FREEZABILITY AND FERTILITY OF FROZEN-THAWED KABASHI ECO-TYPE DESERT RAMS SEMEN PROCESSED WITH TRIS-SOYBEAN AND TRIS-EGG YOLK BASED EXTENDERS

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المستخلص

صمم هذا البحث لدراسة اثر الموسم ونوع المخفف على قابلية الحيوانات المنوية للخراف الكباشية للتجميد وقياس معدل خصوبتها بعد التجميد والإحماء. استخدم في تخفيف السئل المنوي مخفف تجاري (جاهز للإستعمال) ويحتوي على دهون من فول الصويا (بدلاً عن صفار البيض) ومخفف آخر تقليدي (محلي التجهيز) يحتوي على صفار البيض. كلا المخففين يحتويان على التريس كمنظم للأس الهيدروجيني. اختيرت 60 نعجة كباشية وتم تكثيف الشياع فيها باسفنجات مهبلية مشبعة بهرمون البروجيستيرون ولمدة 12 يوم. عند ازالة الإسفنجات حقنت النعاج بهرمون مصل الفرسة الحامل بمعدل 500 وحدة عالمية ، في العضل ، لكل نعجة. لقحت النعاج اصطناعياً في عنق الرحم بعد 56 ساعة من ازالة الإسفنجات. أثبتت النتائج عدم وجود أي أثر معنوي للموسم على حركة الحيوانات المنوية بعد التجميد والإحماء بالرغم من تسجيل معدلات أكبر للحركة خلال موسمي الخريف والشتاء.

وقد كان هناك تفاعل معنوي بين أثر الموسم ونوع المخفف على حركة الحيوانات المنوية بعد التجميد والإحماء.أفضل أداء للمخفف التجاري كان في موسم الخريف بينما كان أفضل النتائج للمخفف التقليدي في فصل الشتاء. أدنى النتائج كانت للمخفف التقليدي وسجلت في موسم الصيف تم قياس معدلات الحمل بمعدلات عدم الرجوع للشبق. لم يلحظ أي فرق معنوي بين معدلات الحمل للسائل المنوي المجمد في المخففين (67.9% للتريس/فول الصويا و74.1% للتريس/صفار البيض)، كما لم يكن هناك أي فرق معنوي في معدلات الولادة (25% و22.2% على التوالي) أو معدل التوائم (1.14 و 1.0 على التوالي).

دلت نتائج هذا البحث إلى عدم وجود تأثير معنوي للموسم على حركة الحيوانات المنوية بعد التجميد والإحماء وأن هناك تفاعل معنوي بين الموسم ونوع المخفف المستخدم . كما اثبتت النتائج إمكانية استبدال صفار البيض في مخففات السائل المنوي المجمد ببدائل نباتية مثل فول الصويا دون ان يكون هناك تأثير كبير على معدلات الخصوبة.

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Abstract

The present study was desinged to examine the effect of season and type of extender on the freezability and fertility of Kabbashi ram semen. Freezability was assessed by comparing the motility and viability of the frozen-thawed semen processed with a commercial soybean-based semen extender (AndroMed) with home-made Tris-based glucose/egg yolk extender. Fertility of the frozen-thawed semen processed with the two extenders was measured by the non-return rate to oestrus (NRR) after cervical insemination of ewes (n=60) treated with an intravaginal sponge containing 40 mg fluoroprogesterone acetate (FGA) for 12 days and an im injection of 500 IU eCG at the time of FGA sponge removal. Insemination was carried out 56 hours after removal of the FGA sponge. Season had no significant effect on post-thawing motility. Significant interaction between the effects of season and the type of extender on the post-thaw motility was observed ($P < 0.001$). The highest post-thawing motility for AndroMed was recorded in autumn while that for the home-made extender was in winter. The lowest value was observed in summer for the home-made extender . The NRR were not significantly different among semen samples processed with AndroMed (67.9%), and the Tris (74.1%) extenders. The overall conception and lambing rates were 71 and 25%, respectively. The prolificacy rates from ewes inseminated with frozen-thawed semen processed with AndroMed and the Tris (1.14, and 1.0, respectively) were also not significantly different. The present results indicated no significant seasonal effect on post-thawing motility. Significant interaction between season and type of extender was observed with consistent performance for AndroMed in all seasons. Thus, an egg yolk-containing semen extender can be replaced with the lecithin-based extender (AndroMed), which could be used for cervical insemination using frozen-thawed ram semen without reducing its fertility.

Key words: Desert ram, semen, freezability, cervical insemination, lecithin, soybean,

Introduction

Artificial insemination (AI) is probably the most important technique ever devised to facilitate the genetic improvement of animals. The widespread use of AI and the realization of its full potential depend essentially on the use of frozen semen, and on the adoption of techniques that result in acceptable fertility levels. However, very low level of fertility (10 - 40%) were obtained when frozen-thawed semen is used for cervical insemination in sheep (Salamon and Maxwell, 1995; Maxwell *et al*., 1999). Alternatively, laparoscopic AI, is an effective method of insemination with frozen-thawed semen, but the stress inflicted to the animal and the high cost limits its use (Fukui, 2008).

Egg yolk-based semen extenders have been widely utilized for cryopreservation of semen from farm animals including sheep (Watson and Martin, 1975; Watson, 1981). However, it is not always easy to prepare semen extenders consistent with quality standards because of the individual quality differences inherent in egg yolk. Also, addition of egg yolk reduces the acrosome integrity of goat spermatozoa **(** Aboagla and Terada, 2004), and high egg yolk concentrations reduce the post-thawing viability of ejaculated spermatozoa in several species, such as goats (Ritar and Salamon, 1991) and rams (Watson and Martin, 1975). Recent trends advocate the elimination of all animal ingredients in extenders, including egg yolk, milk or bovine serum albumin (BSA), in order to design standardized semen extender. Removal of chicken egg yolk from semen extenders would provide several advantages, such as improved consistency in the components of semen extenders and elimination of hygienic risks.

Even though, Sudan Desert sheep are not seasonal breeders, the high ambient temperature may reduce the reproductive efficiency of the animals during the hot dry summer season. Hot summer adversely affects quality of semen with a decrease in the motility of sperms and appearance of high percentage of abnormal and dead sperms (Makawi et al., 2007). The present study aimed to examine the effect of season (autumn, winter and summer) and type of extender (a soybeen lecithin-based, and Tris-based / egg yolk glucodse extenders) on the post thawing motility of semen from Kabbashi eco-type Desert rams. The fertility of ewes cervically inseminated with frozen-thawed semen using AndroMed or the egg yolk- based extender was investigated. Lambing rate (number of ewes lambed/number of ewes inseminated) and prolificacy (number of lambs born/number of ewes lambed) were recorded.

Materials and Methods

Site of study: This experiment was conducted from September 2009 to June 2011 at the Animal Production Research Center (APRC), Department of Small Ruminant Research (Kuku, Khartoum North, Sudan) and semen evaluation and processing were done at the neighboring laboratory of the National Artificial Insemination Center.

Latitude and climate: In Sudan the year is divided into three seasons, winter (November-February), summer (March- June) and autumn (July –October). In khartoum (15° 36 N, 32° E) the summer is dry and very hot (maximum temperature between 38 and 44° C).

Experimental Animals: Ten Kabbashi eco-type Desert rams were purchased at the age of 7 months with an average body weight of 22.95± 2.23 kg. Dentition, by counting the number of permanent incisors that have erupted on the lower jaw of the mouth, was used to determine the age of rams. They were maintained under uniform condition of feeding and management. Semen collection was conducted from the rams at 10 months of age. 60 mature and empty multiparous ewes (with average age of 5.5 y and average body weight of 48 kg) were selected from a flock affiliated to the APRC. These ewes were divided equally into two groups for insemination with frozen-thawed semen processed with the two extenders.

Feeding: The experimental animals were kept under zero-grazing system, where forages were cut and carried to the pens. The forage was comprised of alfalfa (*Medicago sativa*) and Abu 70 (*Sorghum bicolor*). Sorghum grass was offered daily while Alfalfa was offered once weekly. Moreover, a concentrate diet (10.9 MJ, ME/kg^{-1}; 19.2% CP) was offered at a rate of 1 kg/animal/day. Blocks of salt lick and drinking water were available ad lipitum.

Semen collection: Semen was collected at weekly intervals from each ram using standard artificial vagina (AV) for small ruminants. The collecting tubes of AVs containing the semen samples were removed, labeled and placed in water bath at 30^{O}C. The semen was then evaluated for colour, volume, consistency, mass activity and individual motility, according to the methods described by Evans and Maxwell (1987). Sperm cell concentration was counted by using an improved Neubauer haemocytometer counting chamber (Evans and Maxwell, 1987). Semen collection was continued for one year covering the three seasons (autumn, winter and summer). A total of 233 ejaculates of semen were collected during the whole period of the study.

Extender preparation: Two different extenders were used, a commercial ready-made Andromed^{R} (lecithin-based, Minitüb, Tiefenbach, Germany); and home-

made Tris-based /egg yolk-glucose extenders. These extenders were prepared a day before collection according to the instructions of the manufacturers. AndroMed was emptied into a graduated flask and pure water was added in several steps (stock solution). The Tris-based /egg yolk-glucose extender was prepared in the laboratory by mixing: 3.634 gm Tri-hydroxymethylaminoethane (Tris), 1.99 gm citic acid, 05 gm glucose, 5 ml (v/v) glycerol and 15% (v/v) egg yolk, 1000 IU penicillin, 100 mg streptomycin and distilled water added to the level of 100 ml (9, Evans and Maxwell, 1987). Fresh egg yolk was separated completely from the egg white and yolk membrane and measured out. This extender was made into two parts, Part A (non-glycerolated portion) and Part B (glycerolated portion). The two parts of the extender were stored at 4^0C in a refrigerator.

Semen dilution: Only ejaculates with good wave motion, (scoring $\geq$ 3, on a scale of 0 to 5) and containing more than 2.5 X 10^9 sperm cell/ml, were used for further processing and freezing (Evans and Maxwell, 1987) . With Andromed the semen was diluted within the first 10-15 minutes following collection and evaluation in a one-step procedure at 37^oC with a rate of 1 semen: 4 diluent to provide approximately final sperm concentration of 200 X 10^6/ml. With the Tris extender, semen extension was performed at 37°C with part A, then the tube of extended semen was transferred in a water jacket to a cold cabinet and cooled to 5°C over two hours. The glycerolated portion (part B) was then added, gradually in 5 aliquots, to the cooled semen extended in the non-glycerolated part and left in the cold room for 2 hours to equilibrate. After equilibration and adaptation the semen was aspirated into 0.5 ml French straws and sealed with polyvinyl alcohol powder.

Semen freezing: The filled straws were placed horizontally on a cold rack (at 5°C) and placed 3 - 4 cm above the surface of liquid nitrogen in Styrofoam container for 8 minutes. Finally the semen straws were plunged into liquid nitrogen , packaged in plastic goblets and stored in a liquid nitrogen container.

Post- thawing motility: After 48 hours two random straws were drawn from each semen batch and thawed in a water bath at 37°C for 20 seconds (Evans and Maxwell, 1987). Assessment of the frozen-thawed semen for motility and viability was done on a pre-warmed slide (37°C) with a cover-slip, using light phase contrast microscope (400X). The straws for an individual ram in a particular freeze were retained for use only if the sample straw yielded a viability score $\geq$ 40% and only if the progressive motility was judged to be satisfactory (Berg, 1999).

Oestrous synchronization: A total of sixty multi-Parous ewes were treated (20 ewes each season)with progesterone impregnated intravaginal sponges containing 40 mg fluoroprogesterone acetate (FGA; Intervet Internatinal,

Boxmeer, Holland). Synchronization involved placement of the sponges for 12 days followed by intramuscular injection of 500 IU equine chorionic gonadotropin (eCG, Lyophilized serum gonadotropin) at the time of FGA sponge removal.

Insemination procedure: Ewes were cervically inseminated in a standing position after 56 hours from sponge removal. The cervix was located, via a vaginoscope and the semen deposited as far as possible into the cervix without using force by means of an insemination pipette.

Non-Return Rate (NRR): Ewes not returning to estrous 25 days after AI, were considered pregnant. Lambing rate (number of ewes lambed/number of ewes inseminated) and prolificacy (number of lambs born/number of ewes lambed) were recorded.

Statistical analysis: The effect of season and extender on the post-thawing motility of spermatozoa was compared by two ways ANOVA. NRR and lambing rates were compared by the Chi square test. Data analysis was performed using StatSoft 2010 system.

Results

As shown in Table 1, the season of the year in Sudan was found to have a significant effect on the post-thawing motility of Kabbashi rams. However, the best post-thawing motility was obtained in autumn and winter and the worst was in summer. Significant interaction between the effects of season and the extender on the post-thaw motility was observed ($P < 0.001$). When Andromed was used as the diluent, ejaculates collected in autumn gave the highest post-thaw motility (56.43±6.63) compared to semen samples collected in the other two seasons ($P < 0.05$). Ejaculates diluted in Triladyl showed no singnificant difference in post-thaw motilities between samples collected in autumn (54.38±6.29) or winter (56.05±7.56)). However, both extenders recorded significantly higher ($P < 0.05$) post-thaw motility compared to those obtained in summer (46.39±9.52). Using Tris-based/egg yolk-glucose extender (Tris), the result indicated that there were no significant differences between the different seasons in post-thawing motility. Post-thawing motility of semen samples collected in summer and autumn and diluted with AndroMed (47.37±15.58 and56.43±6.63) were significantly higher ($P < 0.05$) than those processed with the Tris extender (40.75±11.72 and 48.532±11.15). In winter, the post-thawing motility of semen samples diluted with Tris (56.67%), were significantly higher than that diluted with AndroMed (46.43%).

Table 1: Effect of season and type of extender on freezability of Kabbashi ram semen.

Type of extender	No. of samples per season	Post-thawing motility		
		Autumn	Winter	Summer
AndroMed	18	56.43 ± 6.63^{Aa}	46.43 ± 12.62^{Bb}	47.37 ± 15.58^{Bb}
Tris/egg yolk	18	48.53 ± 11.15^{Ba}	56.67 ± 6.64^{Aa}	40.75 ± 11.72^{Bc}

Values (mean ± sd) with different lower case letters in the same row and with upper case letters in the same column differ significantly ($p < o.o5$).

As shown in Table (2), the season of the year had no significant effect on the NRR and lambing rates. In this experiment, 5 ewes out of 60 lost their FGA impregnated sponges and were excluded from the experiment. The overall NRR and lambing rates were 67.9% and 25% for AndroMed and 74.1% and 22.2% for the Tris- egg yolk based extenders with no significant differences. The prolificacy rates for ewes inseminated with frozen-thawed semen processed with AndroMed, and Tris/egg yolk (1.14, and 1.0, respectively) were also not significantly different.

Table 2 : Fertility after cervical insemination with frozen- thawed Kabbashi ram semen.

Type of extender	Fertility parameters			No. of ewes inseminated
	NRR	Lambing rate	Prolificacy	
AndroMed	19 (67.9%)*1	7 (25%)*2	8 (1.14)	28
Tris/egg yolk	20 (74.1%)	6 (22.2%)	6 (1.00)	27
Total	39 (71.0%)	13 (23.6%)	14 (1.07)	55

*1 $\chi^2 = 0.281$, P value = 0.8688
*2 $\chi^2 = 0.215$, P value =8.983
NRR : Non return rate

Discussion

It is well known that frozen-thawed spermatozoa are greatly damaged during the freezing and thawing process. This damage may reduce the sperm motility and fertilization rate after artificial insemination (Gillian *et al*., 1997). Quality tests are routinely used to determine acceptability of processed semen for breeding

purposes. As shown in Table (1), summer had an adverse effect on semen processed in Tris-egg yolk- based diluent, and this is in agreement with Makawi *et al*. (2007). Significant interaction between the effects of season and the extender on the post-thawing motility was observed ($P < 0.001$). Post- thawing motility (freezability) in AndroMed was significantly higher during autumn, while those for the Tris-egg yolk-based diluent were higher in winter. Although the least post-thawing motility was observed for the Tris-egg yolk- based diluents in summer, but the overall results for both diluents revealed no significant difference. This result demonstrated that an egg yolk-containing semen extender could be replaced with the soybean lecithin-based extender without reducing the post- thawing motility. Similar result was reported by Fukui *et al(* .2008)who stated that an egg yolk-containing semen extender can be replaced with the synthetic extender, AndroMed. Complete elimination of egg yolk , milk or BSA from semen extenders and the use of a non-animal derived compound, such as a soybean lecithin, instead, is a better choice for reducing the hygienic risks of semen contamination with pathogenic microorganisms (Bousseau *et al*., 1976, Bielanski, 2007).

In sheep the conception rates after cervical artificial insemination using fresh or chilled semen are good (65- 75%). However, NRR is usually low (10 - 30%, according to Salamon and Maxwell (1995) or 20 to 40%, according to Maxwell *et al*.(1999) when frozen-thawed semen was used and this conforms well with the obtained results. Lightfoot and Salamon (1970) attributed the low results to impaired sperm transport through the cervix and a short duration of survival in the female reproductive tract. Central to the problem is the anatomy of the ovine cervix, which forms a natural barrier to the uterus. In the non-pregnant ewe the funnel-shaped rings of the cervix, which average around five in number (Dobson, 1988), are not concentrically aligned, and their openings are constricted in most instances to less than 3 mm (Halbert *et al*., 1990). Generally, it is only possible to deposit the semen in the first fold of the cervix. Alternatively, laparoscopic uterine insemination, is an effective method of insemination with frozen-thawed semen, but it has problems related to surgical stress, high cost equipment, the need for trained technicians and other problems related to animal welfare (Anel *et al*., 2006).

In conclusion, the present study have indicated no significant seasonal effect on post-thawing motility, however, a significant interaction between season and the type of extender was encountered . AndroMed had a better performance in autumn while the Tris-egg based extender recorded better values in winter . AndroMed had more consistent results throughout the three seasons including summer. Thus, an egg yolk-containing semen extender could be replaced by the synthetic soybean lecithin-based extender (AndroMed) which could be used for cervical insemination using frozen-thawed ram semen with promising fertility

results. Further research to overcome the cervical barrier is needed to facilitate intrauterine insemination and improve fertility rates.

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