Figure 1: Restriction patterns from Sac 1 digested P1-5EZ and P2-3EZ- PCR products of sheep embryos Lane1: 100 bp ladder (marker).

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

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

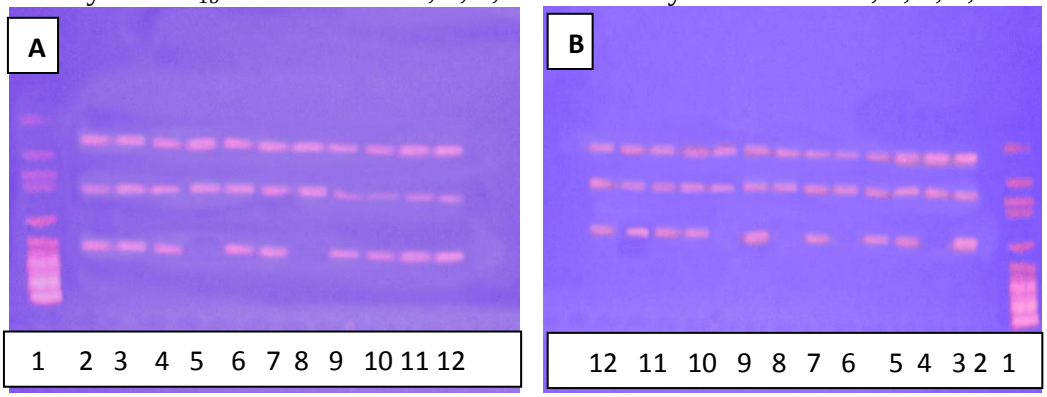

Figure 2: Restriction patterns from Sac 1 digested P1-5EZ and P2-3EZ- PCR products of sheep embryos Lane1: 100 bp ladder (marker).

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

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

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فصل الحيامن الذكرية بالاكباش بواسطة تقنية الالبومين وتجنيس الاجنة المخصبة خارجيا بواسطة بي سي ار (PCR)

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الخلاصة

ان تجنيس او اختيار جنس الجنين له فوائد وتطبيقات مهمة في مهنة الطب البيطري. في البحث الحالي تم جمع 400 مبيض اغنام من مجزرة الشعلة لغرض جمع البيوض. وقد وجدت فروقات معنوية بين عدد الجريبات بين المبيضين الايمن والايسر.كانت نسبة جمع البيوض عالية من النماذج حيث كان عدد البيوض من النوع الجيد 42,35%،المتوسط37,54% والردئ17.84%. تم فصل (عزل) الحيامن الذكرية لثلاثة كباش بواسطة التقنية التي تستعمل مصل دم الابقار، اما باستعمال طبقة واحدة 8% او طبقتين8و16% من البومين مصل الابقار. تم الاستعمال هذه الحيامن في الاخصاب الخارجي للبويضات ثم تم تحديد جنس الجنين بواسطة تقنية الPCR حيث كانت النتيجة الحصول على اجنة الذكرية بنسب72.7%و54.5% عند استعمال طبقة واحدة BSAوبقوة g×200وg× 300على التوالي. في حين ان استعمال طريقة العزل بطبقتين منBSA وكانت نسب الاجنة الذكرية 81.8%و63.6%عن قوة طرد مركزي تعادلg×200وg×300على التوالي.