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TITLE: Evaluation of Quail and Turkey egg yolk for cryopreservation of Nili-Ravi Buffalo bull semen

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24**Original Research Paper****Evaluation of Quail and Turkey egg yolk for cryopreservation of Nili-Ravi Buffalo bull semen****S. Akhter^a, B.A. Rakha^b, M.S. Ansari^c, A. U. Husna^a, S. Iqbal^d M. Khalid^e**^a *Department of Zoology, ^bDepartment of Wildlife Management Pir Mehr Ali Shah Arid Agriculture University Rawalpindi-46300, Pakistan*^c *Department of Zoology, University of Sargodha-Lyallpur Campus-38000, Pakistan*^d *Semen Production Unit, Qadirabad, Sahiwal, Pakistan*^e *Department of Production and Population Health, Royal Veterinary College, London***Running Title:** Quail or Turkey egg yolk in extender for buffalo semen

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25 ABSTRACT

26 Egg yolk is used as a cryoprotectant for semen in different mammalian species including
27 buffalo. Egg yolk from different sources may affect freezability of buffalo bull semen. Quail
28 egg yolk (QEY) and Turkey egg yolk (TEY) in *Tris* citric acid extender was evaluated for
29 post-thaw quality and *in vivo* fertility rate of cryopreserved Buffalo bull semen. Ejaculates
30 were collected on weekly basis from 6 Nili Ravi buffalo bulls (12 ejaculates/bull) for a period
31 of 6 weeks and diluted at 37 °C with TCEY extender (50×10^6 motile spermatozoa ml⁻¹)
32 containing different levels of QEY or TEY (5%, 10%, 15% and 20%) or 20% chicken egg
33 yolk (CEY; controls) and cryopreserved. Percent post-thaw sperm motility (48.3 ± 3.8), plasma
34 membrane integrity (PMI; 67.9 ± 5.3), live/dead ratio (68.2 ± 5.0) and viability (50.5 ± 3.7)
35 were recorded higher ($P < 0.05$) in extender containing 5% QEY compared to control.
36 However, the TEY at 10% in extender improved ($P < 0.05$) the post thaw sperm motility
37 (57.5 ± 5.2), PMI (53.5 ± 4.5), livability (75.3 ± 6.0) and viability (73.5 ± 6.5) compared to
38 higher concentrations of turkey egg yolk and controls (20% CEY). The chromatin damage
39 (2.0 ± 0.9) and intracellular enzymes GOT (24.8 ± 3.5), LDH (77.7 ± 4.5) release was lower
40 ($P < 0.05$) in extender containing 10% TEY compared to the controls. *In vivo* fertility was
41 compared after AI with semen from two buffalo bulls that was cryopreserved in extenders
42 containing 5% QEY, 10% TEY or 20% chicken egg yolk. A total of 600 inseminations (200
43 inseminations/extender) were recorded; the overall fertility rate was significantly higher ($P <$
44 0.05) with semen cryopreserved in extender containing 5% QEY (57.5 vs. 42%), and 10%
45 TEY (57.5 vs. 42%). compared to 20% chicken egg yolk. In conclusion, quail egg yolk at 5%
46 and turkey egg yolk at 10% offers advantages over 20% chicken egg yolk in terms of *in vitro*
47 post-thaw semen quality and *in vivo* fertility of buffalo.

48 **Keywords:** *Coturnix coturnix*; *Meleagris gallopavo*; chromatin damage; extender; sperm
49 motility; viability

50 1. Introduction

51 Artificial insemination using cryopreserved semen is the optimal way of
52 disseminating germplasm of the superior sires to a large number of females. It also facilitates
53 sanitary, quarantine and international exchange of germ-plasm [1, 2]. However in buffalo,
54 fertility rates following AI with cryopreserved semen are quite low and not commercially
55 acceptable. These low fertility rates are attributed to the low quality of cryopreserved buffalo
56 semen [3]. There are studies to show that buffalo spermatozoa are damaged heavily during
57 freezing and thawing process [4,5]. The freezing-thawing process exerts physical and
58 chemical stress to the sperm which ultimately renders the frozen-thawed semen to have
59 reduced motility, viability and fertilizing ability when compared with fresh semen [6-8]. This
60 has led to a continuous effort to improve the post-thaw semen quality with the objective to
61 achieve promising results after insemination with frozen-thawed semen [9].

62 The cholesterol/phospholipid ratio determines the sensitivity of the sperm to cold
63 shock damage [7]. Therefore, sperm with high cholesterol to phospholipid ratio such as rabbit
64 and human sperm [10] are more resistant to the “cold shock” damage than sperm having low
65 cholesterol to phospholipid ratio like boar, ram and bull sperm [11]. Buffalo bull sperms have
66 comparatively lower cholesterol content in their membranes [12-14] that is further decreased
67 during freeze-thaw process. Since egg yolk from different avian species has different ratios
68 of fatty acids, phospholipids and cholesterol, it could have different effects on freezability of
69 the sperm [15-21].

70 Quail egg yolk has higher amount of phosphatidylcholine, less
71 phosphatidylethanolamine and a smaller ratio of poly-unsaturated fatty acids to saturated
72 fatty acids that could contribute additional protective effect to spermatozoa during
73 cryopreservation than chicken egg yolk [15]. It is relevant to mention that saturated fatty
74 acids are less vulnerable to lipid peroxidation than unsaturated fatty acids and this

75 characteristic makes quail egg yolk a more suitable cryoprotectant than chicken egg yolk as
76 has been reported previously for jackass [15], and rooster [22] sperm. In the same context,
77 Turkey egg yolk has a higher content of cholesterol compared to chicken egg yolk and has
78 been reported to result in a better post-thaw semen quality in boar and stallion [21,23-24].

79 Considering the role of cholesterol to phospholipids ratio in the freezability of semen,
80 the present study was conducted to determine if the addition of Quail (QEY) and Turkey egg
81 yolk (TEY) in extender improves the spermatozoa after cryopreservation. The objective of
82 the study was to investigate if Quail or Turkey egg yolk in tris-citric acid extender improve
83 the post-thaw quality and fertility of Nili Ravi buffalo bull spermatozoa.

84 **2. Materials and methods**

85 All experimental procedures and animals used in this study were approved by the
86 ethical committee of the Department of Zoology, PMAS-Arid Agriculture University,
87 Rawalpindi-Pakistan.

88

89 *2.1 Animals and local*

90 Nili-Ravi buffalo breeding bulls (n = 6) of known fertility and similar age (7–
91 8 years) with clinically normal reproductive tracts, kept under uniform feeding and handling
92 conditions at Semen Production Unit, Qadirabad, Sahiwal, Pakistan were used in this study.

93

94 *2.2 Preparation of extenders*

95 Tris-citric acid buffer was used for the semen extender. It was prepared by dissolving
96 1.56g citric acid (Fisher Scientific, UK) and 3.0g *Tris*–(hydroxymethyl)–aminomethane
97 (Research Organics, USA) in 73 mL distilled water. The pH of buffer was 7.0 and the
98 osmotic pressure was 320 mOsmol kg⁻¹. Apart from the buffer, the semen extender contained

99 0.2% (wt/v) Fructose (Scharlau, Spain); 7% (v/v) glycerol (Riedel-deHaen, Germany) and a
100 combination of antibiotics consisting of streptomycin sulphate (1 mg/mL), procaine penicillin
101 (300 IU/mL) and benzyl penicillin (Sinbiotic[®], China) (100 IU/mL). The experimental egg
102 yolks (QEY or TEY) were added at 5%, 10%, 15% and 20%, while 20% CEY in extender was
103 kept as control.

104

105 2.2. *Semen collection and evaluation*

106 Semen was collected with artificial vagina (42 °C) and transferred to the laboratory
107 for initial evaluation (volume, sperm motility and sperm concentration). Semen volume was
108 measured using graduated glass collection tube.

109 Sperm progressive motility was assessed with phase contrast microscope at 400X at
110 37 °C by placing a drop semen sample on a pre-warmed glass slide and covered with a cover
111 slip [25]. Sperm concentration was measured by taking 1 µL of semen and 200 µL of formal
112 citrate solution (1 mL of 37% formaldehyde in 99 mL of 2.9% sodium citrate) with Neubauer
113 haemocytometer (Marienfeld, Germany). Only those ejaculates that qualified a minimum
114 standard of 1 mL volume, 60% motility and 0.5 billion spermatozoa mL⁻¹ of semen were
115 selected for further processing. The qualifying ejaculates (n=36/experiment; 2
116 ejaculates/bull/collection) were split into five aliquots for dilution in experimental extenders
117 containing Quail or Turkey egg yolks (5%, 10%, 15% and 20%) or 20% chicken egg yolk
118 (controls) and were cryopreserved.

119

120 2.3. *Semen processing and cryopreservation protocol*

121 Semen from experimental animals was collected during the peak breeding season
122 (September-November) at weekly intervals for a period of six weeks [three weeks (replicates)

123 for each of the separate experiments on Quail egg yolk (QEY) and Turkey egg yolk (TEY)]
124 during early morning (before sunrise) with the help of an artificial vagina (IMV, France)
125 connected with a rubber cone and graduated glass collection tube at a temperature of 42 °C,
126 using an intact bull as a teaser. Semen aliquots were diluted in a single step at 37 °C with
127 each of the experimental extenders at 50×10^6 motile spermatozoa mL⁻¹. Diluted semen was
128 cooled to 4 °C for 2 hours and equilibrated during 4 hours at 4 °C before being filled in 0.5
129 mL French straws (IMV, France) with suction pump at 4 °C in a cold cabinet (Minitub,
130 Germany). Then the straws were kept 5cm over liquid nitrogen vapours for 10 minutes before
131 being plunged into liquid nitrogen (-196 °C) and stored. The samples from each treatment
132 were thawed at 37 °C for 30 seconds in water bath and assessed for post-thaw quality.

133

134 2.5. *Post-thaw sperm assays*

135 2.5.1 *Sperm motility*

136 Sperm progressive motility was assessed with phase contrast microscope at 400X at
137 37 °C by placing a drop (10 µL) semen sample on a pre-warmed glass slide and covered with
138 a cover slip [25].

139

140 2.5.2 *Sperm plasma membrane integrity*

141 Sperm plasma membrane integrity was assessed by hypo-osmotic swelling (HOS)
142 assay [26]. Solution for HOS assay consisted of 0.73g sodium citrate and 1.35g fructose
143 dissolved in 100 mL distilled water (osmotic pressure ~ 190 mOsmol kg⁻¹). For assessment,
144 50 µL of frozen-thawed semen sample was mixed with 500 µL of HOS solution and
145 incubated for 30-40 min at 37 °C. After that, 5 µL of mixture was placed on a glass slide,
146 covered with cover-slip and examined using phase contrast microscope (400X). Two hundred

147 spermatozoa per experimental extender per replicate were examined for their swelling
148 characterized by coiled tail indicating intact sperm plasma membrane [27].

149

150 2.5.3 *Sperm viability and live/dead ratio*

151 Sperm viability and live/dead ratio were studied by dual staining procedure [28].
152 Equal drops of Trypan-blue (MP Biomedicals, Eschwege, Germany) and semen sample were
153 placed on a glass slide at room temperature, mixed and made into a smear. The smear was
154 air-dried and fixed with formaldehyde-neutral red for 5 min. The slides were then rinsed with
155 distilled water after which 7.5% Giemsa stain (Sigma) was applied for 4 hours. The slides
156 were rinsed with water, air dried and mounted with mounting media. Transparent or light
157 blue sperm were considered as live while those stained dark blue were considered as dead.
158 Transparent or light blue sperm with clear acrosome were considered viable (live with intact
159 acrosome), while sperm having a clear dark blue demarcation and blunt ended acrosome were
160 considered non-viable (dead with damaged acrosome). A total of two hundred spermatozoa
161 per experimental extender per replicate were evaluated in each smear using a phase contrast
162 microscope (1000X; Olympus BX20, Tokyo, Japan) separately for live/dead ratio and sperm
163 viability.

164

165 2.5.4 *Sperm chromatin Damage*

166 Sperm chromatin Damage was assessed using acridine orange assay [29-30]. Smears
167 of semen were prepared on glass slides, air-dried and fixed for overnight in Carnoy's solution
168 (methanol and glacial acetic acid in a 3:1 proportion). The slides were air-dried and incubated
169 in tampon solution (80 mmol/L citric acid and 15 mmol/L Na₂HPO₄, pH 2.5) at 75°C for 5
170 minutes to test DNA integrity. The slides were then stained with acridine orange (0.2
171 mg/mL), washed with water to remove background staining and while still wet, covered with

172 cover slips and evaluated with a epifluorescence microscope (480/550 nm excitation/barrier
173 filter). Sperm with normal DNA presented green, whereas those with an abnormal/damaged
174 DNA presented fluorescence that varied from yellow-green to red in spectrum. One hundred
175 sperm cells were analyzed for each semen sample.

176

177 2.5.5. *Biochemical tests*

178 Sperm cells with damaged membranes lose their essential metabolites and enzymes.
179 To check this damage, the levels of two intracellular enzymes Lactic dehydrogenase (LDH)
180 and Glutamic oxaloacetic transaminase (GOT) were studied as described by Dhimi and Sahni
181 [10]. For this purpose, the 2 mL thawed semen sample was centrifuged at 166g for 20 min
182 and the supernatant was separated to analyze for the extra cellular release of LDH and GOT.
183 For LDH (IU/l) analysis, the 20 μ L of supernatant was mixed with 400 μ L lactate and 100 μ L
184 reagent NDH (Merckmillipore®) in a 5 mL tube and allowed to stand for 10 seconds to
185 complete the reaction. For GOT, 50 μ L of the supernatant was mixed with 400 μ L of TRIS,
186 L-Aspartate of MDH (malate dehydrogenase) and of LDH (lactate dehydrogenase) and 100 μ L
187 of 2-Oxoglutrute and NADH (Merck millipore®) in a 5 mL tube and allowed to stand for 60
188 seconds to complete the reaction. After the completion of reaction, absorbance was measured
189 at 405 and 340 nm for LDH and GOT, respectively, using a spectrophotometer (Microlab
190 300, ELITech Group, France).

191

192 2.6. *Evaluation of best evolved extenders for in vivo fertility rate of buffalo sperm*

193 Based on semen quality assays, the best evolved level of quail or turkey egg yolk in
194 extender was evaluated for in vivo fertility rate of cryopreserved semen. The semen from two
195 buffalo bulls was cryopreserved in *tris*-citric egg yolk extender containing 5% QEY or 10%
196 TEY and 20% chicken egg yolk (control). The inseminations were preformed under field

197 conditions over three months' period during the peak breeding season. All the experimental
198 inseminations were performed approximately 24 hours after onset of heat. Six hundred
199 artificially bred animals (100 buffaloes/bull/extender) were examined for pregnancy through
200 rectal palpation at least 90 days post-insemination.

201

202 2.7. *Statistical analysis*

203 The data on spermatozoa quality parameters are presented as means \pm SEM. ANOVA
204 was used to compare the effect of different types/levels of egg-yolk on different parameters
205 of semen quality. When F-ratio was found significant for a parameter of sperm quality, LSD
206 test was applied to compare the treatment means. The data on *in vivo* fertility rates were
207 analyzed using Chi-square test.

208

209 3. Results

210 The percentages of progressive motility and plasma membrane integrity were higher
211 after the use of 5% quail egg yolk than control ($P < 0.05$; Figure 1). The live/dead ratio and
212 sperm viability were higher when the extender contained 5% and 10% QEY in extender
213 compared to control ($P < 0.05$). A dose dependant decrease in sperm live/dead ratio and
214 viability was observed at 20% QEY compared to extender having 5% QEY in extender ($P <$
215 0.05).

216 The percentage of sperm progressive motility, plasma membrane integrity, livability
217 and viability were higher when extender contained 10% turkey egg yolk compared to the
218 others treatments ($P < 0.05$; Figure 2). Sperm chromatin damage was reduced in extenders
219 having 10, 15 and 20% of Turkey egg yolk. The least damage was observed with 10%
220 Turkey egg-yolk, whereas the chromatin damage caused by 5% Turkey egg-yolk was not
221 different from that of controls which had maximum damage ($P < 0.05$; Figure 2).

222 The effects of different concentrations of turkey egg yolk in the semen extender on
223 the leakage of LDH and GOT have shown that extender containing 10% egg yolk had less
224 GOT leakage compared to the other treatments ($P < 0.05$). LDH leakage was lower in sperm
225 diluted in extender containing 10 and 15% Turkey egg yolk ($P < 0.05$; Figure 2).

226 The fertility rate was higher with semen cryopreserved in extender containing 5% Quail
227 and 10% Turkey egg yolk compared to control in bull I (56 and 58 vs. 42%) and bull II (59
228 and 59 vs. 42%) ($P < 0.05$; Table 1). The overall fertility rate was higher with spermatozoa
229 cryopreserved in extender containing 5 % Quail (57.5% vs. 42.0%) and 10% Turkey egg yolk
230 (57.5% vs. 42.0%) compared to control.

231

232 **4. Discussion**

233

234 Sperm membrane lipids particularly the cholesterol:phospholipid (C/P) ratio
235 determines the sensitivity of sperm to cold shock [31] and sperm having lower C/P ratio
236 (such as buffalo sperm) are more prone to cryo-damage than the sperm having high C/P ratio
237 [32]. The first site of cryodamage is the sperm plasma membrane that becomes transiently
238 leaky and sperm cell loses vital enzymes [33] and membrane lipids [34]. Routinely, the egg
239 yolk is used as cryoprotectant that after disruption of the low density lipoprotein fraction,
240 release the phospholipids that form a protective film at the surface of spermatozoa membrane
241 [35]. It has also been reported that phospholipids from egg yolk could merge with
242 spermatozoa membranes and replace some phospholipids and thereby decrease their phase
243 transition temperatures [36]. Similarly, the cholesterol interacts with the phospholipid
244 hydrocarbon chains [37] at temperatures below the phase transition, forces the chains apart,
245 making the membrane more stable [38].

246 Egg yolk from different avian species such as duck, quail, pigeon, chicken and turkey
247 has different combinations of fatty acids, phospholipids and cholesterol [15-21, 39].
248 Interestingly, the sperm membranes of different species also vary in their cholesterol and
249 phospholipid content that influences their susceptibility to cold shock. Therefore, the
250 differences in sperm membrane composition and the components of the egg yolk from
251 different avian species may culminate in species-specific interactions [20]. Quail egg yolk
252 contained significantly more phosphatidylcholine, less phosphatidylethanolamine and a
253 smaller ratio of polyunsaturated to saturated fatty acids than chicken egg yolk [15], that
254 attributed to higher motilities of frozen thawed boar, jackass and stallion sperm
255 [15,19,17,25]. Similarly, Turkey egg yolk has been reported to contain more cholesterol than
256 chicken egg yolk [17, 24,25] and previously its inclusion in the semen extender has been
257 reported to improve post thaw quality of stallion sperm [40].

258 In buffalo (*Bubalus bubalis*), cold shock and freezing resulted in a significant loss of
259 total lipids and of phospholipids of sperm [41] that may be attributed to production of acetyl
260 CoA through β - oxidation [42] and lipid peroxidation reactions [43]. It is pertinent to mention
261 that buffalo sperm possess high level of polyunsaturated fatty acids and are more prone to
262 lipid peroxidation. Interestingly, the polyunsaturated fatty acid to saturated fatty acid ratio of
263 quail egg yolk was reported to be half of the chicken egg yolk [15]. Therefore, in present
264 study, supplementation of quail egg yolk in extender may have yielded better protection in
265 terms of membrane stabilization through incorporation of saturated fatty acids in sperm
266 membrane. It is pertinent to mention that saturated fatty acids crystalize in a more regular
267 form [15] and are reported to be incorporated more efficiently in the spermatozoal lipids in
268 bovine [44]. Further, quail egg yolk has more phosphatidylcholine than chicken egg yolk that
269 is the more effective phospholipid to protect spermatozoa [15]. It has also been reported that
270 Phosphatidylcholine (PC) to Phosphatidylethanolamine (PE) ratio of quail egg yolk was

271 about twice that of chicken egg yolk [15]. Therefore, improvement in post-thaw parameters of
272 buffalo bull semen at 5% QEY is suggestive of fulfillment of phospholipid requirement at
273 this level. The dose dependent decrease in sperm viability and live/dead ratio using higher
274 concentrations of QEY (15 and 20%) may be explained by the enhanced toxicity associated
275 with increased egg yolk level [45] probably resulting from the elevated levels of substrates
276 available for hydrogen peroxide formation [46]. The use of egg yolk at lower level further
277 has advantages in terms of lesser cryoprotectant antagonists; yolk granules, calcium,
278 progesterone and high density lipoproteins [47-50] that may compromise the freezability of
279 cryopreserved buffalo semen.

280 The present study evaluated the effects of different levels of Turkey egg yolk in
281 extender on post-thaw quality, leakage of intracellular enzymes and fertility of buffalo bull
282 semen. The results revealed that percent sperm progressive motility, plasma membrane
283 integrity, livability and viability, chromatin integrity were all improved ($P < 0.05$) when
284 turkey egg yolk was added at 10% level in Tris-citric acid extender compared to chicken egg
285 yolk (20%). It is a possibility that the turkey egg yolk being rich in cholesterol might have
286 resulted in a better incorporation of cholesterol in the sperm membrane and this would have
287 decreased the susceptibility of sperm to cold shock by lowering the phase transition
288 temperature [37, 51]. It is worth to note that Turkey egg yolk at lower concentrations (5%)
289 was not able to improve the post-thaw semen quality parameters which suggests that it is not
290 just the type of egg yolk *per se* but also the absolute amount of egg yolk (and therefore of
291 cholesterol) in the extender that matters. The high content of cholesterol in Turkey egg yolk
292 has already been reported to increase the progressive motility of stallion spermatozoa after
293 freeze-thawing [52]. However, the exact mechanism of sperm protection by cholesterol
294 during cryopreservation has not yet been established [53]. Nevertheless, in the present study a

295 significant improvement was observed in all the post-thaw semen quality parameters when
296 10% Turkey egg yolk was included in the semen extender.

297 Release of enzymes into the extracellular fluid have been used as an indicator of
298 sperm cells' membrane damage due to cold shock in various species including buffalo [33,
299 54-56]. In the present study, a significantly lower release of GOT and LDH was observed in
300 the extenders containing Turkey egg yolk. This might be due to improved stabilization of
301 sperm membrane resulting from a better incorporation of cholesterol which is present in
302 higher levels in Turkey egg yolk [33]. Thus a comparatively stable sperm membrane not
303 only prevented the leakage of vital intracellular enzymes like LDH and GOT but also
304 protected the sperm nuclei reducing the chromatin damage, and all this seemed to have been
305 reflected in the observed improvement of the functional parameters of sperm like motility,
306 plasma membrane integrity, viability and liveability.

307 While the *in vitro* laboratory tests indicate the extent of damage to sperm during
308 freeze-thawing process, fertility is the ultimate measure to assess the quality of frozen-thawed
309 semen [57]. In the present study, the improved post-thaw semen quality parameters were also
310 supported by the *in vivo* fertility rate that was recorded significantly higher after artificial
311 insemination with semen extender that contained QEY or TEY compared to chicken egg yolk
312 (controls). The higher levels of phosphatidylcholine in QEY and cholesterol in TEY may have
313 provided better protection and ultimately resulted in improved fertility rate of buffalo bull
314 sperm. In our knowledge, this is the first report on the *in vivo* fertility in buffalo after AI with
315 semen containing QEY or TEY in the semen extender.

316 In conclusion, Quail egg yolk at 5% and Turkey egg yolk at 10% offers advantages
317 over 20% Chicken egg yolk in terms of *in vitro* post-thaw semen quality and *in vivo* fertility
318 of Buffalo.

319

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323

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325

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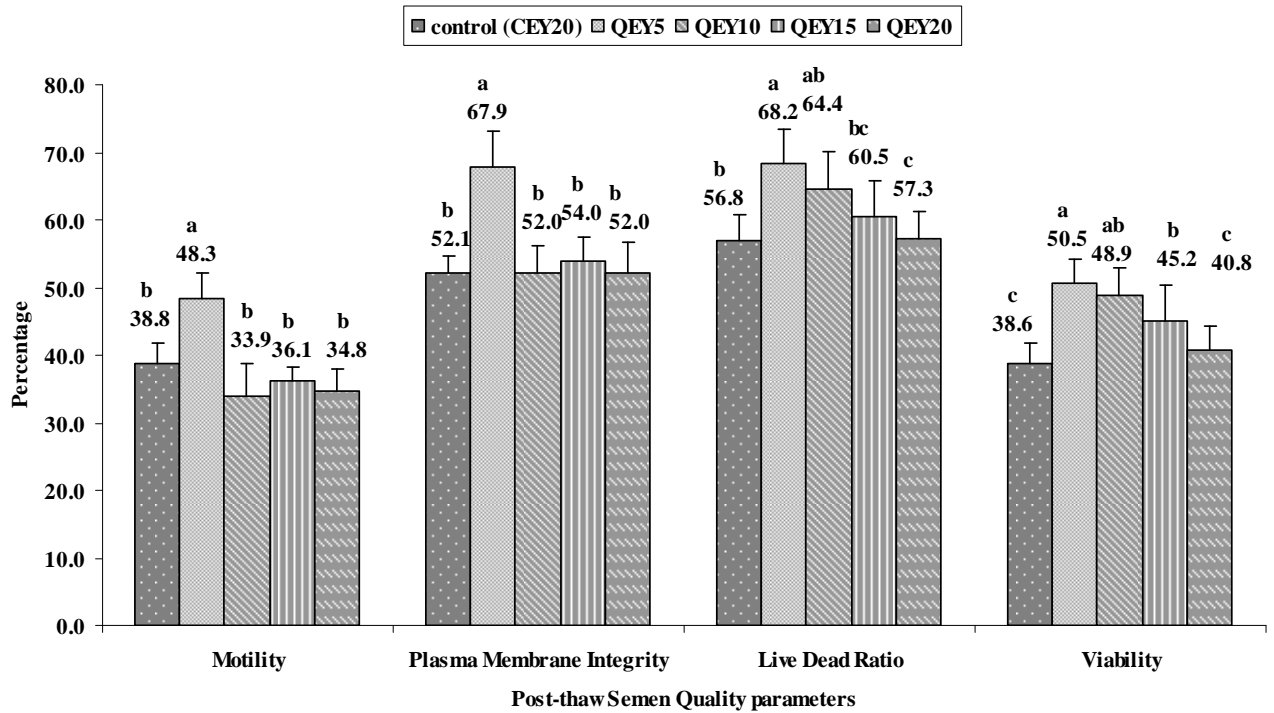
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Table 1: Post-thaw percentage of fertility of the Buffalo bull spermatozoa with Quail, Turkey and Chicken egg yolk in extender

| Bull | Extender | No. of inseminations recorded | Pregnancy rate | Chi- square value | P- value |
|----------------|----------------------|--|-----------------------|----------------------------------|---------------------|
| I | 5% Quail egg yolk | 100 | 56 (56%) | 6.91 | 0.03 |
| | 10% Turkey egg yolk | 100 | 58 (58%) | | |
| | 20% Chicken egg yolk | 100 | 42 (42%) | | |
| II | 5% Quail egg yolk | 100 | 59 (59%) | 7.80 | 0.02 |
| | 10% Turkey egg yolk | 100 | 59 (59%) | | |
| | 20% Chicken egg yolk | 100 | 42 (42%) | | |
| Overall | 5% Quail egg yolk | 200 | 115 (57.5%) | 13.73 | 0.00 |
| | 10% Turkey egg yolk | 200 | 115 (57.5%) | | |
| | 20% Chicken egg yolk | 200 | 84 (42%) | | |

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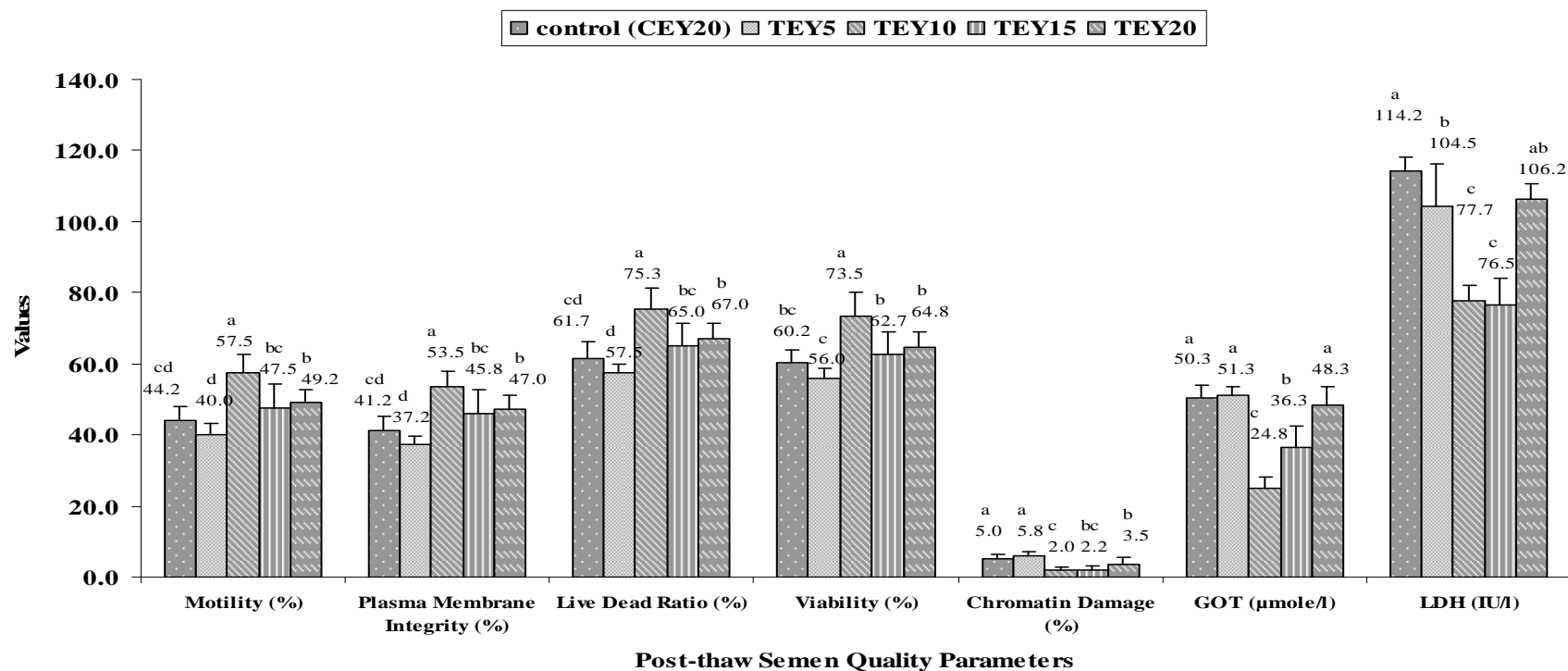


2

3 **Figure 1. Post-thaw semen quality (Motility, Plasma membrane integrity, Live dead ratio**
 4 **and Viability) of buffalo bull spermatozoa frozen with different concentrations of Quail egg**
 5 **yolk in extender. Total numbers of ejaculates were 36 (2 ejaculates/each of 6**
 6 **bulls/collection). Bars with different letters differ ($P < 0.05$) for a given parameter.**

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9

10 **Figure 2. Post-thaw quality (Motility, Plasma membrane integrity, Live dead ratio and Viability) and enzyme release (GOT**
 11 **and LDH) of buffalo bull spermatozoa frozen with different concentrations of Turkey egg yolk in extender. Total numbers of**
 12 **ejaculates were 36 (2 ejaculates/each of 6 bull /collection). Bars with different letters differ ($P < 0.05$) for a given parameter.**

13

HIGHLIGHTS

- Turkey egg yolk and quail egg yolk were evaluated for freezability and fertility of buffalo bull spermatozoa.
- Quail egg yolk at 5 % in extender improved the post thaw quality of buffalo bull spermatozoa compared to 20 % chicken egg yolk.
- Turkey egg yolk at 10 % was better in terms of post thaw quality and enzyme leakage compared to 20 % chicken egg yolk.
- Quail egg yolk at 5 % and Turkey egg yolk at 10 % was efficient to improve the fertility rate in buffalo compared to routinely used 20 % chicken egg yolk.