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Comparison of egg yolks from three avian species in extender for cryopreservation of Sahiwal bull epididymal spermatozoa*

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The present study was designed to compare egg yolks from three avian species in extender for cryopreservation of Sahiwal bull epididymal spermatozoa. The study was conducted on cauda epididymal spermatozoa from ten slaughtered Sahiwal bulls. Semen retrieved from epididymal cauda of each testes pair was pooled and diluted in tris-citric acid-glycerol extender containing either pigeon, guinea fowl or chicken egg yolk semi colon leading to a final spermatozoal concentration of 30×10^6 ml^{-1}. Diluted semen was transferred into 0.5 ml straws, cooled to 4°C and equilibrated for 4 h. The straws were kept on liquid nitrogen vapours for 10 min and then plunged into liquid nitrogen for storage. Motility, plasma membrane integrity and spermatozoa morphology (acrosome ridge; head, mid piece and tail) of each diluted semen sample were assessed at 0, 2 and 4 h post-thawing. All indicators applied and acrosomal integrity were the highest ($P<0.05$) after freezing in extender containing pigeon egg yolk compared to guinea fowl and chicken egg yolk 0, 2 and at 4 h post-thaw. Moreover, spermatozoa tail abnormality percentages were also significantly lower ($P<0.05$) in extender containing pigeon egg yolk compared to guinea fowl and chicken egg yolk at 0, 2 and 4 post-thaw h. In conclusion, pigeon egg yolk used in semen extender improved the post-thaw quality of Sahiwal bull epididymal spermatozoa.

KEY WORDS: bulls / cattle / cryopreservation / egg yolk / epididymal spermatozoa / extender / Sahiwal breed / semen /

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Crossing the indigenous cattle with exotic breeds to improve productive performance of crossbreds reduces populations of the former. Moreover, crossing produces animals of poor adaptability to the local environment [Joshi *et al*. 2001, Garcia *et al*. 2003]. One of the breeds native to South Asia and drastically decreasing in numbers due to such crossing [Dahlin *et al*. 1998] is Sahiwal [Joshi *et al*. 2001, Rehman *et al*. 2006].

Accidental death of a high genetic value Sahiwal bull required exploiting the suitable assisted reproductive technology for conservation of its germplasm. In this regard, cryopreservation of epididymal spermatozoa for future artificial insemination seems to be the best option [Andrabi *et al*. 2006]. Successful cryopreservation of the epididymal spermatozoa of Sahiwal bull needs suitable cryodiluent capable of protecting sperm from cold-shock [Ansari *et al*. 2010].

Traditionally, commercial hen egg yolk has been used in freezing media, probably because of its easy availability [Bathgate *et al.* 2006]. Trimeche *et al.* [1997] found better protection of Poitou Jackass sperm frozen in media containing quail egg yolk compared with chicken yolk. Cryopreservation of stallion semen with duck egg yolk, compared with chicken egg yolk resulted in significantly higher forward motility and livability at post-thaw [Clulow *et al.* 2004]. Substitution of chukar egg yolk for hen yolk improved total motility, progressive motility and straight-line velocity in frozen-thawed stallion semen [Humes and Webb 2006]. More recently, duck egg yolk supplementation in semen extender improved freezability of buffalo bull spermatozoa [Andrabi *et al.* 2008]. This requires screening of different avian egg yolk sources for the cryopreservation of Sahiwal bull epididymal spermatozoa to get optimum post-thaw quality. The present study was, therefore, designed to compare the protective effect of different avian egg yolk addition to extender on the cryopreservation of Sahiwal bull epididymal spermatozoa obtained post slaughtering.

Material and methods

Preparation of extenders

Tris-citric acid was used as a buffer for the experimental extenders. It consisted of 1.56 g citric acid (MERCK, Germany) and 3.0 g *tris*-(hydroxymethyl)-aminomethane (SIGMA, USA) in 73 ml distilled water. The buffer pH was 7.0 and the osmotic pressure 320 mOsmol Kg^{-1}. Fructose (0.2% wt/vol, RIEDEL-DeHAEN, Switzerland) and glycerol (7% vol/vol, MERCK, Germany) were added. Antibiotic combination; streptomycin sulphate - 1mg/ml, procaine penicillin - 300iu/ml, benzyl penicillin - 100 iu/ml available as Sinbiotic® (China) were added to the extender. Egg yolk either from pigeon, guinea fowl or chicken (control) was added at the rate of 20% (vol/vol).

To extract the yolk, cleaned eggs of different avian species were cracked in half and albumen was discarded. The remainder of egg white, which was still adhered to the yolk, was removed by slowly moving the yolk around a 12 cm diameter filter paper (Whatman no. 3). The intact egg yolk was then punctured and the internal yolk was removed and

allowed to flow in a sterile glass beaker. Approximately, 6 eggs of guinea fowl and domestic chicken were used to attain the desired level in the extender. Whereas, about 18 eggs of pigeon were required to make final volume of the extender.

Sampling, epididymal spermatozoa retrieval and initial evaluation

Pair of testes from 10 mature Sahiwal bulls of about 4 years, slaughtered at local abattoir were collected. The samples were transported in Styrofoam box (COOLMAN®, USA) at 25°C to the laboratory within 4 h after slaughtering for the collection of epididymal spermatozoa. Each pair was considered as a single set of experiment. The spermatozoa were obtained by slicing and squeezing the epididymis, in 15 ml plastic tube [Martins *et al*. 2007]. Visual motility was assessed microscopically (×400) with closed circuit television and spermatozoa concentration was assessed by Neubauer hemocytometer (Germany).

Processing of epididymal spermatozoa

Spermatozoa collected from testes of each pair were pooled, divided into three aliquots and diluted with one of the three experimental extenders (30×10^{-6} motile spermatozoa/ml. Diluted semen was packed in straws (0.5 ml), then cooled to 4°C and equilibrated for 4 hours. The straws were kept in liquid nitrogen vapours for 10 min and then plunged into liquid nitrogen (-196°C) for storage. After 24 h, the straws were thawed at 37°C for at least 30 s in water bath and then incubated for 4 hours for assessment of post-thaw semen quality.

Post-thawing spermatozoa functional assays

Epididymal spermatozoa quality was assessed at 0, 2 and 4 h post-thawing.

Visual motility. A 5 µl drop of thawed semen was placed on a warmed (37°C) glass slide and cover-slipped. Visual spermatozoa motility was recorded under phase contrast microscope (×400, 37°C) attached to a closed circuit television.

Plasma membrane integrity. Plasma membrane integrity of epididymal spermatozoa was assessed using supravital stain Trypan blue (0.4% in distilled water) at room temperature [Brito *et al*. 2003]. The test was performed by mixing 5 µl semen sample with equal volume of Trypan blue solution on slide, and air dried for 10 min. A total of 100 spermatozoa per experimental extender per bull were examined under phase contrast microscope (×400). Spermatozoa stained blue were categorized as with damaged plasma membrane, while clear spermatozoa – as with intact plasma membrane.

Acrosomal integrity. To assess the acrosomal intactness, 100 µl semen was fixed in 500 µl of 1% formal citrate (2.9 g tri-sodium citrate dihydrate and 1 ml of 37% solution of formaldehyde in 100 ml distilled water). Intactness of acrosome was indicated by normal apical ridge. Two hundred spermatozoa per experimental extender per bull were studied using phase contrast microscope (×1000) under oil immersion.

Morphologic abnormalities. Semen sample (100 µl), was fixed in 500 µl of 1% formal citrate. Two-hundred spermatozoa per extender per bull were examined using phase contrast microscope (×1000) under oil immersion. The following spermatozoa abnormalities were recorded: head abnormalities incl. micro and macro heads, detached heads and double heads; midpiece abnormalities incl. proximal droplet, distal droplet and abaxial attachment; tail abnormalities incl. tail coiled below the head, tail bent at midpiece, tail without head and double tail.

Statistical analysis

Results are presented as means ±SE. Effects of yolks of different species on spermatozoa quality parameters were analysed by the analysis of variance (ANOVA). When the F-ratio occurred significant ($P<0.05$), Tukey's Honestly Significant Difference was used to compare the treatment means [MINITAB® Release 12.22, 1998].

Results and discussion

Motility is a common indicator of spermatozoa quality, for that reason being used in the laboratory. The data on progressive motility of frozen thawed Sahiwal bull epididymal spermatozoa are presented in Figure 1. Spermatozoa motility (%) was highest ($P<0.05$) in extender containing pigeon egg yolk compared to guinea fowl and chicken egg yolk at 0, 2 and 4 h post-thaw. This is in accordance with Su *et al*. [2008] who reported motility of the Holstein bull ejaculated spermatozoa cryopreserved in

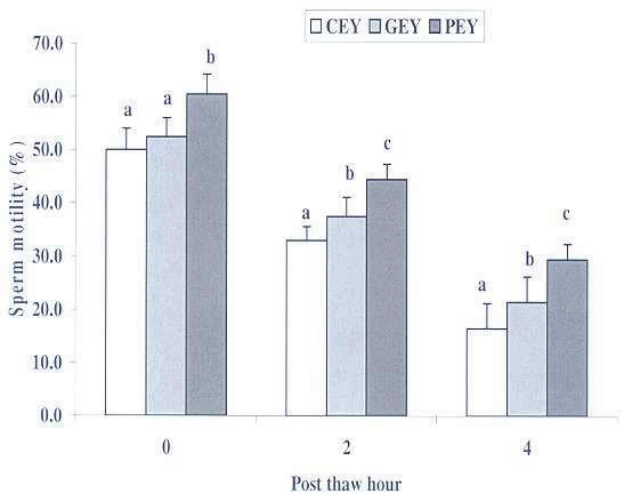

Fig. 1. Effect of chicken, guinea fowl or pigeon egg yolk (CEY, GEY and PEY, respectively) in extender on motility of Sahiwal bull epididymal spermatozoa at 0, 2 and 4 h post-thaw. Different letters above the bars show significant differences ($P<0.05$) between the treatments at a given time.

extender containing 20% pigeon egg yolk compared to chicken egg yolk. It is suggested that lower motility of spermatozoa frozen with chicken egg yolk-containing extender may be due to lower ratio of phosphatidylcholine and phosphatidylethanolamine and higher ratio of polyunsaturated fatty acids [Trimeche *et al*. 1998, Choi *et al*. 2001].

The process of capacitation, acrosome reaction and the oocyte penetration requires a biochemically active plasmalemma [Brito *et al*. 2003]. A supravital stain Trypan blue has been widely used to evaluate the plasma membrane integrity of the bovine spermatozoa. The data on plasma membrane integrity of frozen thawed Sahiwal bull epididymal spermatozoa are presented in Figure 2. Percentage of spermatozoa with intact plasma membrane was significantly ($P<0.05$) higher in semen extender containing pigeon egg yolk compared to guinea fowl and chicken egg yolk at 0, 2 and 4 h post-thaw. Our findings are in line with those of Su *et al*. [2008] who reported a significant increase in spermatozoa with intact plasma membrane after freezing of Holstein bull semen in extender containing pigeon egg yolk as compared to chicken, duck, goose and quail egg yolks. Our own results shown here of spermatozoa plasma membrane integrity are very much supported with the data from this study on motility of Sahiwal bull epididymal spermatozoa.

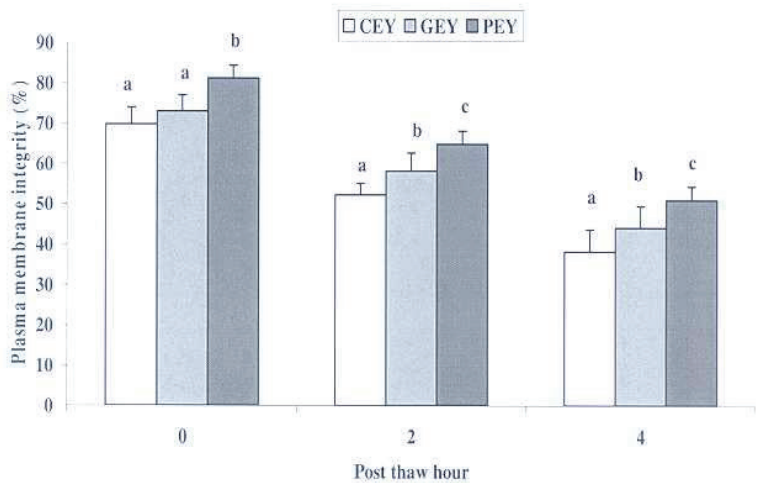

Fig. 2. Effect of chicken, guinea fowl or pigeon egg yolk (CEY, GEY and PEY, respectively) in extender on plasma membrane integrity of Sahiwal bull epididymal spermatozoa at 0, 2 and 4 h post-thaw. Different letters above the bars show significant differences ($P<0.05$) between the treatments at a given time.

Acrosomal intactness is mandatory for the acrosome reaction required at the proper time to facilitate fertilization [Thomas *et al*. 1997]. The change in acrosomal cap is mainly due to sperm aging or cryoinjury, which can be effectively determined by fixing the specimen using phase contrast microscopy [Ansari *et al*. 2010]. The data on normal apical ridge of frozen thawed Sahiwal bull epididymal spermatozoa are presented in Figure 3. Percentage of spermatozoa with intact acrosomes was

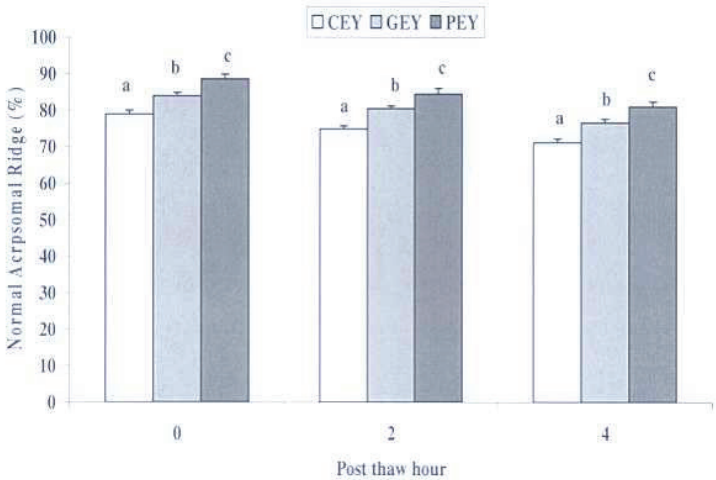

Fig. 3. Effect of chicken, guinea fowl or pigeon egg yolk (CEY, GEY and PEY, respectively) in extender on acrosomal integrity of Sahiwal bull epididymal spermatozoa at 0, 2 and 4 hour post-thaw. Different letters above the bars show significant differences ($P<0.05$) between the treatments at a given time.

significantly higher ($P<0.05$) after freezing in cryodiluter containing pigeon egg yolk compared to guinea fowl and chicken egg yolk at 0, 2 and 4 h post-thaw incubation. Some studies on different livestock species suggest that difference in egg yolk source [Choi *et al*. 2001] affects the percentage of spermatozoa with intact acrosomes after freezing [Andrabi *et al*., 2008; Bathgate *et al*. 2008].

Table 1. Effect of the use of three different avian egg yolks in extender on sperm abnormalities (%) in Sahiwal bull epididymal spermatozoa (head, mid piece, tail) at 0, 2 and 4 h post- thawing.

Abnormality (%)	Post-thaw hour	Egg yolk source		
		chicken	guinea fowl	pigeon
Head	0	0.7±0.5	0.6±0.5	0.7±0.5
	2	0.8±0.4	0.9±0.6	0.7±0.5
	4	1.1±0.7	1.0±0.7	1.0±0.7
Mid piece	0	6.0±0.6	5.9±0.6	6.0±0.6
	2	6.1±0.7	6.2±0.7	6.2±0.5
	4	6.5±6.0	6.4±0.5	6.5±0.5
Tail	0	12.1^{b}±2.1	12.0^{b}±1.6	10.0^{a}±1.3
	2	15.4^{b}±1.6	15.0^{b}±1.1	13.0^{a}±1.2
	4	23.7^{b}±2.2	19.9^{b}±1.6	17.9^{a}±1.5

^{abc}Within rows means bearing different superscripts are significantly different at $P<0.05$.

Evaluation of sperm abnormalities is one of the commonest methods to assess functional status of the semen [Rocha *et al*. 2006]. A significant correlation has been established between the fertilization ability of frozen-thawed bull semen and frequency of some spermatozoa abnormalities, especially spermatozoa head forms and the presence of proximal cytoplasmic droplets [Soderquist *et al*. 1991]. The data on the abnormalities of Sahiwal bull epididymal spermatozoa are given in Table 1. The mean content of tail abnormalities was significantly lower ($P<0.05$) when the semen was frozen with extender containing pigeon egg yolk compared to guinea fowl and chicken egg yolk at 0, 2 and 4 h post-thaw. Su *et al.* [2008] also reported a decrease in percentage of morphological abnormalities of Holstein bull ejaculated spermatozoa frozen in extender containing pigeon egg yolk.

It is well recognized that low density lipoprotein of egg yolk is one of the main factors responsible for the protection of spermatozoa during freezing through different mechanisms. Different proposed mechanisms through which egg yolk protects the spermatozoa include stabilizing the membrane, reducing the spermatozoon membrane phospholipids losses and grabbing the toxic seminal plasma protein [Manjunath *et al.* 2002, Bergeron and Manjunath 2006]. From the data presented in the current study it may be concluded that pigeon egg yolk additive in extender improves the post-thaw quality of Sahiwal bull epididymal spermatozoa by protecting plasma membrane in freeze-thawing process.

Anzar and Graham [1995] reported the strong relationship between fertilizing ability and post-thaw motility, intact plasma membrane, acrosome and spermatozoa abnormalities. As pigeon egg yolk supplement in extender improved the post-thaw spermatozoa motility, protected plasma membrane and reduced the damaged acrosome and spermatozoa abnormalities percentage of Sahiwal bull epididymal spermatozoa, it can be assumed that spermatozoa in question while cryopreserved in extender containing pigeon egg yolk have potentially higher fertilizing ability than when guinea fowl or chicken egg yolk is used.

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