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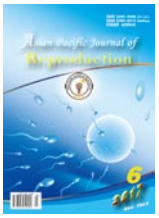

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Cryopreservation of cattle semen using coconut water extender with different glycerol concentrations

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ABSTRACT

Objective: To investigate the effect of coconut water with a lone concentration and different concentrations of glycerol on chilled and cryopreserved cattle semen characteristics. **Methods:** Semen was collected from five mature cattle bulls, at weekly intervals for 5 weeks. The ejaculates were pooled and evaluated for dilution processing. Tris citrate egg yolk fructose was used as control treatment for semen, while 50% (V/V) coconut water, 25% (V/V) bi-distilled water and 25% (V/V, 5% anhydrous monosodium citrate) to 20 mL egg yolk and three different concentrations of glycerol (4%, 6% and 8%) were used as coconut water (CW)-glycerol-yolk extenders (CWCG-4, CWCG-6 and CWCG-8). Extended semen was cooled and cryopreserved. Sperm motility%, sperm membrane integrity%, normal acrosome%, live sperm% and total sperm abnormalities% were recorded after equilibrium and after freeze-thawing. **Results:** The addition of 4% glycerol to coconut water enriched media (CWCG-4) revealed the most effective addition of glycerol on all parameters after equilibrium and after freeze-thawing. **Conclusions:** Coconut water enriched media with 4% glycerol addition is safe to be used as an extender in bull semen preservation because it is a sterile liquid. So, it can be used without addition of antibiotics to the extender, as antibiotics have to some extent hazardous effect on spermatozoa.

1. Introduction

Natural extracts from vegetables and fruits and their seeds maintain life due to their high contents of medicinal compounds essential for health[1,2]. These natural extracts and infusions are used in semen extenders for preserving animal sperms[3]. This cryopreserving property is mainly related to their strong antioxidant capacity, thus protecting spermatozoa from oxidative damage during cryopreservation process[4,5]. Coconut water is characterised by its high contents of antioxidants as expressed by the phytohormones[6], sugar, vitamins, electrolytes and amino acids[7]. The suitability of coconut water-based diluents for processing buffalo semen was shown by Vale *et al*[8,9] and El-Nattat *et al*[10]. Coconut water is capable of *in vitro* sperm capacitation in swine[11] and as a maturation and culture medium for bovine[12], caprine[13] and ovine[14] oocytes and embryos.

The main objective of this study was to evaluate coconut water with different concentrations of glycerol (4%, 6% and 8%) as a semen extender for preserving cattle semen as reflected on sperm motility, sperm membrane integrity, viable and abnormal sperm percent and acrosome integrity of cooled and frozen semen.

2. Materials and methods

2.1. Semen collection and initial evaluation

Semen was collected from five mature cattle-bulls, reared at Buffalo Semen Freezing Centre, General Organization for Veterinary

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Services, Ministry of Agriculture, Abbasia, Egypt, using an artificial vagina at weekly intervals for 5 weeks. The semen was transferred to the laboratory within few minutes. Semen samples were examined for volume, percentage of motile sperm, general sperm morphology and sperm cell concentration. Semen samples with more than 70% motility and 80% morphologically normal spermatozoa and 1×10^9/mL sperm concentration were used for dilution and processing. The ejaculates were pooled in order to have sufficient semen for replicates and to eliminate the bull effect. The semen was given a holding time for 10 min in a water bath at 35-37 ℃ before dilution.

2.2. Semen processing

Tris-citric acid-fructose-egg yolks (TCFY) were used as a control extender[15]. Coconut water obtained from a tender, green, healthy and undamaged coconut fruit coconut water (CW) was filtered two times. CW extender with 20% egg yolk (v/v) and one of three different glycerol concentrations (4%, 6% and 8% V/V) was prepared and used according to Vale *et al*[8] and Cardoso *et al*[16]. All extenders contained 1 000 IU penicillin and 1 mg streptomycin per milliliter.

Pooled semen samples were split into four equal fractions and diluted at 37 ℃ in a single step with the four experimental extenders (TCFY and CW containing 4%, 6% and 8% glycerol) to a final concentration of 60×10^6 spermatozoa/mL. Extended semen fractions were exposed to freezing process according to Ahmad *et al*[17].

2.3. Semen quality assessment

Sperm motility%, sperm membrane integrity%, normal acrosome%, live sperm% and total sperm abnormalities% were assessed after cooling and freeze-thawing (frozen straws were thawed at 37 ℃ for one minute).

2.3.1. Sperm motility

Sperm motility was assessed according to Graham *et al*[18].

2.3.2. Sperm membrane integrity

Plasma membrane integrity of buffalo bull spermatozoa was assessed using the hypo-osmotic swelling test (HOST) as described by Jeyendran *et al*[19] and Ahmad *et al*[17].

2.3.3. Percentage of live sperm and normal acrosome

The dual staining procedure with trypan blue-giemsa stain was performed as described by Kovacs and Foote[20].

2.3.4. Sperm morphology

Abnormal sperm percent was examined in eosin nigrosine stained semen smears[21].

2.4. Statistical analysis

The results expressed as mean± standard errors of the mean. To compare coconut extender with different glycerol concentrations to the control Tris, the data were analyzed by analysis of variance using the ANOVA procedure of SAS program v. 9.2[22]. Differences between means were compared with Waller-Duncan multiple range at Kratio=100.

3. Results

Data output revealed that the cooled semen membrane integrity and acrosome integrity percentages were the only parameters to be affected by the variation of glycerol addition and CW (Table 1). The addition of 4% glycerol to CW enriched media revealed the

Table 1

Coconut enriched media with different concentrations of glycerol effect on cooled cattle semen (Mean± SE) (%).

Parameters	Motility	Live	Abnormality	HOST	Acrosome
TCFY	85.83±3.00^a	83.33±3.33^a	17.33±0.67^a	71.67±1.67^c	81.67±1.67^b
CW CG- 4%	89.17±2.39^a	88.33±1.67^a	11.67±2.03^a	85.67±0.67^a	93.33±1.67^a
CW CG- 6%	85.83±2.70^a	85.00±2.89^a	18.00±3.51^a	81.00±1.00^b	90.67±0.67^a
CW CG- 8%	81.67±1.67^a	83.33±3.33^a	15.33±0.33^a	80.67±0.67^b	91.33±0.67^a
F-cal	1.52	0.67	1.91	29.36	16.66
Sig	0.240 6	0.595 7	0.207 1	0.000 1	0.000 8

Different superscript letters within column (a, b, c) are significantly differed according to Waller-Duncan K-ratio *t* Test (k-ratio=100).

Table 2

Coconut enriched media with different concentrations of glycerol effect on frozen cattle semen (Mean± SE) (%).

Parameters	Motility	Live	Abnormality	HOST	Acrosome
TCFY	40.00±2.83^b	65.00±2.89^b	19.33±0.67^{ab}	65.00±1.10^c	63.33±3.33^b
CW CG- 4%	48.75±1.57^a	81.67±1.67^a	16.67±0.88^c	76.25±0.59^a	76.67±1.67^a
CW CG- 6%	29.38±1.99^c	71.67±1.67^b	18.33±0.33^{bc}	61.63±0.63^d	71.67±1.67^{ab}
CW CG- 8%	30.00±1.34^c	65.00±2.89^b	20.67±0.67^a	70.88±1.14^b	63.33±3.33^b
F-cal	20.87	11.17	6.40	51.29	6.23
Sig	0.000 1	0.003 1	0.016 1	0.000 1	0.017 3

Different superscript letters within column (a, b, c *etc*) are significantly differed according to Waller-Duncan K-ratio *t* Test (k-ratio=100).

most effective addition of glycerol on all parameters. The motility and livability % were apparently increased while the abnormality % was decreased when compared to other treatments. Only HOST and acrosome were significantly (P<0.001) affected.

In the same consent, after freeze-thaw, the addition of variant glycerol concentrations to CW enriched media had significantly affected all parameters (percentage of motility, livability, abnormality, HOST and acrosome) (Table 2). The CW with 4% glycerol revealed highly significant percentage for motility, livability, HOST and acrosome integrity (48.78±1.57, 81.67±1.67, 76.25±0.59 and 76.67±1.67, respectively) compared to the other treatments. While the abnormality percentage was significantly the lowest (16.67±0.88) compared to the other treatments.

4. Discussion

Recently, scientists are interested in the potential health benefits of phytochemicals and the synergistic effects of their multiple compounds compared to the single purified active fractions[4]. Semen cryopreservation leads to biochemical and functional damage to spermatozoa, thus reducing its motility and viability[18], but it is important to preserve the valuable genetic constitution of our local breeds of cattle bulls. Cryodamage induced by freezing and thawing can be decreased by adding lipoproteins, or using the suitable cryoprotectant in the semen extender[23]. Semen freezing is associated with over production of reactive oxygen species and reduction in the antioxidant capacity as manifested by a decrease in intracellular GSH content that induce damage in spermatozoal membrane[24–26]. Seminal plasma has limited antioxidant capacity, so, the use of an extender having strong antioxidant effect is recommended to maintain the viability and fertilizing capacity of frozen spermatozoa[26]. Motility is the most important parameter used for semen evaluation, both before and after preservation[27]. Concannon and Battista[28] suggest that at least 40%-50% sperm motility is necessary for success in artificial insemination. However, Linde-Forsberg and Forsberg[29] postulated that 20%-30% sperm motility is necessary for pregnancy. In our study, CW extender with glycerol 4%, 6% and 8% improved sperm membrane integrity (HOST%) of cooled semen and the superior result was obtained in CW with 4% glycerol. In frozen semen CW extender with glycerol 4% was able to preserve sperm quality as manifested by higher motility, alive, HOST, intact acrosome % and lowered abnormalities as compared to Tris control extender. Silva e*t al*[30,31] concluded that CW containing glycerol is recommended to achieve satisfactory post freezing quality of boar semen. Cardoso *et al*[32] recorded that cryopreservation of canine semen using CW extender resulted in a higher quality of canine sperm. Canine chilled extended semen in CW provided good pregnancy and whelping rates with a higher percentage of female births from French bitches[33]. The inclusion of coconut milk at 15% and 20% in tris-extenders markedly improved sperm viability parameters of goat buck semen[34]. Non-permeable coconut ingredients and permeable glycerol cryoprotectants induce a synergistic beneficial effect on spermatozoa during freezing. The improved results in our study is attributed to the inclusion of CW with high contents of phytohormones[6], sugars[7], vitamins[35], electrolytes[36] and amino acids[37]. All these ingredients in CW have strong antioxidant activities that protect sperm against oxidative damage. CW enriched media with 4% glycerol addition (CWCG-4) is safe to be used as an extender in bull semen preservation because it is a sterile liquid, so, it can be used without addition of antibiotics to the extender, as antibodies have to some extent hazardous effect on spermatozoa.

Conflict of interest statement

We declare that we have no conflict of interest.

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