Effect of Sperm Selection by "Swim-Up" Technique on the Sex Ratio of *In Vitro* Produced Ovine Embryos

Saja Hadi and Ihsan H. AL-Timimi
Department of Surgery and Obstetrics, College of Veterinary Medicine, Baghdad University, Iraq
E. Mail: Saja.1111@yahoo.com
Accepted on:27/1/2013

Summary

The objective of this study was to investigate the percentage of male and female ovine embryos produced after oocytes fertilization by sperm selected with self-migration (swim-up) technique. The sex of *in vitro* produced embryos was determined by Polymerase chain reaction (PCR). Results indicate that the fertilization by sperm selected using Swim-up technique was 81.8% (9/11) male and 18.1% (2/11) female *in vitro* produced embryos when centrifuged at 200×g. While centrifugation at 300×g was 72.7% (8/11) male and 27.2% (3/11) female embryos. It was concluded that the use of "swim-up" technique refer to significant ($P<0.05$) occurred for increasing male embryos ratio compared with normal ratio (50%) which represent the sex, and it is for the first time, preselected sperms used for *In Vitro* fertilization (gender selection) in farm animals in Iraq.

Keywords: ovine, embryos, swim-up, gender, PCR.

Introduction

Predetermining the gender of offspring in the dairy and beef cattle industry allows the breeders to plan their production toward a specific gender. The most effective way to achieve this goal is to separate X- from Y-bearing sperms (1). The only way to effectively separate sperm cell populations is based on their DNA content (1 and 2). Flow cytometry has been widely used for this purpose (3-5), however, this technique presents some specific problems, such as broad fluorescence distribution without a distinct X- and Y- peak (1).

In an attempt to develop a method for the separation of sperm cell populations based on their DNA content, Percoll media density gradient centrifugation has been used on human and bovine sperm (6 - 8).

Percoll is composed of colloidal silica particles (15-30 nm in diameter) coated with polyvinylpyrrolidone (9), which increases the specific gravity of the medium to 1.13 g/ml (10). It is also used to isolate bacteria (11), neutrophils (12), viruses (13) and subcellular particles (14 and 15). It is a simple and low cost method (16) which selects highly motile sperms that reach the medium surface after incubation. Considering the simplicity and practicality of these sperm selection methods, the purpose of the present study was to determine if semen selected by swim-up, 45-90% discontinuous and 67.5% continuous Percoll media density gradient centrifugations alter the gender percentage of *in vitro* produced bovine embryos. In the same time, the five and the ten minutes centrifugation was tested on both Percoll gradients. The aim of this work was to investigate the percentage of male and female ovine embryos produced after in vitro fertilization of oocytes by sperm selected with self-migration (swim-up) technique.

Materials and Methods

Two hundred female genitalia of local non-pregnant 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 Lab. 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, using gage 18 needles attached to a 3ml syringe containing 2 ml of the collecting medium.

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). Only good and fair classified oocytes (A and B) were selected. The oocytes were washed twice in a maturation medium (TALP), incubated in appropriate maturation medium at 39 ° 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) (17).

Three local fertile rams 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.

The procedure of the modified swim-up technique followed in this study is basically the same as that described by (16) with minor modifications, Ham's F10 was used as an alternative media to evaluate the efficiency of the swim-up technique in separating X and Y chromosome bearing sperms. The technique involves the following:-

Semen sample (0.25ml) transferred to 15 ml centrifuge tube. The sperms were washed 2 times using 0.5 ml Ham's F-10 medium and then centrifuged at 200 and 300 ×g for 10min. Supernatant removed and overly sperm pellets with 0.5ml of Ham's F-10 medium with extreme attention in each tube. The tubes must be put in the incubator, inclined at an angle around 45° and incubated at 37°c and 5% CO_2 for 30-60 minutes. By inclining the tubes at 45°, to increase the surface between the medium and the semen and we improve the capability of the sperms to swim out of the pellet and reach the medium. Following incubation, the first 0.25ml was discarded and the final 0.25ml was used for IVF. Selected sperm samples were dilution 1:10 with TALP, then diluted 1:1 with heparin containing media (100 µg/ml Heparin salt) and incubated for 45 minutes at 38 ºC according to (18).

Capacitated sperms suspension were diluted to yield a 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 ° C with 5% CO_2 and 90% relative humidity for 27 hr (19).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 (19).

Cultures of previously fertilized oocytes (zygotes) were performed. Embryos were cultured in (TALP) at 38.5-39 ° C, 5% CO_2, and 90% humidity. Embryonic developments were observed every 24 hrs and 50% of the culture volume was replaced with fresh medium at 24 hrs intervals. According to (20), ova which 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. The cultured embryos were washed twice in culture medium and three times in potassium chloride medium with 2 g/L bovine serum albumin. DNA was isolated from the embryos by the single step method. (21) 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 (my genie 32, thermo block, bioneer, Korey).

Identification of DNA was performed in 20µl reactions containing approximately 5ng of template DNA isolated from embryo, 5 pmole primer, 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 $MgCL_2$.

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).PCR products generated with the primers sets, P1-5EZ and P2-3EZ, were analyzed by restriction analysis. PCR products were subjected to digestion samples were incubated with 20 units Sac 1 (2 µl), (2 µl) of RE 10x buffer, (2µl) DNA, and sterile de ionized water to a total volume of (20 µl). Samples were digested, mix gently by pipetting, close the tube and centrifuge for a few second in a micro centrifuge. Incubate at the optimum temperature for 1- 4 h at 37°C.

The Restriction fragment length polymorphism (RFLP) was then analyzed using 2.5% agarose gel electrophoresis and visualized under UV light (260-280nm). Student t-test and Chi-square test were used for analysis of data according to (22).

Results and Discussion

Four hundred active ovaries collected from the slaughter house, the number and type of oocytes, ratios of maturation and fertilization were shown in (Table, 1). There was a significant (P<0.05) difference in the numbers of oocytes of right compared left ovaries. Similar observations had been revealed by several investigators (17, 23 and 24). Visual assessment of morphological features remains the most important vehicle for selection of oocytes before maturation during oocytes recovery. The results in (Table, 1) showed that a high recovery rate was obtained of good oocyte (Grade A) 43.32% (432/ 997), fair oocyte (Grade B) 38.41% (383/997) followed by and poor oocyte (Grade C) 18.25% (182/997). There was a significant (P<0.05) difference between the 3 different grades. Similar observed have been reported by (25 and 26). 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) (Table, 1). Similar observations had been reported by other workers (20, 27 and 28). 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 (28 and 29).

The in vitro fertilization rate observed was 21.8% (132/604) of matured oocytes. The rate of fertilization was low compared with the results reported by (26, 30 and 31). 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.

Table,1: The number of ovaries, large follicles, oocytes, grades of oocytes collected from slaughtered ewe, maturation and fertilization rate.

No of active ovaries	Mean size of large follicle (mm)	No of oocytes collected	Grade and no of oocyte			Maturation rate for A and B oocytes	Fertilization rate for A and B oocytes
			A	B	C		
400	6.56 ± 1.19	997	432 (43.32%) **A**	383 (38.41%) **c**	182 (18.25%) **b**	704/815 (86.38%)	132/704 (21.8%)

PCR technique has been applied for the identification of sex of embryos with WBC obtained from male and female sheep 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 (Fig.1). This technique

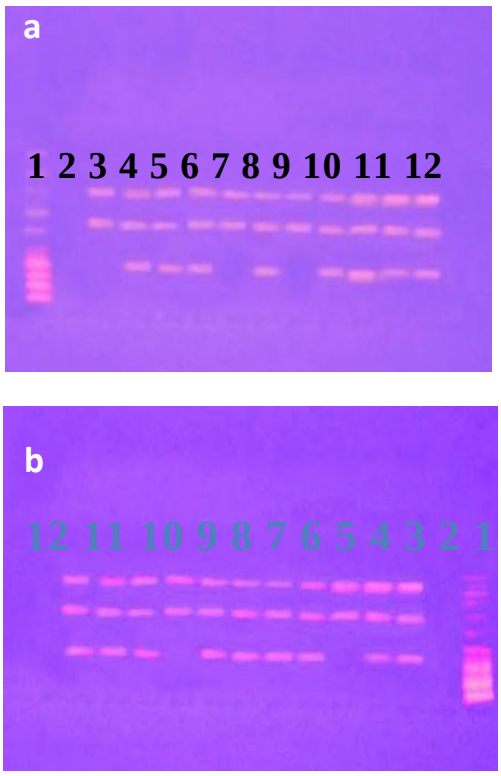
has recently been used as a reliable technique for the quantification of X and Y sperm cells in semen samples, especially to validate techniques for sexing sperm (32- 34).

Swim-up sexed sperms result in more percentage of male embryos by using the 200 × g (S1) and 300× g (S2) which were 81.8% and 72.7% respectively(Table, 2) and shows a deviation ($p<0.05$) from the 50% expected percentage for male and female embryos. While (34) reported 63.6% of male embryos using sperms selected using 200 ×g for 10 min and incubation for 40 min, and (35) who obtained lower % of male embryos (58.45%) using 50 ×g for 10 min and incubation for 1 hr. in bovine. The selection of X or Y bearing sperm might be due to the differences in size and mass of each type of sperms, as described by (3), the Y-chromosome is lighter and smaller than the X-chromosome; the DNA content is greater (3.8%) in X-than Y-chromosome. In addition (36) believes that Y-bearing sperm is faster than X-bearing sperm. Supporting this study, (37) observed a significant male and female ratio deviation (toward male embryos using swim-up for 45 min incubation. Therefore data may suggest that swim-up favors Y-bearing sperm selection, which migrates faster to reach the medium surface. From this result it can concluded that the purity of selected Y-sperms and the percentage of male embryos obtained by IVF were good when it use S_1 protocol than S_2 protocol, and the best result of IVF obtained when it apply lower centrifugation gravity.

Table, 2:- Percentage of male and female embryos obtained after swim-up compared with the expected percentage of 50% of each gender and between treatments.

Treatment	No of embryos	Male		Female	
		No	%	No	%
S1	11	9	(81.80)	2	(18.10)
S2	11	8	(72.70)	3	(27.20)

S1:-swim-up (200xg), S2:-swim-up (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 200×g: Female lanes 4, and 9 only. Male lanes 2, 3, 5, 6, 7, 8, 10, 11 and 12.
b- Sexed embryos at300×g: Female lanes 2, 6 and 8 only. Male lanes 3, 4, 5, 7, 9, 10, 11 and 12.

References

1. Stap, J.; Hoebe, R.A.; Merton, J.S.; Haring, R.M.; Bakker, P.J.M. And Aten, J.A. (1998). Improving the resolution of cryopreserved X- and Y-sperm during DNA flow cytometric analysis with the addition of Percoll to quench the fluorescence of dead sperm. J. Anim. Sci., 76:1896-1902.
2. Cran, D.G. and Johnson, L.A. (1996). The predetermination of embryonic sex using flow cytometrically separated X and Y sperms. Hum Reprod Update, 2:355-363.
3. Johnson, L.A. (2000). Sexing mammalian sperm for production of offspring: the state-of-the-art. Anim. Reprod. Sci., 60-61:93-107.
4. Bodmer, M.; Janett, F.; Hässig, M.; den. Daas, N.; Reichert, P. and Thun, R. (2005). Fertility in heifers and cows after low dose insemination with sex-sorted and nonsorted

sperm under field conditions. Theriogenology, 64(7):1647-1655.
5. Garner, D.L. (2006). Flow cytometric sexing of mammalian sperm. Theriogenology, 65:943-957.
6. Kaneko, S.; Oshio, S.; Kobayashi, T.; Mohri,; H. and Iizuka, R .(1984). Selective isolation of human X-bearing sperm by differential velocity sedimentation in Percoll density gradients. Biomed. Res., 5:187-194.
7. Kaneko, S.; Yamaguchi, J.; Kobayashi, T. and Iizuka, R. (1983). Separation of human X- and Y-bearing sperm using percoll density gradient centrifugation. Fertil. Steril., 40:661-665.
8. Hossepian-de-Lima, V.F.; Ramalho, M.D.; Rodrigues, L.H.; Malheiros, E.B. and Moreira Filho, C.A. (2000). Separation of X- and Y-bearing bovine sperms by Percoll density gradient centrifugation. Theriogenology, 53:480. (Abstract).
9. Samardzija, M.; Karadjole, M.; Matkovic, M.; Cergolj, M.; Getz, I.; Dobranic, T.; Tomaskovic, A.; Petric, J.; Surina, J.; Grizelj, J. and Karadjole, T. (2006). A comparison of BoviPure® and Percoll® on bull sperm separation protocols for IVF. Anim. Reprod. Sci., 91:237-247.
10. Makler, A.; Stoller, J. and Makler-Shiran, E . (1998). Dynamic aspects concerned with the mechanism of separating motile sperm from nonmotile sperm, leukocytes, and debris with the use of high-density Percoll gradients. Fertil. Steril., 70:961-966.
11. Leuschner, R.G.K.; Weaver, A.C. and Lillford, P.J. (1999). Rapid particle size distribution analysis of Bacillus spore suspensions. Coll Surf B-Biointerfaces, 13:47-57.
12. Woldehiwet, Z. Scaife, H. Hart, CA. Edwardset, SW. (2003). Purification of ovine neutrophils and eosinophils: Anaplasma phagocytophilum affects neutrophil density. J Comp Pathol, 128:277-282.
13. Hanabusa, H.; Kuji, N.; Kato, S.; Tagami, H.; Kaneko, S.; Tanaka, H. and Yoshimura, Y. (2000). An evaluation of semen processing methods for eliminating HIV-1. AIDS, 14:1611-1616.
14. Swales, C. and Wright, S. (2000). Evaluation of a continuous flow centrifuge for recovery of Cryptosporidium oocysts from large volume water samples. Wat. Res., 34:1962-1966.
15. Sheoran, A.S.; Feng, X.; Kitaka, S.; Green, L.; Pearson, C.; Didier, E.S.; Chapman, S.; Tumwine, J.K. and Tzipori, S. (2005). Purification of Enterocytozoon bieneusi from stools and production of specific antibodies. J. Clin. Microbiol., 43:387-392.
16. Henkel, R.R. and Schill, W.B. (2003). Sperm preparation for ART. Reprod. Biol. Endocrinol., 1:108.
17. Wani, N.A.; Wani, G.M.; Khan, M.Z. and Salahudin, S. (2000). Effect of oocyte harvesting technique on in vitro maturation and *in vitro* fertilization in sheep. Small Rum. Res., 36:63-67.
18. Fukui, Y.; Glew, A.M.; Gandolfi, F. and Moor, R.M. (1988). Ram specific effects on in vitro fertilization and cleavage of sheep oocytes matured in vitro. J. Reprod. Fertil., 82:337-340.
19. Kharche, S.D.; Goel, P.; Jha, B.K.; Goel, A.K. and Jindal, S.K. (2011). Factors influencing in vitro embryo production efficiency caprine oocytes: A review. Indian J. Anim. Sci., 81(4):344-361.
20. Keskintepe, L.; Simplicio, A. and Brackett, B.G. (1998). Caprine blastocyst development after in vitro fertilization with spermatozoa frozen in different extenders. Theriogenology, 49:1265-1274.
21. Taneja, M.; Appa Rao, K.B.C.; Gangawane, S.; Zawar, S.G. and Totey, S.M. (1998). Rapid sexing of bovine preimplantation embryos using PCR: Production of calves with predetermined sex under field conditions. Ind. J. Exp. Biol. 36:1201-1208.
22. Greyling, J.P.C. (1988). Reproductive physiology in the boer goat doe. PhD Thesis, University of Stellenbosch, South Africa.
23. Greyling, J.P.C. and Van Der Nest, M. (2000). Synchronization of oestrus in goats does affect of progestagen Small Rum. Res. 36:201-207.
24. Hafez, B. and Hafez, E.S. (2000). Reproduction in farm animals. 7th ed.

Lippincott Williams and Wilkins, a Wolters Kluwer Co., Philadelphia U.S.A.
25. Sogorescu, E.; Zamfierescu, S.; Anghel, A. H. and Dorina, N. (2010). The influence of new media on the developmental competence of goat and sheep oocytes. Romanian Biotech. Letter, 15(3):19-25.
26. Wang, Z.G.; Xu, Z.R. and Yu,S.D. (2007). Effect of oocyte collection techniques and maturation media on in vitro maturation and subsequent embryo development in Boer goat. Czech J. Anim. Sci., 52:21-25.
27. Rahman, A.N.M.A.; Abdullah, R.B. and Wan-Khadi Jah, W.E. (2008). Gametogenesis, Fertilization and Early embryogenesis in mammals with special reference to goat: A review. J. Biol. Sci., 8(7):1115-1128.
28. Camargo, L.S.A.; Viana, J.H.M.; Sa, W.F.; Ferreira, A.M.; Ramos, A.A. and Vale Filho, V.R. (2006). Factors influencing in vitro embryo production. Anim. Reprod., 3:19- 28.
29. Khatun, M.; Bhuiyan, M.M.; Ahmed, J.; Haque, A.; Rahman, M.B. and Shamsudin, M. (2011). In vitro maturation and fertilization of prepubertal and pubertal black Bengal goat oocytes. J. Vet. Sci., 12(1):75- 82.
30. Hoque, S.A.M.; Kabiraj, S.K.; Khandoker, M.A.M.Y.; Mondal, A. and Tareq, K.M.A. (2011). Effect of collection techniques on cumulus oocyte complex (COCs) recovery, in vitro maturation and fertilization of goat oocytes. Afr. J. Biotech., 10(45):9177-9181.
31. Joerg, H.; Asa, M.; Graphod, A. and Tskaya, D. (2004). Validating bovine sexed semen samples using quantitative PCR. J. Anim. Breed. Genet., 121:209-215.
32. Parati, K.; Bongioni, G. and Aleandri, R. (2006). Sex ratio determination in bovine semen: A new approach by quantitative real time PCR. Theriogenology, 66:2202-2209.
33. Puglisi, R.; Vanni, R.; Galli, A.; Balduzzi, D.; Parati, K.; Bongioni, G.; Crotti, G.; Duchi, R.; Galli, C.; Lazzari, G. and Aleandri, R. (2006). In vitro fertilisation with frozen-thawed bovine sperm sexed by flow cytometry and validated for accuracy by real-time PCR. Reproduction, 132(3):519-526.
34. Rheingantz, M.G.; Pegoraro, L.M.; Dellagostin, O.A.; Pimentel, A.M.; Bernardi, M.L. and Deschamps, J.C. (2006). The sex ratio of in vitro produced bovine embryos is affected by the method of sperm preparation. Anim. Reprod., 3(4):423-430.
35. McEvoy, J.D. (1992). Alteration of the sex ratio. Anim. Breed, 60:97-111.
36. Pegoraro, L.M.; Rheingantz, M.G. Deschamps, J.C.; Dellagostin, O.A. ; Pimentel, A.M. Bernardi, M.L. and Saalfeld, M.H. (2002). Percoll gradient versus swim-up: effect of sperm preparation method on sex ratio of in vitro produced bovine embryos. Theriogenology 57:751. (Abstract).
37. Schefler, W. C. (2000). Statistics of Biological Sciences. 4th ed., Addison Wesley Pub. Co., Inco.

تاثير عزل النطف بواسطة تقنية "السباحة للأعلى" على نسب جنس الاجنة المخصبة خارجيا في الاغنام

سجى هادي و احسان حمودي التميمي
فرع الجراحة والتوليد- كلية الطب البيطري- جامعة بغداد- العراق

الخلاصة
الهدف من هذه الدراسة هو تحديد نسب الاجنة الذكرية والانثوية بعد اخصاب بيوض النعاج بنطف معزولة بواسطة تقنية "العوم للأعلى" .تم تحديد جنس الجنين بعد الاخصاب الخارجي بواسطة سلسلة تفاعل البلمرة. بينت النتائج بان النطف المعزولة اعطت نسبة اجنة ذكرية بلغت 81.8% (11/9) ونسبة الاجنة الانثوية 18.1% (11/2) عند عزل النطف بقوة طرد مركزي تساوي 200 جاذبية. اما باستعمال العزل بقوة طرد مركزي تعادل 300 جاذبية فكانت النتائج المسجلة 72.7% (11/8) اجنة ذكرية مقابل 27.2% (11/3) اجنة انثوية يمكن الاستنتاج بان استعمال هذه التقنية ادت الى حدوث فرق معنوي ($P<0.05$) باتجاه زيادة نسبة الاجنة الذكرية مقارنة مع النسبة الطبيعية البالغة 50% لكل جنس وهي المرة الاولى التي يتم فيها استعمال الحيامن المعزولة لانتاج اجنة مخصبة خارجيا (اختيار جنس الجنين) في الحيوانات الحقلية في العراق .

الكلمات المفتاحية: اغنام، اجنة، العوم للأعلى، جنس الجنين ، سلسلة تفاعل البلمرة.