Effect of egg yolk of four avian species on the cryopreserved ram spermatozoa

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(Received 10 Apr 2011; revised version 28 Jun 2011; accepted 10 Jul 2011)

Summary

Egg yolk is the most commonly used cryoprotective agent for sperm cryopreservation of several species. The objective of this experiment was to compare the effectiveness of egg yolk from four avian species (domestic chicken, duck, turkey and pigeon) on sperm quality following cryopreservation of the ram semen. Ejaculates were collected, using an electroejaculator, from six fertile rams. Samples were diluted in a tris-citric acid-fructose extender containing egg yolk (15%) and glycerol (5%). Diluted samples were cooled slowly to 5°C over 2 h and equilibrated at that temperature for 2 h. Aliquots of samples were loaded into 0.5 ml straws and frozen in liquid nitrogen vapor for 15 min and stored in liquid nitrogen. Post-thaw progressive motility, live/dead ratio and acrosomal integrity of 200 sperm per slide stained with eosin-nigrosin and giemsa, were evaluated at 0, 2 and 4 h after thawing. Pigeon egg yolk had the most cryoprotective effect in terms of progressive motility, livability and acrosomal integrity ($P<0.05$). There was a significant decrease ($P<0.05$) in progressive motility, livability and acrosomal integrity up to 4 h after thawing. The results indicated that pigeon egg yolk might be superior to chicken egg yolk for cryopreservation of ram spermatozoa in tris-citric acid-fructose yolk extender, however, further experiments are needed to evaluate its effects on fertility.

Key words: Ram sperm, Cryopreservation, Egg yolk, Avian

Introduction

Despite considerable research for optimizing the cryopreservation of ram sperm, it still remains a highly damaging procedure, and deposition of sperm close to the site of fertilization via laparoscopic insemination is the only reliable method to achieve acceptable fertility. However, this technology is not economical in many countries, and there is a need for further improvement of this technology (Leahy *et al*., 2010).

Freezing mammalian spermatozoa offers many advantages to the livestock industry, particularly in conjunction with genetic evaluation and genetic selection, such as in sire reference schemes (Maxwell, 1984), but the processes of freezing and thawing result in certain detrimental effects to the spermatozoa. Cold shock, osmotic stress, ice crystal formation or oxidative damage has been shown to be the main causes of sperm cryoinjury, and loss of sperm viability and fertility (Alvarez and Storey, 1992; Amirat *et al*., 2004). An important constituent of cooling and freezing media for cryopreservation of sperm of several species, including the ram, is the chicken egg yolk. 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 phospholipids 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).

Traditionally, chicken egg yolk has been used as an additive for the freeze preservation of spermatozoa because of its wide availability (Bathgate *et al*., 2006). However, the chemical composition of the

egg yolks of different avian species varies, particularly in terms of the cholesterol, fatty acid and phospholipid contents (Bair and Marion, 1978; Burris and Webb, 2009), which may influence their effectiveness during cooling, freezing, and thawing steps (Bathgate *et al*., 2006). Recently, it has been shown that egg yolk from avian species, other than the chicken, might be more beneficial for cryopreservation of sperm from stallion (Clulow *et al*., 2007; Burris and Webb, 2009), jackass (Trimeche *et al*., 1997), bull (Su *et al*., 2008), and ram (Kulaksiz *et al*., 2010). Further, comparative studies are warranted, especially for the ram spermatozoa for which the only study is that of Kulaksiz *et al*. (2010) which was published during the course of the present investigation. The following experiment was conducted to assess the post-thaw motility, viability and acrosomal integrity after freezing and thawing of sperm of a fat-tailed sheep breed in the presence of the chicken, duck, turkey or pigeon egg yolks.

Materials and Methods

Six fertile fat-tailed Ghezel rams were housed at the Animal Science Farm, College of Agriculture, Shiraz University, Iran. Semen samples were collected by using an electroejaculator. Progressive motility was assessed subjectively under a Zeiss microscope (×400) equipped with a warm stage. Only samples containing more than 80% motile sperm were used. Semen samples were pooled in order to obtain sufficient sperm for the experiment and also to eliminate the ram effect. A tris-based extender [Tris 3.07 g/100 ml, citric acid 1.64 g/100 ml, fructose 1.26 g/100 ml, glycerol 5% (v/v), pH = 6.8) and the antibiotics streptomycin (1 mg/ml) and sodium penicillin G (100 IU per ml)] was used as the basic extender (Kumar *et al*., 2003).

The pooled semen was diluted to a final concentration of 400×10^6 sperm/ml, with the extender containing 15% egg yolk from each of the four avian species i.e. the domestic chicken (*Gallus gallus domesticus*), turkey (*Meleagris gallopavo*), duck (*Anatidae anas platyrhynchos*), pigeon (*Columba livia*), thus resulting in four different extenders. Semen was diluted and kept in a refrigerator at 5°C. Cooling to 5°C took about 2 h; and the suspension was then maintained at this temperature for a further 2 h. At this point, aliquots of samples were loaded into 0.5 ml polyvinyl French straws and frozen by placing them at about 8 cm above the surface of liquid nitrogen for 15 min before plunging them into the liquid nitrogen. After 48 h the frozen samples were thawed in a water bath at 37°C for 40 s. The contents were then poured into a glass tube, and the sperm quality variables assessed.

Progressive motility, percentage of live or dead sperm and percentage of sperm with intact or damaged acrosomes were determined immediately after thawing, and at 2 and 4 h after thawing.

Progressive motility was assessed subjectively according to the method of Evans and Maxwell (1987). Stained sperm smear was prepared in duplicate, by using eosin-nigrosin-giemsa staining (Tamuli and Watson, 1994) and 200 sperm per slide were evaluated. Briefly, the semen and eosin-nigrosin stain were mixed in the proportions 1:3 for 30 s before preparing a smear and dried quickly by blowing warm air over the slide. The dried slides were fixed in 6% formalin for 10 min. The slides were rinsed in slow running water for 7-10 min, and were then finally rinsed once with distilled water and immersed in 10% giesma stain-phosphate buffer solution for 3 h. The slides were dipped in flowing tap water and after rinsing with distilled water, dried in air. The sperm cells were observed under a bright-field microscope (×1000) and categorized into live acrosome-intact, live acrosome-reacted (damaged), dead acrosome-intact, and dead acrosome-reacted groups (Tamuli and Watson, 1994). The giemsa stain, formalin and antibiotics were purchased from local manufacturers (Iran) and other chemicals from the Merck Co. (Germany).

The procedure was replicated three times and their mean values were used for data analysis. The percentage data were transformed into arcsine, but the back-transformed values are reported here. Data on progressive motility were subjected to repeated measures ANOVA using Proc Mixed of the SAS (2002), and other data were analysed by the GLM procedure. Means were compared by the Duncan's

multiple range test (P<0.05).

Results

Significant effects of yolk source, time and their interaction were noted for the parameters investigated (P<0.05). There was a gradual decrease in the percentage of progressively motile sperm (Fig. 1), sperm viability (Fig. 2) and intact acrosome with time (Fig. 3). Pigeon egg yolk had the best cryoprotective effect in terms of the sperm progressive motility, compared with the yolk from other species.

Sperm frozen in the presence of the pigeon egg yolk showed the highest viability rate (%) at 0, 2 and 4 h (P<0.05). Post-thaw sperm viability was higher in the diluents containing turkey or duck egg yolks, compared with the chicken egg yolk. The percentage of spermatozoa with intact acrosome at 0, 2 and 4 h post-thaw was significantly influenced by the yolk source, and was higher for the pigeon egg yolk.

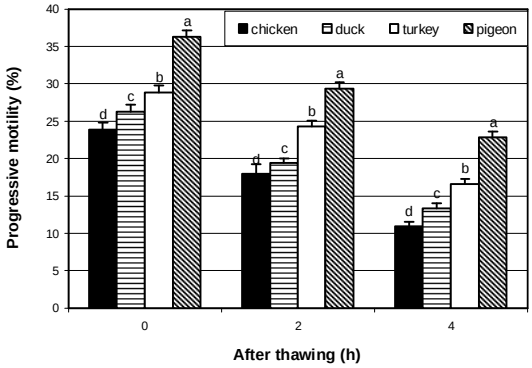

Fig. 1: Effect of yolks of four avian species in the freezing medium on progressive motility of ram sperm at 0, 2 and 4 h post-thaw (mean ± SEM). a, b, c, d: Means with similar letters are not significantly different between yolk sources at a given time (Duncan's multiple range test; P>0.05)

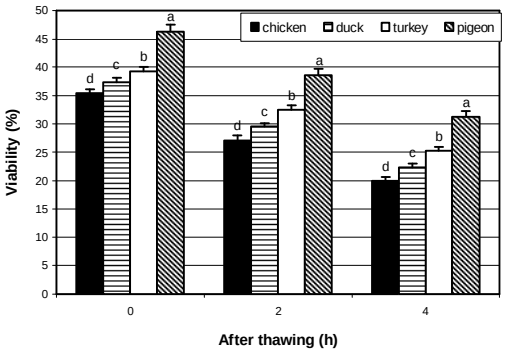

Fig. 2: Effect of yolks of four avian species in the freezing medium on viability of ram sperm at 0, 2 and 4 h post-thaw (mean ± SEM). a, b, c, d: Means with similar letters are not significantly different between yolk sources at a given time (Duncan's multiple range test; P>0.05)

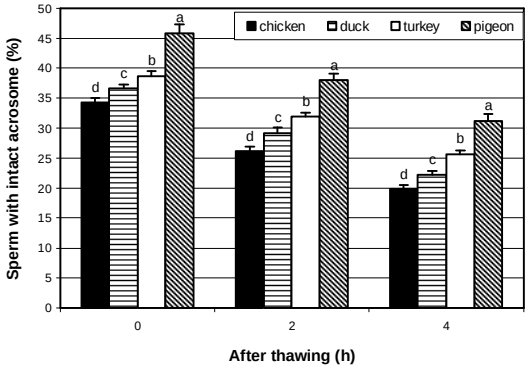

Fig. 3: Effect of yolks of four avian species in the freezing medium on acrosomal integrity of ram sperm at 0, 2 and 4 h post-thaw (mean ± SEM). a, b, c, d: Means with similar letters are not significantly different between yolk sources at a given time (Duncan's multiple range test; P>0.05)

Discussion

The chicken egg yolk is usually included in the freezing extenders for ram spermatozoa. The livability at thawing of spermatozoa in the extender containing chicken yolk in the present experiment was within the range of values reported by other investigators (Bucak *et al*., 2009; da Silva Maia *et al*., 2009; Jafaroghli *et al*., 2010; Kulaksiz *et al*., 2010; Leahy *et al*., 2010; Tonieto *et al*., 2010).

Very little comparative information is available on the effect of the avian egg yolk, other than chicken egg yolk, on freezability of the ram sperm (Kulaksiz *et al*., 2010). The present work showed that replacing the chicken egg yolk in the cryopreservation medium with the duck, turkey or pigeon egg yolk improved the post-thaw motility, viability and acrosomal integrity of the ram sperm. The improvement or decline in post-thaw motility/quality of mammalian spermatozoa with egg yolk of different avian species in the freezing extender may be

attributed to the differences in the biochemical composition of the yolks (Trimeche *et al*., 1997; Surai *et al*., 1999; Bathgate *et al*., 2006).

In the present study, progressive motility, viability and acrosomal integrity, evaluated at 0, 2 or 4 h after thawing were significantly better in the presence of the pigeon egg yolk as compared to the duck, turkey or chicken egg yolk. Su *et al*. (2008) reported that substitution of pigeon egg yolk with egg yolk of other avian species in the diluent improved the freezability of bull sperm. Recently, Kulaksiz *et al*. (2010) reported that chukar egg yolk had the highest cryoprotective effect in terms of ram sperm motility and viability, among six avian egg yolks. It was also reported that substitution of chukar egg yolk for chicken yolk improved the total motility, progressive motility and straight-line velocity in frozen-thawed stallion semen (Humes and Webb, 2006). The constituents of chukar and pigeon egg yolk are similar (Bair and Marion, 1978). The higher levels of cholesterol, lipid and protein present in the chucker egg yolk may confer more protection during the freeze-thaw processes; resulting in higher sperm progressive motility and viability, or acrosomal integrity.

The turkey egg yolk was the second best in terms of progressive sperm motility, viability and acrosomal integrity. Similar results were reported by Humes and Webb (2006), who showed that inclusion of turkey egg yolk produced a higher post-thaw progressive sperm motility compared to egg yolks from duck eggs, omega-3 chicken eggs, and chicken eggs. The high level of cholesterol in the turkey yolk (Kulaksiz *et al*., 2010) could be beneficial, as Purdy and Graham (2004) reported that addition of cholesterol to the bull sperm resulted in better post-thaw sperm quality. Our experiment also showed that the inclusion of duck egg yolk provided a higher post-thaw progressive sperm motility, viability or acrosomal integrity than chicken egg yolk.

The differences in post-thaw progressive motility, viability and acrosomal integrity of ram sperm when frozen in diluents containing the egg yolk of different avian species may be due to the variation in the composition of the yolks.

Pigeon egg yolk compared to yolks of chicken, duck and turkey improved the frozen-thawed quality of the ram sperm. Future studies should be aimed at determining the optimal concentration of pigeon egg yolk in the freezing medium. Studies are also needed to compare the fertility of artificially-inseminated sperm frozen in the diluent containing pigeon egg yolk. Studies are also needed to determine those yolk components, which can help, improve the livability of frozen ram sperm.

References

Alvarez, JG and Storey, BT (1992). Evidence for increased lipid peroxidative damage and loss of superoxide dismutase activity as a mode of sublethal cryodamage to human sperm during cryopreservation. J. Androl., 13: 232-241.

Amirat, L; Tainturier, D; Jeanneau, L; Thorin, C; Gérard, O; Courtens, JL and Anton, M (2004). Bull semen *in vitro* fertility after cryopreservation using egg yolk LDL: a comparison with Optidyl®, a commercial egg yolk extender. Theriogenology. 61: 895-907.

Bair, CW and Marion, WW (1978). Yolk cholesterol in eggs from various avian species. Poult. Sci., 57: 1260-1265.

Bathgate, R; Maxwell, WMC and Evans, G (2006). Studies on the effect of supplementing boar semen cryopreservation media with different avian egg yolk types on *in vitro* post-thaw sperm quality. Reprod. Domest. Anim., 41: 68-73.

Bucak, MN; Tuncer, PB; Sarıozkan, S and Ulutas, PA (2009). Comparison of the effects of glutamine and an amino acid solution on post-thawed ram sperm parameters, lipid peroxidation and anti-oxidant activities. Small Rumin. Res., 81: 13-17.

Burris, C and Webb, G (2009). Effects of egg yolk source on the cryopreservation of stallion semen. J. Equine Vet. Sci., 29: 336-337.

Clulow, JR; Maxwell, WMC; Evans, G and Morris, LHA (2007). A comparison of duck and chicken egg yolk for the cryopreservation of stallion sperm. Aust. Vet. J., 85: 232-235.

da Silva Maia, M; Dimas Bicudo, S; Costa Azevedo, H; Cecilia Sicherle, C; Bartoli de Sousa, D and Rodello, L (2009). Motility and viability of ram sperm cryopreserved in a tris-egg yolk extender supplemented with anti-oxidants. Small Rumin. Res., 85: 85-90.

Evans, G and Maxwell, WMC (1987). *Salamon's artificial insemination of sheep and goats*.

Sydney, Butterworths. PP: 8-21, 107-141.

Humes, R and Webb, G (2006). Use of chicken or chukar egg yolk with two cryoprotectants for preservation of stallion semen. Anim. Reprod. Sci., 94: 62-63.

Jafaroghli, M; Khalili, B; Farshad, A and Zamiri, MJ (2010). The effect of supplementation of cryopreservation diluents with sugars on the post-thawing fertility of ram semen. Small Rumin. Res., 96: 58-63.

Kulaksiz, R; Cebi, C; Akcay, E and Daskin, A (2010). The protective effect of egg yolk from different avian species during the cryopreservation of Karayaka ram semen. Small Rumin. Res., 88: 12-15.

Kumar, S; Millar, JD and Watson, PF (2003). The effect of cooling rate on the survival of cryopreserved bull, ram, and boar spermatozoa: a comparison of two controlled-rate cooling machines. Cryobiology. 46: 246-253.

Leahy, T; Marti, JI; Mendoza, N; Pérez-Pé, R; Muiño-Blanco, T; Cebrián-Pérez, JA; Evans, G and Maxwell, WMC (2010). High pre-freezing dilution improves post-thaw function of ram spermatozoa. Anim. Reprod. Sci., 119: 137-146.

Maxwell, WMC (1984). Current problems and future potential of artificial insemination programmes. In: Lindsay, DR and Pearce, DT (Eds.), *Reproduction in sheep*. Canberra, Australian Academy of Science and Australian Wool Corporation. PP. 291-298.

Pace, MM and Graham, EF (1974). Components in egg yolk which protect bovine spermatozoa during freezing. J. Anim. Sci., 39: 1144-1149.

Purdy, PH and Graham, JK (2004). Effect of adding cholesterol to bull sperm membranes on sperm capacitation, the acrosome reaction and fertility. Biol. Reprod., 71: 522-527.

SAS (2002). *SAS/STAT Software*: SAS (r) 9.1 (TS1 MO), Cary, NC.

Su, L; Li, X; Quan, X; Yang, S; Li, Y; He, X and Tang, X (2008). A comparison of the protective action of added egg yolks from five avian species to the cryopreservation of bull sperm. Anim. Reprod. Sci., 104: 212-219.

Surai, PF; Speake, BK; Noble, RC and Mezes, M (1999). Species-specific differences in the fatty acid profiles of the lipids of the yolk and of the liver of the chick. J. Sci. Food. Agric., 79: 733-736.

Tamuli, MK and Watson, PF (1994). Use of a simple staining technique to distinguish acrosomal change in the live sperm sub-population. Anim. Reprod. Sci., 35: 245-247.

Tonieto, RA; Goularte, KL; Gastal, GDA; Schiavon, RS; Deschamps, JC and Lucia Jr., T (2010). Cryoprotectant effect of trehalose and low-density lipoprotein in extenders for frozen ram semen. Small Rumin. Res., 93: 206-209.

Trimeche, A; Anton, M; Renard, P; Gandemer, G and Tainturier, D (1997). Quail egg yolk: a novel cryoprotectant for the freeze preservation of Poitou jackass sperm. Cryobiology. 34: 385-393.

Watson, PF (1976). The protection of ram and bull spermatozoa by the low density lipoprotein fraction of egg yolk during storage at 5°C and deep-freezing. J. Therm. Biol., 1: 137-141.