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The impact of adding different levels of egg yolk on the motility and morphology pre and post thaw cryopreservation of goat semen

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Abstract

The aim of this study was to examine the effect of different concentrations of egg yolk EY (0%, 10%, and 20%) in the semen extender during the cryopreservation process of goat semen out of the breeding season. A total of 12 ejaculates were collected from six Anglo Nubain dairy bucks as two ejaculates for each buck aged between (1-5) years over a two week period by using Electro-ejaculation (EEJ) during the non-breeding season. Post collection, the semen samples were evaluated for motility and mass activity. Subsequently, the semen samples were initially diluted in Tris solution (without Egg yolk or Glycerol) in order to preserve the motility of sperm cells. The semen samples from each buck were evaluated for pre-freezing motility and morphology then divided into three sub-samples and diluted in Tris extender with T1 (control) 0% EY, T2 10% EY, and T3 20% EY. The semen samples were frozen in liquid nitrogen (-196 C). After thawing, the semen samples were evaluated for sperm motility and morphology. The morphology of sperm did not differ among treatments nor between pre-freezing and post-thawing evaluations. However, the motility of semen diluted with 10% EY was ($P<0.05$) numerically but not statistically higher than semen diluted with 0% and 20% EY. According to the obtained results of this study, it is recommended that a 10% EY level or less be included in the Tris extender during cryopreservation of goat semen for superior motility and morphology results.

تأثير اضافة مستويات مختلفة من صفار البيض في الحركة الفردية ونسبة النطف الطبيعية قبل وبعد التجميد للسائل المنوي في الماعز

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الخلاصة

هدفت هذه الدراسة لاختبار تأثير استخدام مستويات مختلفة من صفار البيض (0٪ , 10٪ و 20%) كجزء من محلول التخفيف بعد عملية الجمع وقبل التجميد خارج موسم التكاثر. تم الحصول على (12) قذفة باستعمال 6 من ذكور الماعز بواقع 2قذفة/ ذكر ماعز سلالة الأنجلو نوبيين (Anglo Nubain dairy) تتراوح اعمارها بين (1- 5) سنة باستعمال جهاز محفز القذف الكهربائي (Electro-Ejaculation) لمدة أسبوعين خارج موسم التكاثر. بعد اجراء عملية جمع العينات ، تم تقييم عينات السائل المنوي واجراء بعض الفحوصات في الحقل من حيث الكثافة والحركة الفردية للنطف. بعد ذلك تمت عملية تخفيف عينات السائل المنوي بشكل اولي في محلول (Tris) بدون صفار البيض أو الجلسرين من أجل الحفاظ على الحركة الفردية للنطف، تم تقييم عينات السائل المنوي مختبريا قبل التجميد ثم قسمت إلى ثلاث مجاميع وتم تخفيفها في محلول (Tris)، المجموعة الاولى (السيطرة) صفار البيض 0٪ ، المجموعة الثانية صفار البيض10 ٪ والمجموعة الثالثة صفار البيض 20 ٪. تم تجميد عينات السائل المنوي في النيتروجين السائل (-196) مئوية. تم تقييم عينات السائل المنوي بعد الاذابة. لم تظهر النتائج أي اختلافات معنوية في نسبة النطف الطبيعية (Morphology) بين المعاملات و قبل التجميد و بعد الاذابة. بينما أظهرت النتائج تحسن في الحركة الفردية للنطف (Motility) في السائل المنوي المخفف بـ صفار البيض 10٪ بدون تسجيل فرق معنوي ($P<0.05$) عند مقارنتها بباقي المعاملات . استنادا الى النتائج التي تم الحصول عليها من هذه الدراسة ، فمن المستحسن أن تستعمل مادة صفار البيض بنسبة 10 ٪ أو أقل في محلول (Tris) أثناء الحفظ بالتجميد للحصول على نتائج أفضل من ناحية الحركة الفردية للنطف ونسبة النطف الطبيعية.

الكلمات المفتاحية : التجميد ، صفار البيض ، نطف الماعز

Key Words : **cryopreservation, egg yolk, goat sperm.**

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Introduction

Throughout the 20th century, two main methods have been used for sperm preservation in terms of storage technology which are slow freezing and verification. It has been reported that the life span of spermatozoa can be affected by a combinations of several key factors such as storage temperature, concentration of chemicals in the extender and the hygienic control(1). It is well known that cryopreservation of sperm cells from all farm animal species is a demanding and difficult task. Despite this fact that there is an association between the cryopreservation techniques and physical stresses which must be tolerated by the spermatozoa from the various animal species. Consequently, these factors can have an influence on the survival of sperm(2). Cryopreservation of sperm is a crucial process in breeding and selection programs for domestic animals due to its contribution in increasing the production factor for these animals. Another important benefit of cryopreservation of sperm is that it can be used in the preservation of genetic material from endangered species (3). It has been reported that the fertility of goat spermatozoa is compromised with cryopreservation process based on pregnancy rates from AI programs (4). Nevertheless, the greatest barrier of using frozen-thawed semen widely is generally due to the decrease in motility and viability of sperm cells post freezing and thawing procedures of goat semen. This is resulted from damage in the membrane integrity and ultrastructure of sperm cell (5).Cryopreservation of semen can contribute to new ways in the development of different reproductive techniques, for example in artificial insemination (AI) which can be used to enhance animal production in a limited timeframe (6). Moreover, cryopreservation can be used for genetic improvement of domestic species by the preservation of the germplasm from animals of rare breeds that are already well adapted to environmental changes (7) .The cryopreservation of domestic animals sperm is a complicated process requiring the balance of many factors to achieve excellent outcomes. Beside the important physiological information of the sperm being required it is also necessary to consider the use of proper diluents, the dilution rate of the sperm, and the process of freezing and thawing the sperm cells. All these factors are important to increase the number of recovered sperm cells after thawing that leads to an improvement in the fertility within the herd. For example, there are several techniques and methods which can be used for cryopreservation of goat (and other farm animals) semen such as using the same cryoprotectants and freezing and thawing techniques; however, unique attention is required in the cryopreservation of goat sperm to improve the post-thaw viability of the spermatozoa. As an illustration, the bulbourethral gland secretions interacts with egg yolk in the goat semen deleteriously, however, this has not been noticed in other farm animals, such as the ram, boar, or the bull (2). The successful use of AI is influenced by the rigorous supervision in terms of collecting and handling of semen, and is achieved by manipulating the cryopreservation media in a way that cannot be deleterious to the goat semen. So these new trends in sperm cryopreservation have given a better solution for the interaction problem between the seminal plasma and the dilution media (8).Over the last five decades, the use of frozen-thawed semen has played a significant role in AI programs in the production of domestic animals. Recently, cryopreserved semen has been used in the establishment of genetic resources especially for threatened animal species (9). Basically, sperm cells are provided with an energy

source, protection against thermal shock, and maintenance of the environmental factors in order to increase the survival rate of sperm cells (2).Hence, the hypothesis of this study is: Does the addition of different egg yolk levels to the semen extender improve the motility and morphology of goat semen pre-freezing and post-thawing?

MATERIALS AND METHODS

1. Time and Location of the experiment
During the month of October, the experiment was conducted at a commercial private farm which is located at Gatton town, Queensland, Australia, approximately 5 Km away from the university of Queensland/ Gatton campus.

2. Experimental animals
Total of six fertile bucks of Anglo Nubain dairy goat breed were selected according to their health records, as well as reproductive history in the recent few years. The age of the six bucks was ranged between one to five years. The bucks were housed in a commercial farm and fed the same amount of feed which is consisted of mixture of barley, pasture and concentrated feed (grain) with water ad libitum on a daily basis.

3. Collection and dilution of Semen

Semen collection was carried out once a week of six bucks by the aid of electro-ejaculation (EEJ). Total of 12 ejaculates were obtained over a period of two weeks from six bucks out of the breeding season. Semen samples were initially diluted post collection at field up to 10 ml with Tris solution extender consisted of 2.42 g Tris (hydroxymethyl) aminomethane, 1.00 g of fructose, 1.48 g citric acid, 25 mg Gentamycin, 30 mg Spectinomycin, 15 mg Lincomycin, 5 mg Tylosin in 100 ml of distilled water (10). Semen samples were evaluated for motility and mass activity. After that, semen samples were transported to private lab for 1.5 h (approx. 90km far from Gatton) by being placed in a container with cotton and ice bags in order to cool them down to 5 °C. Upon arrival to the lab, each semen sample was evaluated again for motility and morphology, then divided into three sub-samples. Only three semen samples from three bucks were freezable due to the poor semen quality that was obtained from the other three bucks in both collections. Semen sub-samples were diluted in a Tris- citric acid- fructose freezing extender (composed of 2.42 g Tris (hydroxymethyl) aminomethane, 1.00 g of fructose, 1.48 g citric acid, 25 mg Gentamycin, 30 mg Spectinomycin, 15 mg Lincomycin, 5 mg Tylosin, and 6.4 ml of glycerol in 100 ml of distilled water) with 0% egg yolk (EY), 10% egg yolk, and 20% egg yolk. Final dilution rate was carried out with (1:1) semen to extender.

4. Freezing and thawing procedures
Plastic straws were labelled for each treatment by using Minitub straws (easy coder thermal printing on straws). After that, diluted semen samples were loaded in 0.25 ml plastic straws and sealed with KLN machine (IMV, France). Straws were equilibrated at 5 °C for 10 minutes, and then straws were suspended on a rack 40 mm above the liquid nitrogen (vapour) for 15 minutes. By using this procedure, straws cooled down from 5 °C to -140 °C. After that, straws were plunged into liquid nitrogen (-196 °C) and stored until thawing. Straws were placed in a water bath (35-37°C) for 20 seconds for thawing. Sperm motility refers to the ability of sperm to move and swim. Sperm morphology refers to the size and shape of individual sperm with normal form. Motility was evaluated by depositing a drop of semen in a glass slide warmed at 37 °C under an optical microscope with 4x magnification and calculate the percentage of motile sperm cells at score ranged (0-100%). Sperm morphology were evaluated by depositing a drop of

diluted semen warmed at 37 °C between slide and coverslip, also warmed at 37 °C, and calculate the percentage of sperm at score ranged (0-100%) with normal form under an optical microscope with 40x magnification (11).

Sperm motility was evaluated post collection, pre-freezing, and post-thawing in all treatments. Whereas, sperm morphology was evaluated pre-freezing, and post-thawing in all treatments.

5. Statistical analysis

Data analysis was performed by using Minitab 16 program which is based on the use of the one way ANOVA test at the 5% significance level in order to compare the means between three treatments(12).

Results

1. Motility

Mean values (±SEM) of semen motility post collection, pre-freezing and post-thaw in different treatments are illustrated in Table 1. The mean values ±SEM of sperm motility recorded at post collection was significantly higher than semen motility pre-freezing with 89.17±2.39 a, 57.5±2.5 b respectively. However, the mean values of sperm motility post-thaw 0% EY, 10% EY; 20% EY were 20.33±8.01 c, 35.83±11.4 bc, 25.83±6.76 c respectively. This indicates that motility of semen samples diluted in 10% egg yolk level was numerically (with $p<0.05$) but not significantly higher than the other concentrations of EY (0% and 20%).

2. Morphology

Mean values (±SEM) of semen morphology pre-freezing and post-thaw in different treatments are presented in Table 2. The mean values ±SEM of sperm morphology pre-freezing recorded at 85.5±3.19 a. Similarly, the post-thaw morphology results of sperm diluted with different concentrations of egg yolk 0%, 10%, and 20% were similar among treatments. Therefore, there was no significant difference between the same manuscript.

Discussion

The main purpose of this study was to examine the effect of different levels of egg yolk in the semen extender on the motility and morphology of goat semen before freezing and after thawing. Semen samples were processed without removing the seminal plasma in all treatments. The motility results in the current study post collection was significantly higher than semen motility pre-freezing with 89.17±2.39, 57.5±2.5 respectively. Besides, the motility results of processed semen samples with the addition of 0% EY, 10% EY; 20% EY were 20.33±8.01, 35.83±11.4, 25.83±6.76 respectively. The morphology results in the current study pre-freezing was 85.5±3.19. Moreover, morphology results of processed semen samples with different concentrations of egg yolk 0%, 10%, and 20% were 86.83 ± 2.43, 88.33 ± 1.67, 88.33 ± 1.56 respectively. The level 0% EY was used to compare the effects of freezing semen with and without the addition of egg yolk cryoprotective agent with the other two treatments 10% EY and 20% EY. There were no significant ($P>0.05$) differences in semen morphology between pre-freezing nor among all treatments. While, there were variations in the semen motility pre-freezing and post thawing due to the interactions with the collection time of the semen and levels of egg yolk at freezing. The remarkable seasonal effect of the egg yolk on sperm motility without removing seminal plasma may be caused by increasing the concentration of EYCE in seminal plasma with the progress of the breeding season into the non-breeding season (13). The authors also stated that the highest

motility rates of spermatozoa were obtained during March to August with declined does rates of egg yolk in the semen extender from 12% EY in March to 1.5% EY in August. However, it was reported that the activity of EYCE reaches the highest level during the breeding season (14). Additionally, Evans & Maxwell (15) revealed that freezing extenders for goat semen should have a low concentration of egg yolk in order to avoid the interaction with the egg yolk coagulating enzyme (EYCE) that present in the seminal plasma of goat semen. The authors also stated that the concentration of EYCE is higher when semen collection is performed by electroejaculation than other techniques, and there are several techniques which can be performed to overcome this problem such as: reducing the egg yolk level in the semen extenders, replacing the egg yolk with anther cryoprotective agent (milk), or washing and centrifuging the semen samples in order to remove its seminal plasma. Similarly, the addition of two concentrations of the egg yolk (20 and 2.5) were examined by (10), the most important finding of the study indicated that using low concentration of egg yolk (2.5%) in the glucose-EDTA extender gave higher motility results than including 20%. The author also stated that lowering the egg yolk level in the freezing extender may have the potential to reduce the toxic effects against sperm cell during cryopreservation process. Furthermore, using low egg yolk level in the semen extender is very essential during non-breeding season in comparison to high concentration in the cryopreservation of goat semen. Another study was carried out by Santiago-Moreno et. al. (16) who compared the addition of two levels of egg yolk (6% and 20%) in the Tris based extender on cryopreservation of Spanish ibex goats. The author recommended including low concentration of EY (6%) which resulted in higher motility than 20% EY. On the other hand, it was stated in another study that the addition of (1.5%) low egg yolk level did not provide spermatozoa with adequate protection during freezing and thawing process. However, including 12% egg yolk in the freezing extender gave better results, indicating that the addition of high level of egg yolk in the freezing extender may stop the activity of the seminal plasma enzymes, which precludes the need to wash semen samples before dilution. This study results supports the finding of the current study which resulted in higher motility rate with the addition of 10% EY in the Tris based extender. In support to this statement, it was revealed that some components of egg yolk may inhibit the enzyme lysophospholipase, and this is based on the fact that the ratio of phospholipase A_2 / lysophospholipase dropped from (1/10) in fresh semen to (1/1.5) post dilution (17). Furthermore, four levels of egg yolk 2.5%, 5%, and 7.5% and 10% were examined to compare the motility of buck semen after being kept at refrigeration temperature, it was indicated by Ranjan et. al.(18) that higher motility rate can be obtained with the addition of 10% egg yolk than the addition of other egg you levels when semen kept for 72 h. Deka, BC & Rao (19) also examined the impact of varying levels of egg yolk (7%, 10%, 20%) during cryopreservation of goat semen. The authors indicated that motility of semen diluted in 10% and 20% egg yolk was significantly higher than semen diluted in 7% egg yolk. Moreover, it has been found

that the source of the egg yolk could improve the post-thaw motility of ram semen regardless the concentration of the egg yolk in the semen extenders. KulaksIz et al (20) pointed out that the inclusion of 15% chucker egg yolk resulted in a higher motility rates compared to using egg from other bird species (chicken, turkey, duck, and Japanese quail). In addition, similar results were obtained by (21) during cryopreservation of stallion spermatozoa with the addition of chucker egg yolk. The reason behind that is due to the higher levels of protein, lipid and cholesterol in the chucker egg. Therefore, low motility rates in the current study may be attributed to the source of egg yolk in the semen extenders. Additionally, it was indicated by Watson, P & Martin (22) that the level of glycerol could be reduced providing with the presence of egg yolk provided in the freezing extender due to the interaction of egg yolk and glycerol in the motility scores during cryopreservation of ram semen. However, it was claimed by Saroff & Mixner (23) that there was no evidence about the interaction of egg yolk and glycerol in terms of protection effects. Another important reason to justify the findings of the current study is that semen collection was performed out of the breeding season which had a significant impact on the motility of semen post collection. As stated before that the concentration of EYCE reaches the highest levels during non-breeding season. In addition, semen samples were transported for a long distance around 90 Km after been initially diluted. The morphology of semen has not been affected according to our study results. Whereas, the motility of semen was decreased significantly 89.17 post collection to 57.5 pre-freezing. This indicated that transporting the semen for a long time may have affected the motility of sperm cells negatively. In support to this statement, a study was carried out in Vietnam indicated that transporting the goat semen for long distances (15-20 Km) may affect the motility and viability of goat semen (24).Another study by Memon, A. A., et al. (25) conducted the influence of different egg yolk levels in Tris based extender on semen of Boer goat pre-freezing and post thaw. The study indicated that better motility was significantly observed in 18% ,12% and 6% egg yolk levels respectively. However, adding egg yolk at 12% and 18% levels showed no significant ($P>0.05$) differences in semen morphology, this supports the findings of the current study. Also, the author pointed out that there were significant differences with better morphology when containing 12% and 18% egg yolk levels than 6% egg yolk level in the semen extender.

NARWADE, B. M., et al. (26) revealed that using Tris based extenders with the addition of 20% and 35% egg yolk levels is proper for cryopreservation of buck semen in terms of motility and morphology. The author also reported that there was a significant improvement observed in semen motility and morphology when using egg yolk based extenders containing trehalose in comparison to other semen extenders. A study carried out by Dhaher, N. N. & Aziz, D. M. (27) evaluated the influence of collection method and egg yolk level on the motility of buck semen. The study showed that motility of sperms collected by using of artificial vagina and 10% of egg yolk was higher compared to other egg yolk levels 5% and 10% .Tar, M., et.

al. (28) studied the impact of egg yolk levels (15% and 20%) on the motility and morphology of semen pre-freezing and post thaw during the breeding season. The author recommended using 20% egg yolk in the semen extender due to positive affect on morphology and motility. In conclusion, it is recommended to include 10% EY egg yolk in Tris-extenders in order to obtain superior motility and morphology results of goat semen pre-freezing and post-thaw during non-breeding season.

Table (1) The Motility rate post collection, pre-freezing and post-thaw in different treatments

Treatment	Motility % (mean ± SE)
Post collection	89.17 ± 2.39 a
Pre-freezing	57.5 ± 2.5 b
Post-thaw 0% EY	20.33 ± 8.01 c
Post-thaw 10% EY	35.83 ± 11.4 bc
Post-thaw 20% EY	25.83 ± 6.76 c

The different letters on the parameters refer to a significant difference between treatments *(p<0.05)*

Table (2) The Morphology rate pre-freezing and post-thaw in different treatments

Treatment	Morphology % (mean ± SE)
Pre-freezing	85.5 ± 3.19 a
Post-thaw 0% EY	86.83 ± 2.43 a
Post-thaw 10% EY	88.33 ± 1.67 a
Post-thaw 20% EY	88.33 ± 1.56 a

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