



The protective effect of egg yolk from different avian species during the cryopreservation of Karayaka ram semen

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ABSTRACT

Egg yolk is one of the most widely used cryoprotective components for sperm preservation and a wide range of factors affect its action on sperm motility, viability and fertilizing ability. The aim of this experiment was to determine the effect of different species egg yolk, namely the domestic chicken (*Gallus gallus domesticus*), the goose (*Anatidae anser*), turkey (*Meleagris gallopavo*), duck (*Anatidae anas platyrhynchos*), Japanese quail (*Coturnix japonica*) and chucker (*Alectoris chukar*) on sperm quality following cryopreservation of ram semen. Ejaculates were collected using the artificial vagina from three Karayaka rams and spermatological characteristics assessed for the pooled semen. Semen samples were evaluated as split ejaculates in the trial and samples extended with a Tris-citric acid-glucose extender containing the different avian egg yolk (15%) and glycerol (5%). The semen straws were equilibrated at 4 °C for 2 h, frozen in liquid nitrogen vapour (for 15 min at –120 °C) and stored in liquid nitrogen (–196 °C). After thawing (37 °C for 30 s), sperm motility, viability, abnormal acrosome and membrane integrity (HOST) were evaluated. Results showed chucker egg yolk to have the best cryoprotective effect in terms of the highest sperm motility (54.0%), compared to the other five avian egg yolks ($p < 0.05$) evaluated. Sperm frozen in chucker egg yolk also showed a higher percentage viability (59%), than sperm stored in quail and turkey egg yolk ($p < 0.05$). The percentage of acrosomal abnormalities after thawing was lower in the chucker egg yolk, than the other species ($p < 0.05$). There was no significant difference in sperm membrane integrity between the egg yolks, except for the quail ($p < 0.05$). Results suggest that chucker egg yolk could be used as an alternative for chicken egg yolk, in a semen extender in cryopreservation, but it warrants further evaluation in fertility trials.

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1. Introduction

Sheep are one of the most important domestic farm animals in Turkey, and amount to 25 million head. The Karayaka, a native sheep breed of Turkey, raised in the Black Sea region makes up approximately 3% of the sheep population in Turkey. The Karayaka breed is renowned for mutton production and meat quality, and is also well adapted to the warm and humid irrigated north semi-arid

regions of Turkey (Ozcan, 1990; Olfaz, 1997; Kaymakci et al., 2001). However, this breed is currently in risk of extinction. In this context, the cryopreservation of gametes would enable producers to keep frozen sperm of the native species in a gene bank – such that it could be used for subsequent AI's over extended periods of time. Cryopreservation as a technique for the storage of ram semen has many advantages, but the process of freezing and thawing induces certain detrimental effects, in terms of sperm structure, biochemical and functional damage – resulting in a reduction of sperm motility, membrane integrity and fertilizing ability (Salomon and Maxwell, 2000; Tekin et al., 2006).

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Table 1

Mean (\pm S.E.) sperm motility, viability, total abnormality, acrosome abnormality and integrity rate in Karayaka ram semen frozen using different avian species egg yolk ($n=7$).

Egg yolk	Motility (%)	Viability (%)	Total abnormality (%)	Acrosomal abnormality (%)	Membrane integrity (%)
Chucker	54 \pm 2.4a	59 \pm 2.4a	35.8 \pm 1.2a	31.6 \pm 0.8a	57.0 \pm 3.5ab
Duck	47 \pm 2.5ac	58 \pm 2.6a	45.2 \pm 2.4bc	40.8 \pm 2.5bc	49.2 \pm 5.0bc
Goose	42 \pm 1.3bc	53 \pm 2.6ab	37.4 \pm 1.3ac	35.4 \pm 1.2ac	45.0 \pm 1.8bc
Turkey	38 \pm 2.6bc	45 \pm 2.4bc	45.2 \pm 1.5bc	42.2 \pm 1.5bc	42.4 \pm 2.4bd
Chicken	35 \pm 1.6bd	50 \pm 3.4ab	51.0 \pm 2.4b	47.4 \pm 2.3b	44.0 \pm 3.3d
Quail	26 \pm 1.9d	36 \pm 3.6c	47.2 \pm 3.1b	43.2 \pm 2.9bc	30.8 \pm 2.1bd

Groups with different letters (a, b, c and d) in the same column are significantly different ($p < 0.05$).

Currently, egg yolk is a common component of most semen cryopreservation extenders for domestic animals. It has been shown to have a beneficial effect on sperm cryopreservation – as a protectant 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 density lipoproteins in egg yolk may be factors that provide protection to sperm against cold shock during the freeze–thaw process. The exact mechanism by which egg yolk helps preserve ram sperm during the freeze–thaw process is however unknown.

There have also been numerous reports that egg yolk from avian species such as the duck, quail, pigeon or chicken have different combinations of fatty acids, phospholipids and cholesterol, which could result in different cryopreservation effects on the sperm (Trimeche et al., 1997; Choi et al., 2001; Andrabi et al., 2007; Bahtgate et al., 2006; Humes and Webb, 2006; Clulow et al., 2007; Moreno et al., 2008; Su et al., 2008). There have however not been many reports comparing the effect of egg yolk of different species (domestic chicken, goose, turkey, duck, Japanese quail and chucker) in the extender on the efficiency of cryopreservation of ram sperm.

The objective of this study was thus to investigate the effect of egg yolk from different avian species on the freeze–thaw sperm quality of ram semen.

2. Materials and methods

2.1. Animals and semen collection

The three rams were housed at the Education Research and Practice Farm, Faculty of Veterinary Medicine, University of Ankara, Turkey at 39°57'N, 32°53'E, at an altitude of 850m. The rams (65–70 kg) were kept under natural light and maintained under a uniform and constant nutritional regime, with each ram being fed a daily diet of 0.9 kg concentrate, dried grass, salt lick and water ad libitum. Semen samples were obtained from the three mature Karayaka rams (aged 2–3 years) and a total of 21 ejaculates collected from the rams, with the aid of an artificial vagina, twice during the non-breeding season (April–May; spring).

2.2. Semen dilution, cryopreservation and thawing

Only ejaculates of between 1 and 2 mL in volume, sperm with >80% progressive motility and an ejaculate concentration of higher than 3×10^9 sperm/mL, were pooled. This was done for uniformity and minimise individual semen differences. In total seven pooled ejaculates were included in the study. A Tris-based extender (375 mM Tris, 124 mM citric acid, 41 mM glucose, pH 7.0) was used as the basic semen diluent (Evans and Maxwell, 1987). The pooled semen was diluted to a final concentration of 400×10^6 sperm/mL, with the extender containing 5% glycerol and 15% egg yolk from each of the six different avian species i.e the domes-

tic chicken (*Gallus gallus domesticus*), goose (*Anatidae anser*), turkey (*Meleagris gallopavo*), duck (*Anatidae anas platyrhynchos*), Japanese quail (*Coturnix japonica*) and chucker (*Alectoris chukar*), thus resulting in six different extenders.

Following dilution, the sperm was drawn into 0.25 mL plastic straws (IMV, Laigle, F-61300, France) and sealed with polyvinyl alcohol (PVA). Straws were equilibrated at 4 °C for 2 h and after equilibration, the straws were suspended on a styrofoam rack 4 cm above the liquid nitrogen (vapour) for 15 min. The straws were then plunged into the liquid nitrogen, where stored until thawing. After storage for a period of 2 months, the semen straws were thawed in a water bath (37 °C for 30 s) for microscopic semen evaluation immediately after thawing.

2.3. Semen evaluation

Sperm motility was assessed using a phase-contrast microscope (400 \times magnification) (Olympus BH-2, Olympus Optical Co. Ltd., Japan), with a warm stage maintained at 37 °C. A wet semen mount was made using 5 μ L semen placed directly on a microscope slide and covered by a cover slip. For each sample, at least five microscopic fields were examined by three trained observers. The mean of the three successive evaluations was recorded as the final motility score (Ax et al., 2000).

The viability of sperm in the sample was assessed by means of an eosin–nigrosin stain (Evans and Maxwell, 1987). The sperm smears were prepared by mixing a drop of semen with two drops of stain on a warm slide and spreading the stain immediately with the aid of a second slide. The viability was assessed by counting 200 sperm cells with bright-field microscopy (400 \times) (Olympus CX21FS1, Olympus Optical Co. Ltd., Japan). Sperm showing partial or complete colorization were considered non-viable or dead. Only sperm showing strict exclusion of the stain were considered to be alive (Chauhan and Anand, 1990).

For the assessment of acrosomal and sperm abnormalities, at least three drops of each sample were added to an Eppendorf container containing 1 mL Hancock solution (62.5 mL formalin (37%), 150 mL saline solution, 150 mL buffer solution and 500 mL double-distilled water) (Schafer and Holzmann, 2000). One drop of this semen mixture was put on a slide and covered with a cover slip. The percentage of the acrosomal and sperm abnormalities was determined by counting a total of 200 sperm cells under phase-contrast, using an immersion objective (Olympus BH-2, Olympus Optical Co. Ltd., Japan).

The hypo-osmotic swelling test (HOST) was used to evaluate the functional integrity of the sperm membrane, based on curled and swollen tails. This was performed by incubating 10 μ L semen in 100 μ L semen of a 100 mOsm hypo-osmotic solution (fructose and sodium citrate) at 37 °C for 30 min. After incubation, 0.1 mL of the mixture was spread with a cover slip on a warm slide. Sperm were evaluated using bright-field microscopy (Olympus CX21FS1, Olympus Optical Co. Ltd., Japan) and all sperm with swollen or coiled tails were recorded (Revell and Mrode, 1994).

2.4. Statistical analysis

The study had seven replications. Data were expressed as the mean \pm standard error of the mean (SEM). One-way analysis of variance (ANOVA) with a subsequent Tukey's test was used to compare the mean values resulting from the various treatments at a significance level of $p < 0.05$. All analyses were carried out using the SPSS 11 for Windows statistical software package.

3. Results

The one-way analysis of variance indicated differences to be significant between egg yolks regarding sperm motility, viability, abnormality and membrane integrity (Table 1). According to the results, chucker egg yolk had the best cryoprotective effect in terms of the highest sperm motility (54.0%), compared to the other five avian egg yolks ($p < 0.05$) evaluated. Sperm frozen in chucker egg yolk recorded a higher viability rate (59%) than sperm preserved in quail and turkey egg yolk diluent ($p < 0.05$). The percentage of acrosomal abnormalities after thawing was lower in chucker egg yolk, than the others ($p < 0.05$). There was no significant difference in the membrane integrity between the egg yolks, except for the quail ($p < 0.05$). Sperm extended in chicken egg yolk recorded lower percentages regarding sperm motility, viability, acrosomal abnormalities and membrane integrity, except for quail yolk ($p < 0.05$).

4. Discussion

Limited data are available for ram semen cryopreservation using different egg yolk sources. Egg yolk is the one of the most commonly used components of cryoprotectants utilised during the freeze–thawing process. The beneficial effect of egg yolk in the cryopreservation of sperm can be attributed to a resistance factor, which helps to protect the sperm against cold shock, and a storage factor, which helps to maintain viability. The phospholipid, cholesterol and the low density lipoprotein content of chicken egg yolk specifically have been identified as the protective components (Pace and Graham, 1974; Watson, 1976; Foulkes, 1977).

Egg yolk of other bird species have successfully been used as an additive for the cryopreservation of sperm in certain species, especially equine (Clulow et al., 2007).

The results of the present study are in agreement with other studies. The most important finding of the present study was that semen frozen in the extender containing 15% chucker egg yolk recorded higher sperm quality than semen frozen in the other egg yolks. Similar results have been reported by Humes and Webb (2006), who found chucker egg yolk to improve the percentage of motile stallion sperm after the freeze–thaw process, when compared to chicken egg yolk. This may be attributed to the higher levels of protein, lipid and cholesterol present in the chucker egg. These components have been demonstrated to actively protect sperm during the various stages of the cryopreservation process (Prasard et al., 1988; Maurice et al., 1994). The higher levels of these components present in the chucker egg yolk may improve the protection of the sperm during the freeze–thaw processes – resulting in higher progressive sperm motility after thawing. Trimeche et al. (1997) found that after the freeze–thawing process of donkey sperm, quail egg yolk improved the percentage of motile sperm, compared to chicken yolk. Contrary, Moreno et al. (2008) reported quail egg yolk in the diluent to have no advantage over chicken egg yolk in the cryopreservation of Spanish ibex epididymal sperm. However in the current study, quail egg yolk gave the worse sperm

motility and viability results post thawing. The discrepancies in the present results may also be related to the freezing method used and specie-specific factors. A relationship generally exists between the fatty acid ratio of the phospholipids of sperm and their susceptibility to cold shock (Poulos et al., 1973). Similarly, ram and bull sperm, which have a high specific ratio, are more susceptible to cold shock than for example rabbit and human sperm, which have a lower fatty acid ratio (Darin-Bennett and White, 1977). The cholesterol in the membranes of sperm, which varies between species; also influences their susceptibility to cold shock. These differences in membrane composition and the components of different egg yolks may culminate in specie-specific interactions (Moreno et al., 2008).

Su et al. (2008) demonstrated pigeon egg yolk to have the best cryoprotective effect in terms of bull sperm progressive motility and viability between five avian egg yolks evaluated in the extenders. In the present study, pigeon egg yolk was not studied, but the constituents of pigeon and chucker egg yolk are said to be very similar (Bair and Marion, 1978). Previous studies reported the substitution of chicken with duck egg yolk to improve the post thawing motility in stallion and bull sperm. Burris and Webb (2009), reported that although the values were not significant, the inclusion of duck egg yolk in the diluent resulted in the second highest progressive sperm motility. The inclusion of turkey egg yolk provided a higher ($p < 0.05$) post thawing progressive sperm motility than any of the three extenders – which included chicken egg yolk. Andrabi et al. (2007) and Clulow et al. (2007) found duck egg yolk to compare favourably with other avian egg yolks in extenders used to improve the frozen–thawed quality of buffalo bull and stallion sperm. Similarly, duck egg yolk provided better sperm quality in terms of motility, viability, abnormal sperm and membrane integrity than other avian egg yolks – except chucker egg yolk in the present study. This may be attributed to the higher levels of protein, lipid and cholesterol present in the duck, compared to chicken egg yolk (Choi et al., 2001). Similarly, the lipid profile of chucker egg yolk may have affected the current results.

The present trial shows that different avian egg yolks (chicken, goose, and duck) do not have different cryoprotective actions on ram sperm cryopreservation; This is in accordance with the study of Su et al. (2008), who showed no differences between the same avian egg yolks. In the current study however, acrosome abnormalities in sperm frozen using goose egg yolk were lower than in the others. Therefore goose egg yolk may be more acceptable than chicken and duck egg yolks.

The inclusion of turkey egg yolk produced a higher post-thaw progressive sperm motility than any of the three extenders – which included chicken egg yolk (Burris and Webb, 2009). The analysis of the fat content and fatty acid profile of the various egg yolks used in this experiment did not help in the explanation of observed treatment differences. Turkey contained the highest levels of cholesterol and in contrast, the data of the present study demonstrated turkey egg yolk to have no advantages.

5. Conclusion

Based on the results of this study, it is recommended that ram sperm should be cryopreserved using a Tris-based extender containing chucker egg yolk, instead of chicken egg yolk. This conclusion, however, is based only on sperm characteristics, and ultimately, a full fertility trial will be necessary to confirm the beneficial effects of the inclusion of chucker egg yolk in ram semen cryopreservation protocols.

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