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Evaluation of pigeon egg yolk for post thaw quality, enzyme leakage and fertility of buffalo (*Bubalus bubalis*) bull spermatozoa

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Running Title: Pigeon egg yolk for cryopreservation of buffalo semen

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ABSTRACT

Egg yolk is used as a cryoprotectant in semen preservation. However, its composition varies according to the species which may influence its effectiveness during the freeze-thaw process. Therefore, study was conducted to identify the optimum level of pigeon egg yolk (PEY) in *Tris* citric acid (TCA) extender for freezability and *in vivo* fertility of buffalo semen. Semen was collected at weekly intervals for a period of three weeks (replicates) from 6 Nili Ravi buffalo bulls (2 ejaculates/bull/ replicate) and diluted with TCA extender (50×10^6 motile spermatozoa ml^{-1}) containing 5%, 10%, 15% and 20% PEY or 20% CEY (control) and cryopreserved. Post-thaw sperm quality and extracellular enzymes leakage was assessed after thawing. Sperm motility, plasma membrane integrity, livability and viability was significantly higher in extenders containing 10% and 15 % PEY compared to 5% PEY, 20% PEY or 20 % CEY (controls). A dose-dependent decrease was recorded in the chromatin damage for the PEY, being lowest for the 15% and 20% PEY which was significantly less compared to controls (20%CEY). The extracellular GOT and LDH leakage was significantly lower ($P < 0.05$) in extender containing 10% and 15 % PEY compared to the controls. Semen collected from 2 bulls and cryopreserved in extenders containing 15% PEY or 20% chicken egg yolk was assessed after AI. A total of 400 buffaloes were inseminated (100 inseminations/extender/bull). The overall fertility rate was significantly higher ($P < 0.05$) with semen cryopreserved in extender containing 15% PEY (56 %) compared to 20% CEY (42 %; controls). In conclusion, pigeon egg yolk at 15 % offers advantages over 20% chicken egg yolk in terms of in vitro post-thaw semen quality and *in vivo* fertility of buffalo.

Keywords**:** *Columba livia domestica*; *bubalus bubalis*; extender; Lactic dehydrogenase, Glutamic oxaloacetic transaminase

1. Introduction

Egg yolk is a very valuable source of vital nutrients usable in medical, pharmaceutical, nutraceutical and biotechnological industries. Its cryoprotective properties were identified for the first time by Philips and Lardy [1] and since then it has been used as impermeable cryoprotectant for sperm across the species. The cryoprotectant role of egg yolk has been attributed to its low density lipoproteins (LDLs) fraction that includes proteins, lipids, phospholipids, cholesterol, and both saturated and unsaturated fatty acids [2]. In addition to LDLs, the other vital components in the egg yolk include vitamins, minerals/ trace elements, antibodies and antioxidants [3,4], all of which are of immense significance for sperm.

Egg yolks from different avian species differ in their composition, especially in the content of cholesterol, fatty acids, phospholipids [5,6], amino acids [7] and trace elements [8]. Levels of omega-3 polyunsaturated fatty acids, vitamin E, carotenoids, and Se in eggs not only show profound interspecies variations, but they are also markedly higher in free-living species compared with their domesticated or captive counterparts [8,9]. Moreover, a substantial (up to three fold) variation for the trace element content has been reported in eggs from different domestic avian species that were offered same feed [8]. As, such differences in the composition of egg yolks from different species may potentially affect their effectiveness for sperm cryoprotection [10], attempts have been made to identify egg yolk sources most suitable for sperm cryopreservation. It is imperative to mention that egg yolk from avian species other than chicken were found more beneficial for cryopreservation of stallion [6,11], jackass [12], bull [13,14], ram [15] and buffalo sperm [16]. Very recently turkey and quail egg yolk have been tested as cryoprotectants for buffalo sperm cryopreservation; they not only improved the sperm cryopreservation but were also able to improve in vivo fertility compared to traditionally used chicken egg yolk. Moreover, the results obtained in this study showed that in the presence of turkey and quail egg yolk there was a significant decrease in the extracellular enzyme leakage

from buffalo sperm which led the authors to suggest that the cryoprotective abilities of the turkey and quail egg yolk are linked to their variable cholesterol or phospholipid contents [17].

The principal protective component of the egg yolk is the lecithin that can replace some of phospholipids in sperm plasma membrane and form a protective film thereby decreasing its damage during cooling [18]. Pigeon egg yolks have higher lecithin content compared to chicken egg yolk (4.86 % Vs 2.94%) [19]. In addition, the high density lipoproteins (HDLs) that are antagonistic to cryoprotection and the yolk granules that hinder sperm motility are less in pigeon compared to other egg yolks [18]. When the trace elements were studied in different avian egg yolks, interestingly, the pigeon egg yolk was found superior in having Selenium (Se) [8] that has been positively correlated with fertility in humans and other species [20,21]. Pigeon egg yolk LDLs had the best cryoprotective effects on frozen-thawed boar sperm than the hen, duck, quail, pigeon and ostrich egg yolks' LDLs [18] and when whole egg yolk was compared with other avian species, pigeon egg yolk offered better protection to bull [13] and ram sperm [22]. Conventionally, chicken egg yolk is used at 20 % in TCA extender for buffalo semen [23-26], while few studies investigated optimal levels of other avian egg yolks in comparison to 20 % chicken egg [17,27]. Therefore, present study was designed to investigate the optimal level of pigeon egg yolk in in *Tris* citric acid extender by assessing the post thaw sperm quality, extracellular enzyme leakage and *in vivo* fertility of cryopreserved buffalo bull semen.

2. Materials and methods

2.1. Preparation of extenders

Tris-citric acid buffer was used for the semen extender. It was prepared by dissolving 1.56g citric acid (Fisher Scientific, UK) and 3.0g *Tris*–(hydroxymethyl)-aminomethane (Research Organics, USA) in 73 mL distilled water. The pH of buffer was 7.0 and the osmotic pressure was 320 mOsmol kg^{-1}. Apart from the buffer, the semen extender contained 0.2%

100 (wt/v) Fructose (Scharlau, Spain); 7% (v/v) glycerol (Riedel-deHaen, Germany) and a
101 combination of antibiotics consisting of streptomycin sulphate (1 mg/mL), procaine penicillin
102 (300 IU/mL) and benzyl penicillin (Sinbiotic®, China) (100 IU/mL). The pigeon egg yolk
103 (PEY) was added in the semen extender at 5%, 10%, 15% and 20%, while CEY was added at
104 20% which served as controls.

105 *2.2. Semen collection and evaluation*

106 Semen from buffalo bulls were collected with artificial vagina (42°C), and transferred
107 to the laboratory for initial evaluation (volume, sperm motility and sperm concentration).

108 Sperm progressive motility was assessed with phase contrast microscope at 200X at 37
109 °C by placing a drop of diluted semen sample on a pre-warmed glass slide covered with a cover
110 slip [25]. Sperm concentration was measured by taking 1 µL of semen and 200 µL of formal
111 citrate solution (1 mL of 37% formaldehyde in 99 mL of 2.9% sodium citrate) using Neubauer
112 haemocytometer (Marienfeld, Germnay). Only those ejaculates were selected for further
113 processing that qualified a minimum standard of 1 mL volume, 60% motility and 0.5 billion
114 spermatozoa/ml.

115 *2.3. Semen processing and cryopreservation protocol*

116 The qualifying semen ejaculates were split into five aliquots for dilution in
117 experimental extenders containing PEY (5%, 10%, 15% and 20%) or 20% chicken egg yolk
118 (CEY) (controls). Semen aliquots were diluted in a single step at 37°C with each of the
119 experimental extenders at 50×10^6 motile spermatozoa mL^{-1}. Diluted semen was cooled to 4°C
120 for 2 hours and equilibrated for 4 hours at 4°C before being filled in 0.5 mL French straws
121 (IMV, France) with a suction pump at 4°C in a cold cabinet (Minitub, Germany). The semen
122 filled straws were then kept over liquid nitrogen vapours about 5cm above the level of liquid
123 $nitrogen_2$ for 10 minutes before plunging into liquid nitrogen (-196 °C). The frozen-straws

for each treatment were thawed at 37 °C for 30 seconds in water bath and assessed for post-thaw semen quality parameters.

2.5. Post-thaw sperm assays

2.5.1. Sperm motility

Sperm progressive motility was assessed with phase contrast microscope at 200X at 37°C by placing a drop (5 µL) of semen sample on a pre-warmed glass slide and covered with a cover slip [25].

2.5.2. Sperm plasma membrane integrity

Sperm plasma membrane integrity was assessed by hypo-osmotic swelling (HOS) assay [28]. Solution for HOS assay consisted of 0.73g sodium citrate and 1.35g fructose dissolved in 100 mL distilled water (osmotic pressure ~190 mOsmol kg^{-1}). For assessment, 50 µL of frozen-thawed semen sample was mixed with 500 µL of HOS solution and incubated for 30-40 min at 37 °C. After that, 5 µL of mixture was placed on a glass slide, covered with cover-slip and examined using phase contrast microscope (400X). Two hundred spermatozoa per experimental extender per replicate were examined for their swelling characterized by coiled tail indicating intact sperm plasma membrane [25].

2.5.3. Sperm viability and live/dead ratio

Sperm viability and live/dead ratio were studied by dual staining procedure [26]. Equal drops of Trypan-blue (MP Biomedicals, Eschwege, Germany) and semen sample were placed on a glass slide at room temperature, mixed and made into a smear. The smear was air-dried and fixed with formaldehyde-neutral red for 5 min. The slides were then rinsed with distilled water after which 7.5% Giemsa stain (Sigma) was applied for 4 hours. The slides were rinsed with water, air dried and mounted with mounting media. Transparent or light blue sperm were considered as live while those stained dark blue were considered as dead. Transparent or light

148 blue sperm with clear acrosome were considered viable (live with intact acrosome), while
149 sperm having a clear dark blue demarcation and blunt ended acrosome were considered non-
150 viable (dead with damaged acrosome). A total of two hundred spermatozoa per experimental
151 extender per replicate were evaluated in each smear using a phase contrast microscope (1000X;
152 Olympus BX20, Tokyo, Japan) separately for live/dead ratio and sperm viability.

153 *2.5.4. Sperm chromatin damage*

154 Sperm chromatin damage was assessed using acridine orange assay [29,30]. Smears of
155 semen were prepared on glass slides, air-dried and fixed in Carnoy's solution (methanol and
156 glacial acetic acid in a 3:1 proportion) overnight. The slides were air-dried and incubated in
157 tampon solution (80 mmol/L citric acid and 15 mmol/L Na_2HPO_4, pH 2.5) at 75°C for 5
158 minutes to test DNA integrity. The slides were then stained with acridine orange (0.2 mg/mL),
159 washed with water to remove background staining and while still wet, covered with cover slips
160 and evaluated with a epifluorescence microscope (480/550 nm excitation/barrier filter). Sperm
161 with normal DNA presented green, whereas those with an abnormal/damaged DNA presented
162 fluorescence that varied from yellow-green to red in spectrum. One hundred sperm cells were
163 analyzed for each semen sample.

164 *2.5.5. Biochemical tests*

165 Sperm cells with damaged membranes lose their essential metabolites and enzymes. To
166 check this damage, the levels of two intracellular enzymes Lactic dehydrogenase (LDH) and
167 Glutamic oxaloacetic transaminase (GOT) were studied as described by Dhami and Sahni [31].
168 For this purpose, the 2 mL thawed semen sample was centrifuged at x 166g for 20 min and the
169 supernatant was separated to analyze for the extra cellular release of LDH and GOT. For LDH
170 (IU/l) analysis, 20 µL of supernatant was mixed with 400 µL lactate and 100 µL reagent NDH
171 (Merckmillipore®) in a 5 mL tube and allowed to stand for 10 seconds to complete the reaction.
172 For GOT, 50 µL of the supernatant was mixed with 400 µL of TRIS, L-Aspatate of MDH

(malate dehyrogenase) in a 5 mL tube. For LDH (lactate dehyrogenase) 50 µL of the supernatant was mixed with100 µL of 2-Oxoglutrate and NADH (Merck millipore®) in a 5 mL tube. Both the mixtures were then allowed to stand for 60 seconds to complete the reaction. After the completion of reaction, absorbance was measured at 405 and 340 nm for LDH and GOT, respectively, using a spectrophotometer (Microlab 300, ELITech Group, France).

2.6. Evaluation of the best evolved extenders by assessing the in vivo fertility rate of buffalo sperm

Based on the *in vitro* sperm quality assays, the best evolved levels of pigeon egg yolk in extender were evaluated for in vivo fertility rate of cryopreserved semen. The semen was collected from two buffalo bulls of similar age and known fertility. The ejaculates were split, with one portion cryopreserved in the chicken egg yolk (20%, Control) containing extender and the other portion in the pigeon egg yolk (15%) containing extender. The inseminations were performed under field conditions over the three months of period during the peak breeding season. All the experimental inseminations were performed approximately 24 hours after onset of oestrus. Four hundred artificially bred animals (100 buffaloes/bull/extender) were examined for pregnancy through rectal palpation at least 90-days post-insemination.

2.7. Experimental design and data analysis

Semen was collected from 6 Nili Ravi buffalo bulls (2 ejaculates/bull/ replicate) at weekly intervals for a period of three weeks (replicates). Semen from each bull was processed separately and each ejaculate was split into five aliquots and cryopreserved with TCA extender containing 5%, 10%, 15% and 20% PEY or 20% CEY (controls). Total numbers of ejaculates were 36 (three replicates per each of the six bulls and two ejaculates per replication). The data on semen quality parameters and enzyme leakages were analyzed using analysis of variance in randomized complete block design. When F-ratio was found significant ($P<0.05$), Least significant difference (LSD) test was used to compare the treatment means. For *in vivo* fertility

198 rates: semen ejaculates collected from two buffalo bulls was split into two aliquots and
199 cryopreserved in the PEY (15%)
200 containing extender or CEY (20 %) containing extender (control). Four hundred (100
201 buffaloes/bull/extender) were examined for pregnancy through rectal palpation at least 90-days
202 post-insemination. The data on conception rate were analyzed using Chi-square test.
203

204 **3. Results**

205 The data on effect of different levels of pigeon egg yolk in extender on post-thaw
206 quality of buffalo bull spermatozoa are presented in figures 1-5. The percentages of progressive
207 motility, plasma membrane integrity, live/dead ratio and sperm viability were higher ($P<0.05$)
208 in extenders having 10% and 15% PEY compared to 5% PEY, 20% PEY or 20% CEY
209 (controls;). Sperm chormatin damage did not differ in extenders having PEY (5%, 10%, 15 %)
210 and CEY (20%; controls). However, the least damage was observed with 20% PEY egg-yolk
211 compared to CEY (controls;$P < 0.05$).

212 The data on the effects of different concentrations of pigeon egg yolk in extender on
213 the leakage of LDH and GOT are shown in figures 6-7. It is evident that the extender containing
214 10% PEY and 15% PEY egg yolk had less LDH and GOT leakage compared to 5%PEY, 20%
215 PEY and 20 % CEY (controls; $P < 0.05$).

216 The extender having 15% PEY yielded higher ($P<0.05$) fertility rate in bull 1 (57% vs.
217 41%), while the difference was not significant in bull 2 (55vs. 43%; $P>0.05$). The overall
218 fertility rate was higher ($P<0.05$) with spermatozoa cryopreserved in extender containing 15%
219 PEY (56% vs. 42.0%) compared to 20% CEY (controls) ($P < 0.05$; Table 1).
220

221 **4. Discussion**

The pigeon egg yolk possesses higher lecithin content [19], lower cryoprotectant antagonists [18] and higher levels of useful trace elements. Therefore, present study was conducted to investigate the optimum levels of pigeon egg yolk for cryopreservation of buffalo bull semen.

The sperm membrane is considered to be primary site of cryodamages owing to its higher PUFA content that makes it more vulnerable to the intracellular ice crystals formed during the freezing process. The damage to the membrane results in the loss of lipids, phospholipids [32], cholesterol and ultimately the ratio of PUFAs: SFAs is disturbed. Since egg yolks from different avian species vary considerably in their fatty acid, cholesterol and phospholipid content, their use in the semen extender may result in to different levels of cryoprotection for the sperm. In the present study, pigeon egg yolk at 10% and 15% levels in tris citric acid extender improved the post thaw quality of buffalo sperm compared to 20% CEY. In a similar study, quail egg yolk that is high in saturated fatty acids and turkey egg yolk that have higher cholesterol content were reported to have better cryoprotective effect on buffalo sperm compared to chicken egg yolk [17]. It is relevant to mention that the major cryoprotectant viz; lecithin content being higher in the pigeon compared to the chicken egg yolk [19] might have played a role to ameliorate the cryodamages possibly through replacing the phospholipids in sperm plasma membrane and/or by forming a protective film around the sperm membrane as reported earlier [18]. On the basis of results obtained in the present study it is clear that 10-15% of pigeon egg yolk in the semen extender had enough lecithin to provide better protection to the sperm plasma membrane compared to 20% chicken egg yolk. Further, decreased levels of cryoprotectant antagonists like HDLs and yolk granules that are interestingly present in low concentration in pigeon egg yolk [18] might also have contributed to the better sperm protection observed in this study.

246 The avian egg yolks have different levels of micronutrients such as vitamins, minerals/
247 trace elements [8], antibodies [33] and antioxidants [34]. Among trace elements, Selenium (Se)
248 is reported to have significant impact on male reproduction in rats, mice, chickens, pigs, sheep,
249 and cattle [35]. Selenium is an integral part of glutathione peroxidase (GSH-PX), an enzyme
250 which has a major role in providing protection to cell internal structures and cell membrane
251 lipids against free radicals [36,37]. In the present study, improvement observed in sperm
252 motility, plasma membrane, livability, and viability and especially chromatin damage is
253 considered might be associated with the higher levels of Se present in the pigeon egg yolk
254 along with a reduction in oxidative stress induced during cryopreservation. Not only Se
255 supplementation of the semen extender results in a dose-dependant increase in the total
256 antioxidant capacity and post thaw quality of buffalo sperm [38] but also dietary Se
257 supplementation in mice, sheep and cattle has been reported to improves the semen quality [35,
258 39-41].

259 The cryopreservation induces extracellular enzymes leakages possibly by damaging/
260 deteriorating the sperm membrane [17]. The extracellular enzyme leakage is considered to be
261 a marker of fertilizing ability of ram [42], buck [43] bull [44] and buffalo bull sperm [17].
262 Lactate dehydrogenase (LDH) is involved in metabolic processes which provide energy for
263 survival, motility and fertility of sperm [17,43]. Its extracellular leakage has negatively been
264 correlated with fertilizing ability of sperm and could be used as a marker of membrane damage
265 during freezing. Similarly, the release of glutamic oxalacetic transaminase (GOT) is associated
266 with cryodamages in buffalo sperm [45]. In the present study, extracellular leakages of LDH
267 and GOT were recorded lowest in buffalo sperm cryopreserved in the extender with 10% and
268 15 % pigeon egg yolk compared to 20 % chicken egg yolk (controls). The higher levels of
269 lecithin present in the pigeon egg yolk might have formed a protective layer and/or replenished
270 the phospholipids damaged/lost during cryopreservation resulting into comparatively less

271 enzyme leakage [19] and therefore, a better protection of the sperm. Moreover, higher levels
272 of Se in pigeon egg yolk might have enhanced the antioxidant potential that resulted in
273 reduction of lipid peroxidation of sperm phospholipids [8,38]. It is pertinent to mention that
274 the data on motility, plasma membrane integrity and viability are very much supported with
275 that of enzyme leakages, i.e., a similar pattern of effects of egg yolk levels are observed on
276 motility, viability and plasma membrane integrity of cryopreserved buffalo semen.

277 The higher fertility rates were recorded with extender having 15% pigeon egg yolk
278 compared to 20% chicken egg yolk. The real test to evaluate a semen sample is to check the
279 fertility rate after its *in vivo* insemination in a routine artificial insemination programme under
280 field conditions [46-48]. The higher fertility rate observed with 15% pigeon egg yolk observed
281 in the present study are in line with the higher sperm quality viz; motility, plasma membrane
282 integrity, livability, viability, chromatin integrity and extracellular enzyme leakage suggesting
283 that all the sperm quality parameters used to assess the semen quality do have essential role in
284 the fertilization process [49].

285 On the basis of existing information on the PEY, better sperm quality and higher
286 fertility rates observed post-insemination in this study could be linked to the higher lecithin
287 contents and higher Se and Mo concentrations present in the PEY. However, for proper
288 evaluation, a precise bio-chemical composition of the pigeon egg yolk is required along with
289 its mechanism for sperm membrane protection. In conclusion, the results of this study have
290 shown that 15% pigeon egg yolk in the semen extender significantly improves the post-thaw
291 semen quality and *in vivo* fertility in the buffalo.

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295

296 **References**

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