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CENTRIFUGAL INTERMITTENT DENSITIES COLUMN OF SUCROSE SOLUTION TO ASSORT OF RAM SPERMATOZOA

By

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ABSTRACT

Centrifugal intermittent densities of sucrose solution column were used in person study. Dry sucrose was weighted at 1.5, 2.0, 3.0 and 3.5 *g* then, dissolved in one ml of sodium citrate buffer to prepare the sucrose density layers as SDL1,SDL2,SDL3 and SDL4 (2000 µL/density layer), respectively. Hence, four tubes of densities column were put in the four caves of centrifugation rotor then, 1000 µL of raw semen / tube was placed on the top layer (SDL1) and centrifuged at 300 *g* /20 minutes. Post-centrifugation, the density layers (SDL1, SDL2, SDL3 and SDL4) of each sucrose column tube were individually separated from bottom to top in empty four test tubes (2000µL/layer/tube) using micropipette with a tiny hole. The tubes that contained SDL1, SDL2, SDL3 and SDL4 were incubated at 37^{o}C for 60 minutes then, percentages of sperm motility, dead spermatozoa and abnormal spermatozoa were recorded. The fertility rates and sex ratio of lambs were also recorded with SDL1, SDL2, SDL3 and SDL4 layers using eighty ewes (20ewes/density layer). The results showed that sperm quality was not significantly different among SDL1, SDL2, SDL3 and SDL4 during incubation at 37^{o}C for up to 60 minutes post-centrifugation. Advancing of incubation time for up to 60 minuts caused significantly ($P<0.05$) lower motility (%) however, significantly ($P<0.05$) higher dead spermatozoa and abnormal spermatozoa than 0 minutes with SDL1, SDL2, SDL3 and SDL4 post-centrifugation. The fertility rate of SDL1, SDL2, SDL3 and SDL4 layers; was 80.00, 80.00, 76.00 and 80.00%, respectively. The newborn female lambs were 14.29, 33.33, 73.68 and 85.00% while, the newborn male lambs were 85.71, 66.67, 26.32 and 15.00% in SDL1, SDL2, SDL3 and SDL4 layers, respectively.

In conclusion from the previous results it could be concluded that centrifugal technique of sucrose gradient density column could conserve sperm livability and modulation the sex ratio of either newborn as male or female lambs.

Keywords:

Rams*, Semen, Centrifugations, Sex ration, Sucrose density.*

INTRODUCTION

Recently sexed lambs become available for ram spermatozoa post-extension to alter the sex ratio of offspring. Sex ratio is depended on the major difference between the X- and Y- chromosomes through solutions. In this context, **Johnson (2000)** reported that percentage of deoxyribonucleic acid (DNA) was higher in X- spermatozoa by 4.2% than Y- spermatozoa in ram spermatozoa. Moreover**, Prakash *et al.* (2014)** reported that, the size of X- sperm is larger than Y- sperm, so sperm motility to be faster in Y chromosome than X chromosome sperm and the surface charges of X sperm is negative but Y sperm has a positive.

Many technologies have been developed to sorting sperm such as discontinuous Percoll gradient centrifugation **(Oliveira *et al.*, 2012),** spermatozoon swim-up **(Lucio *et al.*, 2012)**, modification of mating time **(Khalifa *et al.*, 2013*)***,* sperm centrifugation in albumin **(Hadi and Al -Timimi, 2013),** differential protein profile **(De Canio *et al.*, 2014)** and surface charge **(Karabinus *et al.*, 2014).** The centrifugation of sucrose densities column have been shown the potential criteria for sorting either X- and Y- bearing spermatozoa.

Thus, **Houch and Foote(2002)** reported that separation of sperm differing by about 0.005g/ml in gradient density; this is sufficiently sensitive to detect differences in density of sperm. **Gosálvez *et al.* (2011)** confirmed that centrifugation of spermatozoa increased abnormal spermatozoa up to 18.60 %, but viability of spermatozoa and their fertility were still active. On the other hand, **Rath *et al.* (2013)** reported that centrifugation of semen in gradient density had success rate ranged from 86 % to 94%. In addition, sucrose prepared with discontinuous gradient density resulted in sedimentation of X- bearing spermatozoa that settled in the bottom **(Kanesharatnam *et al.*, 2012)**. Furthermore, **Stefanov *et al.* (2015)** concluded that semen diluted with disaccharides and trisaccharides can be used for successful insemination and enhance pregnancy rate after artificial insemination.Generally, selection of gender in farm animals has great economic advantages; sperm cells sexing namely the possibility to pre-selection the sex of offspring prior to conception **(Stephen *et al.*, 2015).** Then, farmers prefer female offspring for dairy industry and greater efficiency in production of lambs while, selection for male is the first choice for mutton meat industry **(Pindaru *et al.*, 2016).**

This study is intended to throw put light on an attempt for separation of ram spermatozoa bearing X- and Y- chromosomes' by centrifugal counter. Therefore, fertility rates and sex ratio of newborn lambs were investigated with centrifugal gradient density column of sucrose solution.

MATERIAL AND METHODS

This study was carried out at El-Serw Experimental Farm, belonging to Animal Production Research Institute (*APRI*), Egypt. The experimental work through a period from May, to November, 2015.

1. Ram semen collection.

Eight sexually mature healthy Rahmani rams (33 months old) will be used to determine fertility rate. Semen samples were collected using an artificial vagina. One or two pooled consecutive ejaculates from each ram were used every day to evaluate sperm quality as progressive motility (%),dead spermatozoa(%) abnormal spermatozoa morphology (%). Rams were received daily the clean fresh water freely and concencerate feed mixtur and roughage according **NRC (2007).**

2. Semen evaluation.

The sperm motility (%), dead spermatozoa (%), abnormal spermatozoa (%) and sperm cell concentration (x 10^9 /ml) were evaluated as the method described by **Salisbary *et al.* (1978)**.

3. Sodium citrate buffer, dry sucrose and different gradient density of sucrose solutions

3.1. Preparation of Sodium citrate buffer (SCB).

The ingredients of SCB-based buffer were dissolved up to 100 ml of distilled water which included 2.37g sodium citrate, 0.50 g fructose and 0.05g citric acid. In order to control microbial contamination 1.00 ml of antibiotic was added; it contains 200 mg Procaine penicillin and 250 mg Dihydrostreptomycin sulphate. All chemicals were purchased from Sigma Chemical Co. (St. Louis, MO, USA).

3.2. Preparation of dry sucrose.

Sucrose sugar was placed in the oven at 60°C for up to 2-3 hours to dry the sugar surface of moisture. Then, the dry sucrose was packaged in dry jar and stored in dry place for preparing different gradient density of sucrose solutions.

3.3. Preparation of different gradient densities of sucrose solutions / test tube.

Four weights from dry sucrose (1.5, 2.0, 3.0 and 3.5 g) were dissolved in SCB to produce different sucrose density layers (SDL) at 1.5, 2.0, 3.0 and 3.5 g/ml to perform SDL1, SDL2, SDL3 and SDL4, respectively. Then, each different density layers as SDL1, SDL2, SDL3 and SDL4 were filtered post- preparation through filter paper to make sucrose solution cleanly. The SDL1, SDL2, SDL3 and SDL4 had Osmolality (determined by freezing point depression ohmmeter) at 185, 225, 295 and 329 mOsm/kg^{-1} and also pH value (measurement by pH Metter, pH-009, I) at 6.92, 6.94, 6.95 and 6.97, respectively.

4. Preparation of intermittent densities column of sucrose solutions /test tube.

The intermittent densities column of sucrose was prepared by using 2000 µL / density layer. Then, four clean and sterile tiny hole micropipettes were decanted the different density layers on the wall of clean test tube which arranged sucrose denisity layers from bottom to top as SDL4, SDL3, SDL2 and SDL1.

5. Centrifugation of intermittent densities column of sucrose solutions with raw semen / test tubes.

Four test tubes contained the gradient densities column were inserted into four caves of centrifugation rotor. Then, 1000 µL of raw semen / tube were loaded on the surface of the top layer (as SDL1) of each tube. The sucrose densities column tubes exposed to centrifugation at 300 *g* /min. for 20 minutes. Post-centrifugation, the tubes were removed from centrifugation rotor carefully without any disturbance of the sucrose densities column. The distribution of spermatozoa through the different densities (each tube) were separated together using micropipette with a tiny hole (like France straw 0.25 ml) allowed to make contact with the inside wall of the test tube. The tiny hole was introduced carefully into the bottom layer (SDL4) and one layer per collection was collected individually and transferred into four sterile tubes (2000 µL/ layer/ tube).

6.Evaluationofspermatozoa distributionin densitiesduringincubation post-centrifugation.

Thirteen times were used to evaluate incubation effect on semen parameters post-centrifugation using four individually (contained SDL1, SDL2, SDL3 and SDL4) test tubes / time. The spermatozoa distribution through SDL1, SDL2, SDL3 and SDL4 layers were individually collected as 2000 µL / density layer. Then ,the tubes were incubated at 37°C to assay semen quality as sperm motility (%), dead spermatozoa (%) and abnormal spermatozoa (%) for up to 0, 30 and 60 minutes.

7. Fertility trial.

Eighty Rahmani ewes were divided into four equal groups (20 ewes / group). Each group was inseminated by spermatozoa siwming in SDL1, SDL2, SDL3 and SDL4 layer. Ewe showed estrous was inseminated by 500 µL contains >100x10^6 fertile spermatozoa. The semen dosage was ejected at os-cervix (using open speculum) after 12 hours of heat inception. After 14 -19 days, the returned ewe to heat was inseminated by the same dose and same layer as described in the previous insemination techniques. Fertility rate after 1st and 2nd inseminations was determined on the basis of pregnancy by non-returns to heat again. Hence, fertility rate,

lambing rate and sex ratio of each sucrose density layer were calculated as following: fertility rate (number of ewes conceived / number of ewes inseminated). Lambing rate as single birth (number of ewes lambing single/ number of ewes lambed) and twins' rate (number of ewes lambing twins/ number of ewes lambed). Sex ratio calculated as No. of born lambs in particular sex / total No. of lambs born.

8. Statistical analysis.

The data from incubation experiment were subjected using SPSS (2013) program for social sciences version 22 Inc., Chicago. Significant differences among the means ± SE were determined by using Duncan's multiple-range test at $P < 0.05$ within the same SPSS program. Also, the fertility rate results were analyzed using Chi-Square test.

RESULTS AND DISCUSSION

Evaluation of spermatozoa distribution in densities column during incubationpost-centrifugation.

The effects of SDL1, SDL2, SDL3 and SDL4 layers on sperm quality as sperm motility (%), dead spermatozoa (%) and abnormal spermatozoa (%) during incubation time at 37°C for up to 60 minutes post- centrifugation are presented in Fig. 1, 2 and 3, respectively. The sperm quality was similar ($P<0.05$) among SDL1, SDL2, SDL3 and SDL4 layer. These results are in agreement with those of **Sureka *et al.* (2013)** who found that semen centrifuged in sucrose could be protected sperm without any effect on sperm survival ability. Also, **Jafaroghli *et al.* (2011)** reported that disaccharides displayed protective effect on the function of sperm membrane and intracellular mitochondria that generate energy to improve sperm lifespan. Moreover, **El-Sheshtawy *et al.* (2015)** stated that sugars interact with phospholipids in the plasma membrane and increasing of sperm survival by stabilizing biomembrane bilayers. In the present study, SDL1, SDL2, SDL3 and SDL4 layer were safe to protect sperm membrane and function of sperm mitochondria. Such findings were obtained by **Soylu *et al.* (2007)** who showed that ram spermatozoa could normally live without sperm dysfunction in extenders with an osmotic pressure at 400 $mOsm/kg^{-1.}$ Regarding the forward effect of incubation time, at 37°C for up to 60 minutes on sperm quality, it caused decreased significantly ($P<0.05$) on sperm motility (%) while, increased significantly ($P<0.05$) on dead (%) and abnormal (%) spermatozoa in SDL1,SDL2,SDL3 and SDL4 layer post-centrifugation. Generally,advancing of incubation time had negative effect on sperm parameters which were

close shown in the present study. Such finding strongly supports the findings of **Aitken *et al.* (2014)** who stated that during progressive time of incubation a toxic enzyme namely AAAO (membrane-bound aromatic amino acid oxidase) which released from dead spermatozoa. Similarly, **Gibb *et al.* (2014)** reported positive correlation between incubation and sperm quality and production of free radical. However, other study confirmed that, the depressing of sperm parameters during incubation is usually accompanied by accumulation of lactic acid which lowers pH **(Bohlool *et al.*, 2015**). Furthermore, **Acharya *et al.* (2016)** reported that, quality of ram spermatozoa is greatly reduced with storage time and it is dependent on factors such as type of extender and storage temperature.

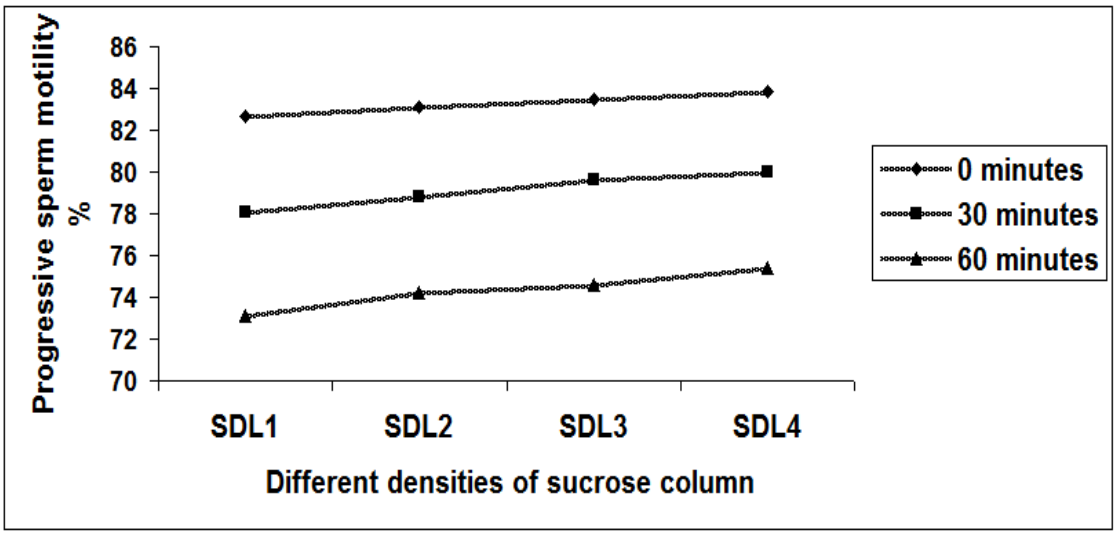

Fig. (1): Progressive ram sperm motility of different sucrose density layers post-centrifugation during incubation at 37ºC for 0, 30, 60 minutes.

SDL1: sucrose density 1.5g/ml, **SDL2:** sucrose density 2.0g/ml, **SDL3:** sucrose density 3.0g/ml and **SDL4:** sucrose density 3.5g/ml.

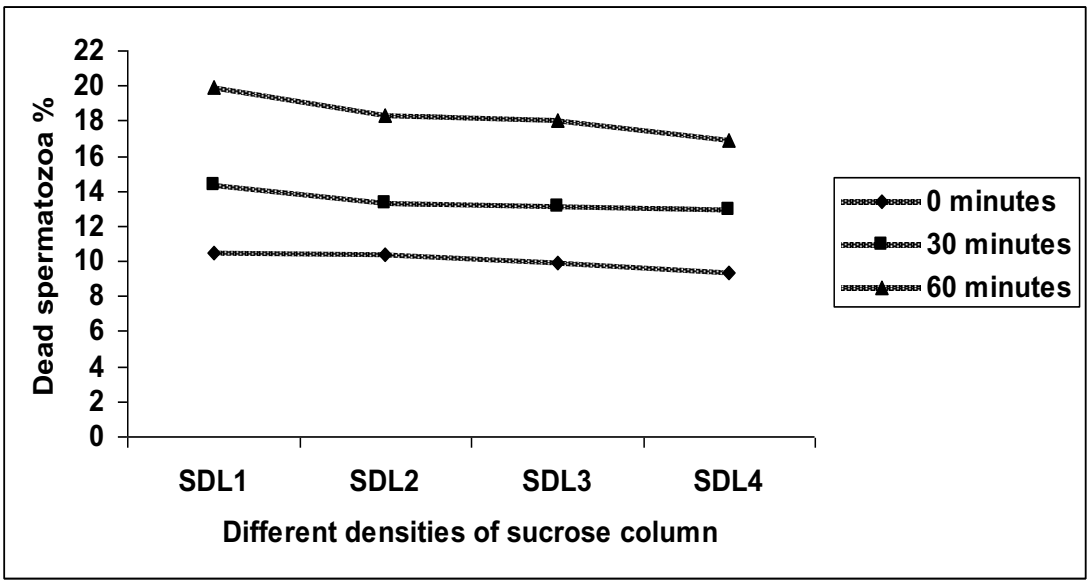

Fig. (2): Dead ram spermatozoa (%) of different sucrose density layers post-centrifugation during incubation at 37ºC for 0, 30, 60 minutes.

SDL1: sucrose density 1.5g/ml, **SDL2:** sucrose density 2.0g/ml, **SDL3:** sucrose density 3.0g/ml and **SDL4:** sucrose density 3.5g/ml.

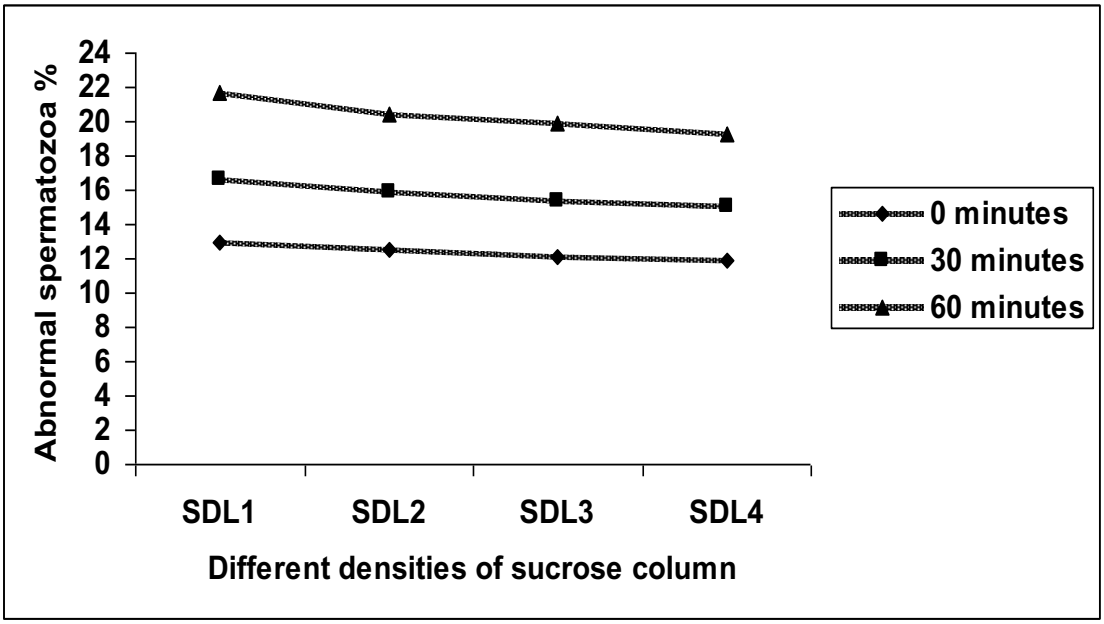

Fig. (3): Abnormal spermatozoa (%) of different sucrose density layers post-centrifugation during incubation at 37ºC for 0, 30, 60 minutes.

SDL1: sucrose density 1.5g/ml, SDL2: sucrose density 2.0g/ml, SDL3: sucrose density 3.0g/ml and **SDL4:** sucrose density 3.5g/ml.

Evaluation of Fertility rate and sex ratio of different sucrose densities.

The calculation of fertility rate of ewes and sex ratio as affected by insemination of SDL1, SDL2, SDL3 and SDL4 layers are presented in (Table1). The total fertility rates after 1st and 2nd services were 80.00, 80.00, 76.00 and 80.00% for ewes inseminated with SDL1, SDL2, SDL3 and SDL4 layers, respectively. Such results are almost similar to that reported by **Donovan *et al.* (2004)** who found that percentage of pregnant ewes after insemination with fresh semen was ranged from 70.00 % to 82.00%. The pregnancy difference during two services may be due to early fetal mortality or unobserved abortions that may take place after pregnancy. Similarly, **Dixon *et al.* (2007)** reported that approximately 19.9% of the ewes experienced late embryonic loss, fetal loss, or both and 21.2% of the embryos or fetuses were lost from day 25 to term. It is well known that proportions of fetuses lost were associated with breed type, concentrations of progesterone, estradiol and vascular endothelial growth factor **(Kumar and Naqvi 2014)**. Regarding to the sex ratio of ram spermatozoa, the present study revealed that using spermatozoa with SDL4 layer could give higher percentage of female lambs (85.00%) than SDL1 (14.29%). Contrariwise, the SDL4 and SDL1 layer could observe of male lambs at 15.00 and 85.71%, respectively. **Ollero *et al.* (2000)** reported that centrifugal counter current distribution in a sensitive-charge aqueous two-phase system achieved the separation of a sperm population enriched in Y- chromosome-bearing ram spermatozoa (75%) with a high viability rate (57%). Similarly**, Kanesharatnam *et al.* (2012)** indicated that centrifugal intermittent density column of sucrose at 15, 20, 30 and 35% could fractionate X- spermatozoa significantly at 15.55, 14.00, 26.33 and 31.85% in cattle (using 2% of orcein red stain to investigate chromosomes), respectively. In addition, **Sureka *et al.*(2013)** confirmd that separation of X- sperm in billy goats significantly at 41, 45, 55 and 65% among gradient sucrose densities column adjusted at 1.0, 1.1, 1.12 and 1.13g/ml, respectively. Surprisingly, in this study the female lambs were increased from high to low density sucrose solution which may attributed to greater number of X- bearing chromosomes in the bottom layer than Y-bearing chromosomes in the top layer. Also, the present results have given an indication that SDL2 layer gave 33.33% female and 66.67% male lambs, but SDL3 layer resulted in 73.68 % female and 26.32% male. According to scientific theories, X- and Y- spermatozoa have 1 % difference in head radius, which would affect swimming and sedimentation velocities **(Shum and Eamonn, 2015).**

Table (1): Calculation of fertility rate and sex ratio of gradient sucrose density layers.

Items	Gradient sucrose density layers			
	SDL1	SDL2	SDL3	SDL4
No. of ewes inseminated at 1st service	20.00	20.00	20.00	20.00
No. of ewes conceived at 1st service	15.00	16.00	15.00	16.00
No. of ewes inseminated at 2nd services	5.00	4.00	5.00	4.00
No. of ewes conceived at 2nd services	5.00	4.00	4.00	4.00
Total ewes inseminated throug1st and 2nd services	25.00	25.00	25.00	25.00
Total ewes conceived at 1st and 2nd services	20.00	20.00	19.00	20.00
Fertility rate at 1st and 2nd services (%)	80.00	80.00	76.00	80.00
No. of ewes lambed at 1st and 2nd services	20.00	20.00	19.00	20.00
No. of lambs born at 1st and 2nd services	21.00	21.00	19.00	20.00
Lambing rate:				
No. of ewes lambing single lambs	19.00	19.00	19.00	20.00
Single rate (%)	95.00	95.00	100.00	100.00
No. of ewes lambed twins	1.00	1.00	0.00	0.00
Twins rate (%)	5.00	5.00	0.00	0.00
No. of male lambs	18.00	14.00	5.00	3.00
Male lambs' rate (%)	85.71	66.67	26.32	15.00
No. of female lambs	3.00	7.00	14.00	17.00
Female lambs (%)	14.29	33.33	73.68	85.00

SDL1: sucrose density 1.5g/ml, SDL2: sucrose density 2.0g/ml, SDL3: sucrose density 3.0g/ml and SDL4: sucrose density 3.5g/ml.

CONCLUSION

The obtained results showed that, the centrifugal method that attempted was efficient for sexing of ram spermatozoa by using intermittent densities column of sucrose sugar. The lowest density layer at 1.5g/ml could separate Y sperm with male rate at 85.71%, but the highest density layer at 3.5 g/ml produced female lambs' rate at 85.00 %. Therefore, this technique could be primary considered as low-cost tool to modify sex ratio of lambs as either male or female in Rahmani sheep.

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تعديل النسبة الجنسية للحيوانات المنوية للكباش باستخدام الطرد المركزي لعمود محلول السكروز متقطع الكثافة

اد/ عز الدين ابراهيم خليفة – د / طارق مسلم محمود مهدي

استاذ الفسيولوجي-قسم بحوث الاغنام والماعز- باحث فسيولوجي-قسم بحوث الاغنام والماعز

مركز البحوث الزراعية - معهد بحوث الانتاج الحيواني- قسم بحوث الاغنام والماعز

الملخص العربي

تعديل النسبة الجنسية للحيوانات المنوية للكباش باستخدام الطرد المركزي لعمود محلول السكروز متقطع الكثافة فيما بين العديد من التقنيات لفصل الحيوانات المنوية استخدام الطرد المركزي لعمود محلول السكروز متقطع الكثافة. وتم وزن1.5، 2.0، 3.0، 3.5جم من السكروز الجاف لتكوين الكثافات التالية 1.5، 0و2 3.0، 3.5جرام/ ملى بعد اذابة السكروز في 100 ملى مخفف سترات الصوديوم لتعطى المعاملات **SDL1, SDL2, SDL3 , SDL4 (2000)** ميكرون/ طبقة على التوالي. وتم عمل عمود من السكروز متقطع الكثافة في انبوبة اختبار وكان ترتيب الكثافة من القاع الى القمة كالتالي **SDL4 , SDL2 , SDL3, SDL1** وتم وضع 1000ميكرون من السائل المنوي الخام للكباش على سطح طبقة **SDL1** وتم الطرد المركزي300 لفة /دقيقة لمدة20دقيقة . وبعد الطرد المركزي تم فصل الطبقات الأربعة كل في انبوبة اختبار من اسفل الى أعلى. وتم فحص جودة السائل المنوي %] الحركة ، % الميت ، % الغير طبيعي [خلال التحضين على درجة 37م5 لمدة صفر،30، 60دقيقة . وتم حساب معدل الخصوبة ، والنسبة الجنسية باستخدام 80 نعجة قسمت الى اربع مجاميع 20نعجة /كثافة من عمود السكروز. والنتائج اوضحت عدم وجود فروق معنوية بين **SDL4, SDL3, SDL2, SDL1** في جودة السائل المنوي اثناء التحضين. ولكن هناك فروق معنوية فيما بين أوقات التحضين من صفر الى 60 دقيقة. وكانت معدلات الخصوبة بعد التلقيح بجرعتين 80%، 80%، 76%، 80%لكلا من **SDL2, SDL1**و **SDL4, SDL3** على التوالي. وعدل عمود السكروز النسبة الجنسية فيما بين الإناث 14.29، 33.33، 73.68، 85.00و%للذكور15.00،26.32،66.67،85.71%عند التلقيح باستخدام **SDL1**و **SDL2**و **SDL4, SDL3** على التوالي. وبناء على النتائج المتحصل عليها نخلص ان الطرد المركزي لعمود محلول السكروز متقطع الكثافة يمكنه تعديل النسبة الجنسية و فصل الحيوانات المنوية مع الحفاظ على حيويتها.