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RESEARCH ARTICLE

Effects of Different Levels of Pigeon Egg Yolk in Extenders on the Post-Thaw Semen Quality of Sahiwal Bulls

Hafez Jamil-ur-Rahman, Nazir Ahmad*, Najib-ur-Rahman, Salman Waheed, Maqbool Ahmad, Muhammad Younis[1] and Tanveer Ahmad[2]

Department of Theriogenology, University of Agriculture, Faisalabad, Pakistan; [1]Semen Production Unit, Qadirabad, Sahiwal, Pakistan; [2]Department of Clinical Medicine & Surgery, University of Agriculture, Faisalabad, Pakistan
*Corresponding author: profnazir53@hotmail.com

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ABSTRACT

In this study, effects of replacing chicken egg yolk (CEY) with pigeon egg yolk (PEY) in extenders on post-thaw semen quality in Sahiwal bulls were investigated. Attempts were also made to see if post thaw semen quality was affected by reducing PEY level in the extender. Twenty four semen samples were diluted with five Tris-based extenders. Extender A contained 20% CEY and was used as control, while extenders B, C, D and E contained 5, 10, 15 and 20% PEY, respectively. After freezing and storage for 24 hrs in liquid nitrogen, these samples were evaluated for post-thaw semen quality parameters.

The difference in post extension sperm motility between extenders A (20% CEY) and E (20% PEY) was non significant. Post extension sperm motility decreased as the level of PEY in the extender was decreased. A similar trend was recorded for post thaw sperm motility, livability, absolute index of livability and sperm with intact plasma membrane. The percentages of spermatozoa with abnormal head, or tail were lower ($P<0.01$) in control extender A and extender E compared to extenders B, C and D. However, for abnormal mid-piece, extenders A and E showed lower values than extender C only. It was concluded that replacing CEY with PEY in same concentration (20%) did not improve post thaw semen quality. Moreover, reducing the concentration of PEY in semen extender from 20 to 5% had adverse effects on post-thaw quality of Sahiwal bull semen.

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INTRODUCTION

Livestock play an important role in the agricultural economy of Pakistan. They are the main source of milk, meat, hides and skins. Cattle are one of the most important farm animals in the country (Siddique *et al*., 2010). The gross production of milk in Pakistan is 46,440 tons, where the contribution of cows is 16,133 tons (Hameed *et al*., 2010; Anonymous, 2010-2011), which comes to be about 35% of the total milk produced in the country. However, the average daily milk yield of our dairy cows is much less compared to that for most of the exotic breeds. Besides other factors, low genetic potential of our dairy animals is an important factor blamed for their low productivity.

The improvement in the productive and reproductive performance of livestock can be brought about by two ways. Firstly, by improving the genetic make up and secondly, by improving feeding and management conditions of animals. The improvement brought through genetic improvement is slow but tends to be permanent when achieved. However, improvements achieved through better feeding and management are rapid but are temporary and animals return to their previous low producing state as soon as improvements of feeding and management are withdrawn.

Artificial insemination (AI) is the best available tool for rapid genetic improvement of livestock within the minimum possible time. However, for the success of this technique, it is necessary that spermatozoa are preserved for long periods without damaging their fertilizing ability. Preservation of semen in frozen form in liquid nitrogen has been routinely used, as the spermatozoa can be stored for long time and can even be transported to long distances.

Scientists have made different experiments for the improvement in fertility of frozen semen through different

dilution rates, different diluters and supplements together with use of various cooling rates, freezing and thawing protocols (Watson, 1995; Holt, 2000). However, the viability and fertility of frozen–thawed spermatozoa is generally low compared to the liquid semen, which seems to be due to sperm damage during freezing and thawing processes. The main causes of sperm injury during cryopreservation include: the oxidative damage, osmotic stress, ice crystal formation and cold shock resulting in the damage to spermatozoa viability and decrease in their fertilizing ability (Alvarez and Storey, 1992; Amirat *et al.*, 2004; Li *et al.*, 2005).

Now-a-days, egg yolk is commonly used in semen extenders for deep freezing. It protects the sperm plasma membrane and acrosome against the cold shock (Amirat *et al.*, 2004). Its main beneficial effect is thought to be because of its low-density lipoproteins (LDL), which stick to sperm plasma membrane during the cryopreservation process and stop the leakage of membrane phospho-lipids, enhancing their tolerance to cryopreservation (Parks and Graham, 1992).

Commonly, chicken egg yolk (CEY) has been used in semen extenders due to its easy and wide availability. Many scientists evaluated the use of yolk from eggs of various avian species other than that of chicken for cryopreservation of semen from various animal species and found improvements in the semen quality in the boar (Bathgate *et al.*, 2006), Piotou jackass (Trimeche *et al.*, 1997), stallion (Clulow *et al.*, 2007) and buffalo (Andrabi *et al.*, 2008). It has been hypothesized that replacement of yolk from chicken eggs with that from eggs of other birds in semen extender can improve post thaw quality of cattle bull spermatozoa. A 20% concentration of egg yolk is generally used in semen extender for bovines. However, Bispo *et al.* (2011) observed that low egg yolk concentration (2.5%) gave superior fertility results in goats compared to high concentration (20%). Therefore, this project was designed, i) to investigate if the replacement of CEY with that from pigeons in extenders can improve the post-thaw semen quality in Sahiwal bulls, and ii) to study if reducing the level of pigeon egg yolk (PEY) in extender affects the freezability results.

MATERIALS AND METHODS

Experimental animals: Three adult Sahiwal bulls with clinically normal reproductive tract and donating semen of acceptable quality, aged 4-6 years and kept at the Semen Production Unit (SPU), Qadirabad, District Sahiwal, Pakistan were used in this study. This region is situated at an altitude of about 173m and lies between longitudes 73° and 74°E and latitudes 30° and 30.15°N (Ahmad *et al*., 1981).

The experimental bulls were kept under naturally prevailing climatic conditions. For housing individually, North-South directionally situated pens having sufficient cross ventilation and protection against heat during summer and an open space for sun-bath in winter were used. Bulls were fed good quality seasonal green fodder at the rate of 10% of body weight. In addition, two to three kg of concentrate was offered per bull per day. Clean water was provided for 24 hours. Vaccination against Hemorrhagic Septicemia (HS) and Foot & Mouth Disease (FMD) was done as per schedule. Preventive measures against worm infestation were also undertaken.

Semen collection and initial evaluation: Semen from experimental bulls was collected once a week using an artificial vagina during April-May, 2011. Before collection, sufficient time was allowed for sexual preparation and 1-2 false mounts were allowed for proper sexual stimulation (Younis *et al.*, 1998). Two ejaculates were collected on each collection day from each bull. Immediately after collection, ejaculates for individual bull were pooled and evaluated for sperm motility and concentration. Samples with at least 60% progressively motile sperm were selected. In this way, a total of 24 pooled semen samples, with eight samples for each bull, were used for further processing.

Extension and freezing of semen: Five experimental extenders containing tris, citric acid, fructose, egg yolk, glycerol and antibiotics were prepared (Table 1). Extender A contained 20% CEY and was used as control, while different levels i.e. 5, 10, 15 and 20% of PEY were added into extenders B, C, D and E, respectively (Table 1).

Each pooled semen sample from each bull was divided into five portions and extended with one of the five experimental extenders by one step dilution method at 37°C (Younis *et al.*, 1998). After recording post-extension sperm motility, samples were cooled to 22°C, filled in 0.5 ml French straws and equilibrated for 4 hours at 4°C. Then samples were frozen in the liquid nitrogen first by holding the straws in vapors 5 cm above the surface of the liquid nitrogen for 8 minutes (Fiaz *et al.*, 2010). Then straws were dipped in the liquid nitrogen.

Post-thaw evaluation of semen: After 24 hrs of storage in liquid nitrogen, the straws were thawed for 30 seconds in a water bath at 37°C and evaluated for sperm motility, livability and absolute index of livability, plasma membrane integrity and morphology. In order to determine the livability of spermatozoa, the thawed semen samples were kept at 37°C and the motility was observed at hourly intervals, till the death of the last sperm. Absolute index of livability for each sample was computed as described by Younis *et al*. (1999).

The plasma membrane integrity of spermatozoa was assessed through hypo-osmotic swelling test (HOST), as described earlier (Jeyendran *et al.*, 1984). Briefly, 500 µL of hypo-osmotic solution was mixed with 50 µL of frozen-thawed semen and was incubated at 37°C for 1 hour. Then a drop of well mixed semen was placed on a glass slide and was covered with a cover slip. At least 200 spermatozoa were counted in different fields under phase contrast microscope. Sperm with swollen/coiled tail were regarded as having intact plasma membrane.

For determination of sperm with morphologically abnormal head, mid-piece or tail, frozen-thawed semen sample (100 µl) was fixed in 500 µl of 1% formal citrate (2.9g tri-sodium citrate dihydrate and 1 ml of 37% solution of formaldehyde, dissolved in 100 ml of distilled water). Morphological abnormalities of at least 200 spermatozoa were assessed using phase contrast microscope under oil immersion and percentages of spermatozoa with abnormal head, mid piece or tail were determined for each group.

Table 1: Composition of experimental extenders

Ingredients	Extender A (Control)	Extender B (5% PEY)	Extender C (10% PEY)	Extender D (15% PEY)	Extender E (20% PEY)
Tris (g)	2.42	2.42	2.42	2.42	2.42
Citric acid (g)	1.34	1.34	1.34	1.34	1.34
Fructose (g)	10.00	10.00	10.00	10.00	10.00
Chicken egg yolk (ml)	20.00	-	-		
Pigeon egg yolk (ml)	-	5.00	10.00	15.00	20.00
Glycerol (ml)	7	7	7	7	7
Distilled water (upto ml)	100	100	100	100	100
Penicillin (IU/ml)	1000	1000	1000	1000	1000
Streptomycin (µg/ml)	1000	1000	1000	1000	1000

Statistical analysis: Mean values (± SE) for various parameters of semen quality for five experimental extenders were calculated. In order to see the magnitude of variation in these parameters among different groups, the data were analyzed statistically through analysis of variance technique; following completely randomize design (Steel *et al.*, 1997). Duncan's Multiple Range Test was applied for multiple means comparison, where necessary.

RESULTS AND DISCUSSION

The first objective of the present study was to investigate the effects of replacing CEY with PEY on post-thaw semen quality in Sahiwal bulls. As shown in Table 2, the post extension sperm motility was higher in extender E containing 20% PEY compared to control extender A with 20% CEY (70.21±0.88% versus 69.58±0.67%), the difference was non significant. A similar trend was seen for post thaw sperm motility, where the values were 51.67±0.58 and 50.42±0.60%. Similarly, post thaw livability of sperm at 37ºC did not differ between control extender A and extender E having 20% PEY, although value was higher in extender E (5.08±0.15 hrs) than in extender A (5.00±0.14 hrs; Table 2). The same was true for post thaw absolute index of livability of spermatozoa at 37ºC. Hypo osmotic swelling test showed that percentage of sperm with intact plasma membrane was lower in extender E containing 20% PEY (87.57±0.38%) compared to control extender A (88.20±0.24%), the difference was non significant. Regarding sperm with abnormal heads and mid pieces, there was no difference between the two extenders A and E; although the mean values were lower in extender E than from control extender A (Table 2). However, extender E showed significantly ($P<0.05$) lower percentage of sperm with abnormal tails (8.58±0.22%) compared to extender A (11.38±0.24%). Thus, there was no difference in post thaw semen quality parameters between the two extenders, indicating that replacement of 20% CEY with 20% PEY in extender had no beneficial effects on most parameters of post thaw semen quality of Sahiwal bulls.

These results are partially supported by those of Santiago-Moreno *et al.* (2008), who concluded that quail egg yolk offers no advantages over CEY in the cryopreservation of Spanish ibex epididymal spermatozoa. However, results of the present study are not supported by those of Su *et al.* (2008) and Akhter *et al.* (2011), who stated that replacement of CEY with PEY in extender significantly improved post thaw sperm motility percentage in cattle bulls. Kulaksız *et al.* (2010) observed that chucker egg yolk had the best cryoprotective effect in terms of the highest post-thaw sperm motility (54.0%), compared to the other five avian egg yolks ($P<0.05$) including domestic chicken, goose, turkey, duck and Japanese quail. According to Andrabi *et al.* (2008), duck egg yolk in extenders improved the freezability of buffalo bull spermatozoa compared to CEY. Differences in the quality of post thaw semen diluted in extenders having egg yolks from different avian species were attributed to differences in the biochemical composition of yolks from different birds (Trimeche *et al.*, 1997; Bathgate *et al.*, 2006).

The second objective of the present study was to investigate if reduction in the concentration of PEY in extender affects the freezability results of Sahiwal bull semen. The results on post extension sperm motility (Table 2) revealed that the value was highest (70.21±0.88%) in extender E with 20% PEY and lowest (56.46±0.64%) in extender B having 5% PEY, the difference was significant ($P<0.05$). The post extension sperm motility decreased as PEY concentration in extender was decreased from 20 to 5%. An almost similar pattern was recorded for post thaw livability and absolute index of livability of spermatozoa at 37ºC. The same was true for percentage of sperm with intact plasma membrane as revealed by hypo osmotic swelling test. The post thaw sperm motility decreased significantly as the PEY level was decreased from 20 to 10%, with no further decrease when PEY was decreased from 10 to 5%. The percentage of sperm with abnormal heads or abnormal tails increased significantly with decrease in concentration of PEY in the extender. The percentage of sperm with abnormal mid piece increased as PEY in extender was decreased from 20 to 10%.

In general, the quality of frozen-thawed semen declined as the concentration of PEY in the extender was decreased. This indicates that reduction in the level of PEY in the extender adversely affected the post thaw semen quality of Sahiwal bulls. This effect was evident after dilution of the semen with the experimental extenders and persisted even after freezing and thawing. These results are in line with those reported by Su *et al.* (2008), who noted that the progressive motility and viability of the frozen-thawed bull spermatozoa was highest using 20% PEY in the extender when compared with concentrations of 5, 10, 30 and 40% PEY ($P<0.05$).

However, Bispo *et al.* (2011) evaluated low and high egg yolk concentrations and concluded that the use of glucose–EDTA extender with a low egg yolk concentration (2.5%) gave superior fertility results in goats compared to high concentration (20%). Dong and Vandevoort (2009) stated that the concentration of egg yolk (2–50%, vol/vol), the dilution method, and the delay

Table 2: Effects of different extenders on post extension and post-thaw quality parameters of Sahiwal bull spermatozoa

Semen Quality Parameters	Experimental Extenders				
	A (control; 20% CEY)	B (5% PEY)	C (10% PEY)	D (15% PEY)	E (20% PEY)
Post extension sperm motility (%)	69.58±0.67^{A}	56.46±0.64^{C}	58.75±0.86^{BC}	60.83±0.98^{B}	70.21±0.88^{A}
Post thaw sperm motility (%)	50.42±0.60^{A}	39.79±0.56^{BC}	37.92±1.08^{C}	41.04±0.60^{B}	51.67±0.58^{A}
Sperm livability (hrs)	5.00±0.14^{A}	3.17±0.13^{C}	3.63±0.10^{BC}	4.00±0.12^{B}	5.08±0.15^{A}
Absolute index of livability of sperm	101.77±2.84^{A}	60.88±0.79^{D}	64.79±0.97^{C}	70.40±0.78^{B}	103.23±1.71^{A}
Sperm with intact plasma membrane (%)	88.20±0.24^{A}	80.90±0.17^{D}	82.80±0.17^{C}	84.63±0.17^{B}	87.57±0.38^{A}
Sperm with abnormal heads (%)	6.50±0.22^{D}	10.37±0.20^{A}	9.67±0.22^{B}	8.58±0.22^{C}	6.42±0.18^{D}
Sperm with abnormal mid pieces (%)	1.50±0.10^{B}	1.79±0.12^{AB}	2.17±0.08^{A}	1.50±0.10^{B}	1.46±0.10^{B}
Sperm with abnormal tails (%)	11.38±0.24^{D}	16.04±0.42^{A}	14.17±0.54^{B}	12.13±0.26^{C}	8.58±0.22^{E}

Values with different letters in a row differ significantly from one another (P≤0.05);
CEY = Chicken egg yolk; PEY = Pigeon egg yolk

(1–5 hrs) in addition of egg yolk had non significant effect on post thaw motility of ejaculated Rhesus monkey sperm. In the present study, post-thaw semen quality of Sahiwal bulls was adversely affected with the decrease in the concentration of PEY in the extender. This could have been due to differences in osmotic pressure between extenders having lower concentrations of PEY and semen. This idea is supported by the fact that a significant decrease in sperm motility of semen diluted in extenders containing lower concentrations of PEY was observed immediately after extension. Unfortunately, the osmotic pressure of the experimental extenders used in the present study could not be recorded.

Conclusions: Based on the findings of the present study, it was concluded that replacing CEY with PEY in the same concentration (20%) did not improve post thaw quality of Sahiwal bull semen. Moreover, reducing the concentration of PEY in semen extender from 20 to 5% adversely affected the post thaw semen quality.

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