

The Viability of Local Ram Semen in Tris Buffer With Three Different Egg Yolks

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Abstract. Egg yolk consisted of lecithin and phospholipids are one of the most commonly used components that will protect spermatozoa against cold shock during cooling and freezing. The aim of this study was to evaluate the effect of different hen egg yolk on Tris extender on the freezability of local ram semen. Semen from six sexually matured local rams was collected weekly using artificial vagina. Collected semen was evaluated macroscopically and microscopically and extended using tris extender consisted of 20% (v/v) regular egg yolk (TRCEY), native egg yolk (TNCEY), omega-3 hen egg yolk (TOEY) and 6% (v/v) glycerol. Those were packed in 0.25 ml straws, equilibrated at 5°C for 3 hours, frozen and stored in nitrogen tank for 24 hours, and thawed at 37°C for 30 second. The result of the experiment showed that there were no significant differences on the sperm motility and the number of living sperm. Percentage of plasma membrane intact in TOEY (60.3%) was significantly higher compared to TREY (56.9%) and TNEY (55.6%). In conclusion, the addition of omega 3 egg yolk in Tris extender protects plasma membrane better than the regular or native hen egg yolk.

Key Words: ram semen, egg yolk, frozen semen

Introduction

Cryopreservation as a technique for the storage of ram semen has many advantages although the process of freezing and thawing induces certain detrimental effects, in terms of sperm structure, biochemical and functional damage. The sperm plasma membrane damage results in the irreversible loss of its functions. Due to the high content of unsaturated fatty acids in the plasma membrane, mammalian sperm is sensitive to oxidative stress (Salomon and Maxwell, 2000; Tekin et al., 2006).

The survival of spermatozoa during freezing and thawing was affected by many factors, including the composition of the cryo-extender (Curry, 2000; Leibo and Songsasen, 2002). Egg yolk is a common component of the most semen-cryopreservation extenders for domestic animals. It has been shown to have a beneficial effect on sperm cryopreservation as a protection of the plasma membrane and acrosome against temperature-related injury, in

association with the other components (Amirat et al., 2004). It is believed that the phospholipids, cholesterol and low dense lipoproteins in egg yolk may be the factors that provide protection to sperm against cold shock during the freeze-thawing process. Nevertheless, the exact mechanism by which egg yolk helps preserve ram sperm during the freeze-thawing process is unknown.

The egg yolk fatty-acid profile was clearly affected by the fatty acid profile of hen diets (Meluzzi et al., 2000; Butarbutar, 2004). Therefore, the ratio of phospholipid and cholesterol content in yolk of regular or native and chicken eggs that were enriched with omega-3 fatty acids has a different combination which may result in different cryopreservation effects on the ram sperm.

The following experiment was designed to compare the freezability of ram sperm in Tris base extender added with 20% of native, regular or omega-3 egg yolks.

Materials and Methods

Media preparation

All chemicals were obtained from Merck, Germany. Tris buffer (TB) was prepared by mixing 2.42 g Tris-hydroxymethyl-aminomethane with 1.28 g monohydrate citric acid and 2.16 g D-fructose dissolved in 100 ml. The freezing extender composed of 74% TB, 20% (v/v) native, regular or omega-3 egg yolk and 6% of glycerol concentration (Table 1). All extenders were added with antibiotics (500 IU penicillin and 500 µg streptomycin per ml). A total of three extenders were prepared i.e. tris native hen egg yolk (TNEY), tris regular hen egg yolk (TREY) and tris omega-3 hen egg yolk (TOEY).

Semen collection and evaluation

Five local rams were used as sperm donors. Semen was collected using an artificial vagina once a week during four weeks. The semen samples were assessed for macroscopic evaluation including volume, colour, consistency, and mass activity. The microscopic evaluation was conducted under 100-400 time magnifying microscope (Olympus CH20) including mass activity, percentage of progressive motility [0 (not motile) -100% (100% motile)], ratio of living and dead sperms using eosin-negrosin (Barth and Oko, 1989), and sperm concentration using Neubauer

counting chamber (Kirkman-Brown and Björndahl, 2009). Sperm morphology was assessed with carbolfuchsin-eosin (Al-Makhzoomi et al., 2008) and intact membrane with hypo osmotic swelling (HOS) test (Fonseca et al., 2005). Only ejaculations with concentration greater than 2500×10^6 sperm/ml, >75% progressive motile sperm and >90% of the sperm with normal morphology were selected for this study. Total of 20 ejaculations were individually processed for cryopreservation.

Semen processing and thawing

After evaluation, each of raw semen was equally divided into three tubes and extended in one of three extenders; TNEY, TREY and TOEY to reach the total semen concentration of 400×10^6 per ml (100×10^6 per straw). Extended semen was individually packed in 0.25 ml straws and equilibrated at 4°C within four hours. The straw was frozen in a Styrofoam box at 5 cm above the liquid nitrogen level for 10 minutes. The frozen semen was stored for 24 hours in liquid nitrogen for further evaluations.

Frozen semen was thawed using warm water (37°C) for 30 seconds. Semen evaluation was focused on the percentage of motile, life and intact membrane sperms. The motile sperm was evaluated by mixing the semen gently and placing a 10 µl drop of extended semen on a warm slide and covered with a glass

Table 1. Extender composition

Composition	Cryo-extender		
	TREY	TNEY	TOEY
Buffer Tris (mL)	7.4	7.4	7.4
Regular hen Egg yolk (ml)	2	-	-
Native hen Egg yolk (ml)	-	2	-
Omega-3 hen Egg yolk (ml)	-	-	2
Glycerol (ml)	0.6	0.6	0.6
Total volume (ml)	10	10	10
Streptomycine (mg)	10	10	10
Pennicilline (IU)	10.000	10.000	10.000
pH	6.9	6.8	6.8
Osmotic pressure (mosmol/kg)	1075	1075	1075

TNEY: tris native hen egg yolk; TREY: tris regular hen egg yolk; TOEY: tris omega-3 hen egg yolk

cover slip (18x18 mm) from five selected representative fields. The mean of the five estimations was recorded as final motility score. Living sperm was assessed by placing 10 μ l drop of semen on a slide added with 40 μ l drop of nigrosine-eosin, mixed together and smeared on a slide and dried quickly in heating stage (37°C). Microscopes were selected randomly from ten fields, with total of 200 cells. Individual sperm was recorded as living (unstained) or dead (stained). Hypo osmotic swelling (HOS) test was assessed by placing 10 μ l of extended semen in 2 ml of HOS solution, gently mixed and incubated for 30 minutes in a water bath at 37°C. After incubation, 20 μ l of the solutions containing semen was placed on a microscope slide, covered with a cover glass and evaluated under microscope. Total of 200 spermatozoa were counted from at least five different fields, and sperms with intact membrane were classified as swelled (coiled).

Statistical methods

The data were statistically analyzed for differences among the means by one way analysis of variance. The Turkey's test was applied to compare treatment means using statistical Minitab software version 14.

Results and Discussion

The mean of semen volume was 0.72 ± 0.16 ml, pH was 6.65 ± 0.07 , cloudy to creamy white in colour and thin to thick in consistency. The mean of mass activity was 3 ± 0.0 with the percentages of motile and living sperm were $75 \pm 4.0\%$ and $84.09 \pm 2.28\%$ respectively. The sperms with normal morphology and intact membrane were $94.75 \pm 3.2\%$ and $88.15 \pm 3.21\%$ with 1032.00 ± 232.06 million/ml in sperm concentration.

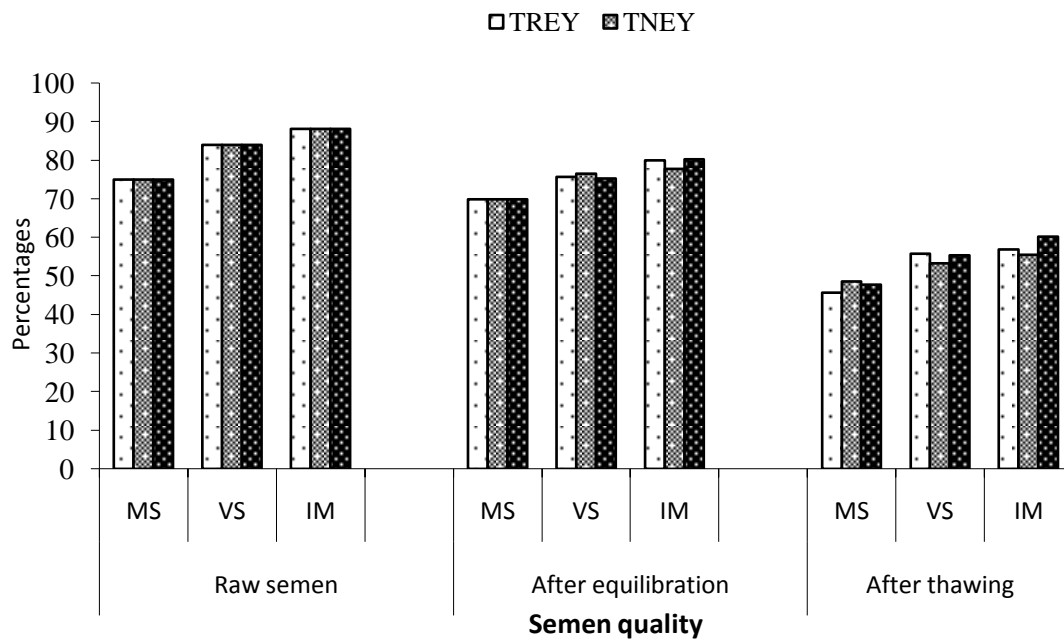
There were no differences ($P > 0.05$) among extenders in forward motility after thawing. The forward motility of sperm in TNEY, TREY or TOEY were 45.7 ± 2.31 , 48.60 ± 3.24 and $47.80 \pm 2.25\%$ respectively. No differences were

detected on the living sperm after thawing. The living sperms were 53.40 ± 3.42 to $55.8 \pm 3.38\%$. The membrane intact of sperm in TOEY extender ($60.30 \pm 1.33\%$) was greater ($P < 0.05$) than those extended in TNEY ($56.20 \pm 2.35\%$) or TREY ($55.60 \pm 1.95\%$) and no differences were found on membrane intact between TNEY and TREY (Figure 1).

Sperm motility had been the most common examined parameter for spermatozoa quality and viability, although its relation to fertilization capacity of sperm was often contradictory (Fonseca et al., 2005). In this study, the sperm motility in all extenders was not affected by egg yolk. The motility of sperm depends of flagellar action whose energy is metabolized by the mitochondrial dense in mid-piece. Flagellar motion is the main energy-demanding process of viable spermatozoa and most energy derived from the hydrolysis of adenine triphosphate (ATP) to adenine di- and mono-phosphates (Garner and Hafes, 2000).

Sperms require nutrients to maintain their cellular activity; the main source of nutrients is simple carbohydrate such as fructose or glucose, in seminal plasma or diluent content (Vishwanath and Shannon, 2000). Fructose was essential for energy utilization by spermatozoa and to support sperm motility and movement patterns. In this study, Tris buffer contained fructose as well as in yolk with 0.6% carbohydrate (Manjunath et al., 2002). It might explain why there were no differences in sperm motility in all extenders.

Egg yolk protects the sperm cell from the damaging effects of low temperature; the beneficial effect of egg yolk in the cryopreservation of sperm can be attributed to a resistance factor, which helps protect the sperm against cold shock to maintain viability. The phospholipid, cholesterol and the low-density lipoprotein contents of hen egg yolk specifically have been identified as the protective components which protect the membrane phospholipid integrity during



(MS = motile sperm; VS=viable sperm; IM=intact membrane; TREY= Tris Regular Egg yolk; TNEY = Tris Native Egg yolk; TOEY=Tris Omega-3 Egg yolk)

Figure 1. The quality of local ram semen during processing in Tris buffer with three different egg yolk.

cryopreservation (Aurich, 2005).

Based on the percentage of sperm with intact membrane, egg yolk of omega-3 seemed to be more effective to protect ram spermatozoa, compared to regular or native hen egg yolk. Omega-3 egg yolks were obtained from hens fed with omega-3 enriched diets. Omega-3 enriched eggs are available in the market. These eggs are the same as the regular egg, except that they contain higher level of the polyunsaturated fatty acid called omega-3. This higher level of the polyunsaturated fatty acid may improve the protection of the sperm during the freeze-thawing processes resulting in higher membrane intact after thawing. Native egg yolks were obtained from pastured hen, without standard diets. Traditionally, regular egg yolk, has been used in freezing media, probably because of its easy availability (Bathgate et al., 2006), and lower price compared to native or Omega-3 egg yolk. Limited report on avian egg yolks used for semen preservation was available. Duck egg

yolk was more favoured than the other avian egg yolks in extenders used to improve the frozen-thawed quality of buffalo bull and stallion sperm (Andrabi et al., 2007; Clulow et al., 2007). Other study proved that pigeon egg yolk has the best cryo-protective effect in terms of bull sperm progressive motility and viability among five avian egg yolks, but no differences found among the avian egg yolks (chicken, goose, and duck) on the composition and different cryo-protective actions on ram sperm cryopreservation (Su et al., 2008).

According to Humes and Webb (2006), chucker egg had a higher level of protein, lipid and cholesterol which improve the percentage of motile stallion sperm after the freeze-thaw process, when compared to chicken egg yolk. Recently Kulaksız et al. (2010) reported that chucker egg yolk also had the best cryoprotective effect in terms of the highest sperm motility compared to the other five avian eggs ram semen cryopreservation.

As stated above, all sperm-processing steps may introduce damage to sperm membranes



(MS: motile sperm; VS: viable sperm; IM: intact membrane; TREY: tris regular egg yolk; TNEY : tris native egg yolk; TOEY: tris omega-3 egg yolk; R-AE: decrease between raw to after equilibration; AE-AT : decrease between after equilibration to after thawing).

Figure 2. The decreasing semen quality during freezing of local ram semen in Tris buffer with three different egg yolks.

and organelles (Silva and Gadella, 2006). The differences in measured semen quality parameters after 4 hours equilibration were not found among extenders. In fact, all extenders demonstrated significant decrease ($P<0.05$) compared to raw semen. Sperm motility decreased by 5% in each extender; on the other hand living sperm and intact membrane decreased from 7.49 to 8.69%, and from 7.85 to 10.35 respectively (Figure 2).

Similarly, all parameter indicated a highly significant decrease ($P<0.01$) after thawing. The decrease of forward motility from post-equilibration to post-thawing was between 21.4, 24.3%, and living sperm between 20 and 23.3% respectively. The membrane intact of sperm from post-equilibration to post-thawing was decreased by 20 to 23.2% (Figure 2).

Conclusions

Based on the results of this study, it is indicated that Omega-3 egg yolk has a better beneficial effect to protect membrane intact of ram sperm than regular or native hen egg yolk. However, these results are based on in vitro

evaluations, so further fertility trials are required.

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