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RESEARCH ARTICLE

Comparison of Cryopreservative Effect of Different Levels of Omega-3 Egg-Yolk in Citrate Extender on the Quality of Goat Spermatozoa

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A B S T R A C T

The objective of the present study was to compare quality of chilled and frozen-thawed goat semen processed in citrate extender containing 3 different levels (2.5, 5 and 10%) of omega-3 egg-yolk (EY). Ejaculates were collected from five adult goats using artificial vagina. Quality of fresh semen, processed semen after 3 hrs of chilling and 24 hrs of freezing was assessed based on live sperm %age, abnormality (determined by eosin-nigrosin stain) and general and progressive motility (evaluated by CEROS computer assisted semen analyzer). The result showed a significant ($P<0.05$) decrease in post-chilled live sperm %age from the fresh sample for extenders using 2.5 and 5% EY but not for the 10%. Moreover, a significantly lower percentage general and progressive motility was recorded using the 2.5% EY compared to the others that showed post-chilled sperm motility non-significantly different from the fresh sample. After chilling, 5% EY showed significantly lower percent sperm abnormalities compared to others. However, the abnormalities increased after freezing to a level non-significantly different from the 10% EY that sustained to demonstrate higher live sperm %age and motility than both 2.5 and 5% EY. An overall increase in post-thawed live sperm %age, general and progressive motility was observed with increase in concentration of EY added. Thus, though the difference with the 5% EY is in magnitude, the 10% omega-3 EY in citrate extender is preferred compared to 2.5% for superior post-thawed goat semen quality, extended without washing seminal plasma.

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INTRODUCTION

Artificial insemination (AI) is a useful assisted reproductive biotechnology to achieve a rapid livestock genetic improvement and production. Its success however, depends on several factors which include quality of frozen-thawed semen used for AI. The ability to produce a good post-thawed semen quality in-turn depends on many other factors which include the composition of the cryopreservation media. The commonly used components of semen extenders for cryopreservation include egg-yolk, buffer, sugars, glycerol and antibiotics, each providing a complex and important role in protecting the spermatozoa (Purdy, 2006).

Cryopreservation of mammalian spermatozoa has been a challenge in many species of animals. The direct utilization of a cryopreservation media tested for one species to another to produce the same result has been difficult due to differences in the physiological characteristics of semen produced. This is particularly more important in goat semen cryopreservation due to an enzyme called egg-yolk coagulating enzyme (EYCE) found in the seminal plasma that interacts with egg-yolk of the cryopreservation media, causing coagulation and production of toxic substances deleterious to spermatozoa. The mechanism by which the enzyme causes damage to the spermatozoa is attributed to the production of lysolecithin by hydrolyzing lecithin in the egg-yolk (Ashmawy *et al*., 2010).

Attempts have been made by several workers to minimize the harmful interaction between EYCE and egg-yolk either by removal of seminal plasma through washing (Cabrera *et al*., 2005; Ari and Daskin, 2010; Naing *et al*., 2011) or by adding lower concentration of

egg-yolk into the extender (Ashmawy *et al*., 2010; Priyadharsini *et al*., 2011; Ramukhithi *et al*., 2011), all with variable outcomes. Despite the possible difference in the type of egg-yolk used, most past research appeared to overlook its importance and impact on the outcome of cryopreservation. Research has continued, yet with no standard cryopreservation media for goat semen. The type of egg-yolk used and concentration added to extenders remains a great concern in the success of goat semen cryopreservation. This study was designed to compare effect of using different levels of omega-3 egg-yolk (2.5, 5 and 10%) in citrate extender on the quality of goat spermatozoa after cryopreservation without removing the seminal plasma.

MATERIALS AND METHODS

Animals: This study was conducted at the National Veterinary Biodiversity Institute in Jerantut, Malaysia. There were five bucks which consisted of two Red Kalahari breed, two local Katjang and one Utan-cross breed, used for semen collection. Their ages ranged between 3 and 4 years, with body weights between 30 to 60 kg. The bucks were under intensive management and were fed with a commercial pellet and *Brachiaria Humidicola* grass, mineral block and water provided *ad libitum.*

Semen collection and evaluation: Sixteen semen samples were collected twice-per-week using artificial vagina for a period of six weeks. Six samples were from the two Red Kalahari bucks (3 from each), seven from the other two Katjang bucks (3 from one and 4 from the other) and 3 from the Utan-cross buck. Collected semen samples from each buck were processed separately and evaluated after collection, following chilling and freezing, for live sperm percentage, abnormality, general and progressive motility. To minimize possible variation due to breed differences, fresh semen collected from all bucks was set to have minimum of 70% sperm motility and 80% normal morphology before further processing and treatment, otherwise discarded. Motility was assessed with CEROS computer assisted semen analyser, using a 4 µL of diluted fresh sample with 3% sodium citrate (1:10). For estimation of live sperm percentage, a 10 µL of fresh semen was mixed with 30 µL of eosin-nigrosin stain. A thin smear was prepared and evaluation was made out of 200 spermatozoa examined under a microscope (400x). The same eosin-nigrosin stained slides were also used to determine sperm abnormality for which 200 sperm cells were examined for defects associated with sperm head, mid-piece and tail region.

Cryopreservation procedures

Extender preparation: The Ladang Ternakan Kelang Omega Plus eggs (LTKM SDN BHD) were used as source of egg-yolk in this study. The eggs contained 400-600 mg omega-3 polyunsaturated fatty-acid per 100g egg-yolk, almost five times more enriched than regular chicken eggs. To minimize the effect of possible variation among individual eggs, egg-yolks collected from a batch of eggs were pooled together and sourced out for preparation of the extenders to process the collected semen samples.

To prepare sodium citrate diluents, 2.37g sodium citrate, 0.50g glucose, and 7% glycerol were dissolved in 70 ml of distilled water. Then, 7 ml of the diluent was transferred into 3 different glass tubes. One ml of antibiotics (100000 i.u penicillin and 100mg streptomycin) was added into each glass tube. After that, 0.25, 0.5 and 1.0 ml of egg-yolk were added into the glass tubes. Distilled water was added to make up the volume upto 10 ml to produce 2.5, 5 and 10% egg-yolk concentrations, respectively.

Dilution of semen: Soon after collection and evaluation, dilution of semen was done at 37^{o}C. Sperm concentration in the neat semen was determined by SpermaCue photometer (Minitub®). The neat semen was then divided equally and put into 3 glass tubes. Based on initial sperm concentration and volume of the neat semen, required amount of the respective prepared diluents was added into each tube with expected final concentration of 200 x 10^6 sperm/ml.

Chilling of semen: The glass tubes with diluted semen were placed in the refrigerator. After 30 minutes, the glass tubes were transferred into another empty beaker and after 3 hours, the quality of semen was assessed.

Freezing, thawing and evaluation: The chilled semen was packed into 0.25 ml straws. After sealing, straws were placed horizontally on a cold rack (5^{o}C) and lowered into nitrogen vapors (-50^{o}C), 3-4 cm above the surface of liquid nitrogen. After 3 minutes, when the temperature reached -100^{o}C, the frozen straws were transferred into goblets of appropriate size and transferred into a liquid nitrogen tank (-196^{o}C). After 24 hours of storage, two straws per-extender were randomly chosen, thawed in water at 37^{o}C for 30 sec and evaluated for post-thaw semen quality (Naing *et al*., 2011).

Statistical analysis: The mean values for fresh, post-chilled and post-thawed semen quality parameters for the 3 extenders are expressed as Mean ± SEM. A one-way analysis of variance (ANOVA) with Tukey's multiple comparison test was applied to determine differences among the extenders using SPSS statistical software version 16.0. Differences with P value <0.05 were considered to be statistically significant.

RESULTS

The quality of spermatozoa after chilling using the 3 extenders is shown in Table 1. There was a general decrease in post-chilled semen quality parameters such as live sperm %age and motility compared to fresh semen for all extenders. Highest sperm abnormality and a significantly lower (P<0.05) live sperm %ge and motility was obtained using 2.5% egg-yolk. The 5% egg-yolk showed significantly higher percentage of general and progressive sperm motility, but not live sperm %age, compared to the 2.5% egg-yolk. Percentage sperm abnormality was lowest for the 5% egg-yolk compared to other extenders. However, 10% egg-yolk showed higher percentages of live sperm and motility compared to 2.5% egg-yolk, with no significant reduction in quality compared to the fresh semen (Table 1).

Similar to chilling, a general further decrease in live sperm %age, general and progressive motility for all extenders compared with fresh sample was recorded after freezing (Table 2). Among the extenders, 10% egg-yolk again showed the highest percentage of live sperm, general and progressive motility, which was significant, compared to the 2.5% EY, the latter showed the lowest quality. In contrast to other sperm quality parameters, percentage abnormality after freezing using all the extenders tended to show improvement compared to the fresh sample with least abnormality resulted from the 5% egg-yolk, though not significantly different from the 10% egg-yolk (Table 2).

Table 1: Fresh and post-chilled semen quality parameters (%, mean ± SE) diluted using 3 different extenders

Parameters (%)	Fresh sample	Egg yolk level (%)		
		2.5	5	10
Live sperm	94.4±1.2^{a}	68.3±5.5^{b}	80.5±3.3^{b}	81.7±3.3^{ab}
General motility	91.5±1.0^{a}	63.2±6.0^{b}	78.7±3.0^{a}	79.8±3.2^{a}
Progressive motility	75.7±1.5^{a}	52.0±5.3^{b}	68.8±2.3^{a}	68.5±2.7^{a}
Abnormality	8.6±1.1^{a}	9.2±1.4^{a}	2.90±0.4^{b}	7.0±0.6^{a}

Values with different superscripts within rows show significant differences (P<0.05).

Table 2: Mean ± SEM of fresh sample and post-thawed semen quality parameters (%) extended using 3 different extenders

Parameters (%)	Fresh sample	Egg yolk level (%)		
		2.5	5	10
Live sperm	94.4±1.2^{a}	37.0±4.8^{c}	55.1±3.9^{b}	57.1±5.0^{b}
General motility	91.5±0.9^{a}	33.8±4.4^{c}	48.6±3.8^{bc}	53.5±4.6^{b}
Progressive motility	75.7±1.5^{a}	28.0±3.9^{bc}	42.6±3.4^{bc}	48.1±4.4^{b}
Abnormality	8.6±1.1^{a}	8.1±0.6^{a}	5.1±0.3^{b}	7.7±0.8^{ab}

Values with different superscripts within rows indicate significant differences (P<0.05).

DISCUSSION

During cryopreservation, sperm cells are subjected to a number of physical and chemical stresses. Because of the deleterious effect of these factors, it is inevitable to have a reduction in the quality of sperm after cryopreservation (Andrabi, 2007*;* Lemma, 2011), which is the same as what was observed in the present study for all extenders tested. How much the quality of semen deteriorates however, depends on several factors including the type of cryopreservation media and its composition, and processing procedure.

The current study revealed that when there was an increase in egg-yolk concentration, semen quality both after chilling and freezing increased in terms of most parameters including live sperm %age, general and progressive motility. The results showed that 10% egg-yolk in citrate extender was most suitable to obtain a higher recovery rate of frozen-thawed live sperm %age and motility compared to the 2.5 and 5% egg-yolk, although the difference with 5% was in magnitude. Though 20% egg-yolk is accepted as standard level in most cases for bull semen cryopreservation (Rahman *et al*., 2012), the level of egg-yolk reported in goat is variable. For instance, using 20% egg-yolk in Tris-based extender with washing of semen resulted in good post-thawed semen quality in goat (Ari and Daskin, 2010). On the other hand, a study by Priyadharsini *et al*. (2011) concluded that 10% egg-yolk in Tris buffer was superior to 20% egg-yolk during cryopreservation of the Jakharana goat semen.

The percentage abnormality of post-thawed spermatozoa was highest for the 2.5% egg-yolk, decreased with 5% egg-yolk, then increased non-significantly with 10% egg-yolk. These might imply that using 2.5% egg-yolk was not sufficient to protect the sperm. The 10% egg-yolk that resulted in highest post-thawed motility and live sperm %age showed slight increase in abnormality from the 5%, possibly indicating the start of the deleterious interaction between the egg-yolk and EYCE. A concentration between 5 and 10% (7.5%) should be further investigated as it may provide optimum protection that yields higher live sperm %age, motility and low abnormality. According to Ashmawy *et al*. (2010), who compared different levels of egg-yolk in Tris extender for goat semen freezing without washing, there was a non significant difference in post-thawed recovery rate up-to 10% level but, with a significant drop in quality for concentrations >10% (15 and 20%). Hence, if a higher omega-3 egg-yolk of the present study is sought perhaps washing seminal plasma before addition might be required. Omega-3 egg-yolk in citrate extender seems to be effective in preserving sperm morphology during cryopreservation as the evaluation showed below 10% abnormality across all the extenders. For bucks of normal fertility, the number of abnormal spermatozoa in a sample can vary from 5 to 15% and a reduction in fertility is not observed until the number of abnormal spermatozoa exceeds 20% (Onifade, 2011).

In the present study, the percentage abnormality after freezing and thawing using all the extenders tended to show improvement compared to the fresh sample with significant reduction observed with the 5% egg-yolk, and non significantly different from 10% egg-yolk. Majority of sperm abnormalities observed in this study were minor or secondary defects such as simple coiled or bent tails (data not shown) that usually encounter in response to change in temperature, but not of the defects of major type such as acrosome defect, defects associated with the sperm head and mid-piece (‘‘Dag’’ defect), which are irreversible and genetically related (Chenoweth, 2005). It can be hypothesized that the fresh semen exposed to the environment before mixing with the diluents is experiencing more shock to have higher minor abnormalities that tend to dwindle and reverse back after dilution during the process of chilling and freezing. This hypothesis, however, needs further investigation to understand how the abnormalities are reversed and the possible role of the omega-3 fatty acid added to the extender.

The mechanism of action by which egg-yolk provides protection is attributed to presence of low-density lipoproteins, which adhere to the sperm membrane during cryopreservation thereby preserving the sperm (Moussa *et al*., 2002). It has been suggested that the phospholipids from the egg-yolk could merge with the sperm membrane replacing some of the sperm phospholipids, decreasing their phase-transition temperature and help preventing cold shock (Huopalahti *et al*., 2007). Moreover, the egg-yolk has an additional property in protecting against toxic actions of seminal plasma constituents including antibacterial cationic peptides that are released from large disaggregating proteins (Huopalahti *et al*., 2007) and cause severe membrane damage (Shannon *et al.*, 1987). The 2.5% egg-yolk used in this study failed to provide

better protection demonstrated by lower live sperm %age and motility and higher abnormality as compared to the 10% egg-yolk probably due to insufficient amount of egg-yolk for protection against damage by the cooling and freeze-thawing process.

An interesting observation in the present study was the ability to obtain a good post-thawed semen quality without washing seminal plasma before processing. This has the advantage of cutting-short the procedure of processing and minimizing exposure of the semen to several external factors for a prolonged time that can decrease sperm quality and ability to survive during cryopreservation. This finding is in disagreement with some earlier studies who reported the benefit of removing seminal plasma as it maximizes post-thaw motility and acrosomal integrity in goat semen (Cabrera *et al*., 2005; Naing *et al.*, 2011). However, when seminal plasma is separated by washing, not only the enzyme-EYCE is removed but also other beneficial components that sperm cells need to survive after ejaculation (Ari and Daskin, 2010). This is also supported by Ramukhithi *et al*. (2011), who used 20% egg-yolk in Tris extender without washing, but still obtained better post-thaw motility rate as compared to washed semen. Thus, as of the present study, washing the semen appeared to be not essential when a concentration of up-to 10% omega-3 egg-yolk is used in citrate extender.

Earlier studies have reported the use of low level of egg-yolk (2.5%) as optimum with diluents such as Tris-based (Ashmawy *et al*., 2010) and glucose-EDTA (Bispo *et al*., 2011) for superior post-thawed goat semen quality. The variation compared to our study might be attributed to the difference in the type of extender and egg-yolk used. In the present study, the use of omega-3 fatty acids enriched egg-yolk probably helped to compromise the possible effect of the harmful interaction expected between EYCE and egg-yolk. Omega-3 fatty acids are essential polyunsaturated fatty acids required for physiological processes including reproduction (Mattos *et al*., 2000). A number of studies have shown the effect of using omega-3 fatty acids on semen quality and fertility of domestic animals. For example, Adams *et al.* (2008) stated that supplementation of omega-3 fatty-acid is beneficial in increasing post-thaw progressive motility of equine spermatozoa. A study in boars by Rooke *et al.* (2001) showed that supplementing diet of boars with tuna oil, which is rich in omega-3 fatty acid, increased the phospholipid fatty-acids of sperm that subsequently resulted in increase in sperm quality. Nalley and Arifiantini (2011) have also reported that addition of omega-3 egg-yolk in Tris extender protects plasma membrane better than regular or native hen egg-yolk. So it is suggestive that the use of omega-3 egg-yolk in the present study might have helped in preserving the live sperm %age and motility of spermatozoa during cryopreservation.

Generally, the present study revealed that better cryopreservation of goat spermatozoa can be achieved using a 10% omega-3 egg-yolk in citrate extender without the need to separate seminal plasma. Further studies might be considered to see effect of using a much higher level of egg-yolk (20%) than the 10%. Comparison of the 10% omega-3 egg-yolk- citrate extender with commercially available egg-yolk-free extenders such as Bioxcell® would be another area of research to be explored.

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