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

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Running Title: Quail or Turkey egg yolk in extender for buffalo semen

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ABSTRACT

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

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

1. Introduction

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

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

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

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

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

2. Materials and methods

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

2.1 Animals and local

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

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

2.2. Semen collection and evaluation

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

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

2.3.Semen processing and cryopreservation protocol

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

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

2.5. Post-thaw sperm assays

2.5.1 Sperm motility

Sperm progressive motility was assessed with phase contrast microscope at 400X at 37 °C by placing a drop (10 µL) 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 [26]. 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 [27].

2.5.3 Sperm viability and live/dead ratio

Sperm viability and live/dead ratio were studied by dual staining procedure [28]. 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 blue sperm with clear acrosome were considered viable (live with intact acrosome), while sperm having a clear dark blue demarcation and blunt ended acrosome were considered non-viable (dead with damaged acrosome). A total of two hundred spermatozoa per experimental extender per replicate were evaluated in each smear using a phase contrast microscope (1000X; Olympus BX20, Tokyo, Japan) separately for live/dead ratio and sperm viability.

2.5.4 Sperm chromatin Damage

Sperm chromatin Damage was assessed using acridine orange assay [29-30]. Smears of semen were prepared on glass slides, air-dried and fixed for overnight in Carnoy's solution (methanol and glacial acetic acid in a 3:1 proportion). The slides were air-dried and incubated in tampon solution (80 mmol/L citric acid and 15 mmol/L Na_2HPO_4, pH 2.5) at 75°C for 5 minutes to test DNA integrity. The slides were then stained with acridine orange (0.2 mg/mL), washed with water to remove background staining and while still wet, covered with

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

2.5.5. Biochemical tests

Sperm cells with damaged membranes lose their essential metabolites and enzymes. To check this damage, the levels of two intracellular enzymes Lactic dehydrogenase (LDH) and Glutamic oxaloacetic transaminase (GOT) were studied as described by Dhami and Sahni [10]. For this purpose, the 2 mL thawed semen sample was centrifuged at 166g for 20 min and the supernatant was separated to analyze for the extra cellular release of LDH and GOT. For LDH (IU/l) analysis, the 20 µL of supernatant was mixed with 400 µL lactate and 100 µL reagent NDH (Merckmillipore®) in a 5 mL tube and allowed to stand for 10 seconds to complete the reaction. For GOT, 50 µL of the supernatant was mixed with 400 µL of TRIS, L-Aspatate of MDH (malate dehyrogenase) and of LDH (lactate dehyrogenase) and 100 µL of 2-Oxoglutrate and NADH (Merck millipore®) in a 5 mL tube and 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 best evolved extenders for in vivo fertility rate of buffalo sperm

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

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

2.7. Statistical analysis

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

3. Results

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

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

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

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

4. Discussion

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

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

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

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

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

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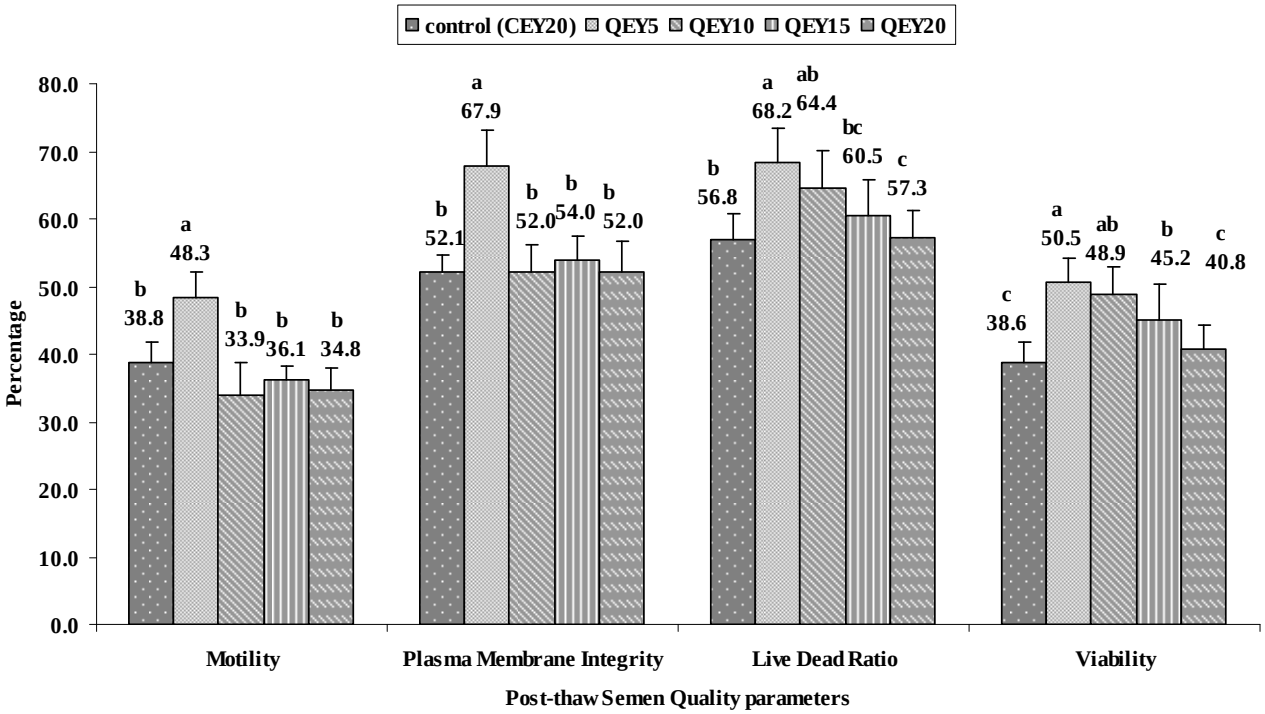
Table 1: Post-thaw percentage of fertility of the Buffalo bull spermatozoa with Quail, Turkey and Chicken egg yolk in extender

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%)		

1



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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.**

7

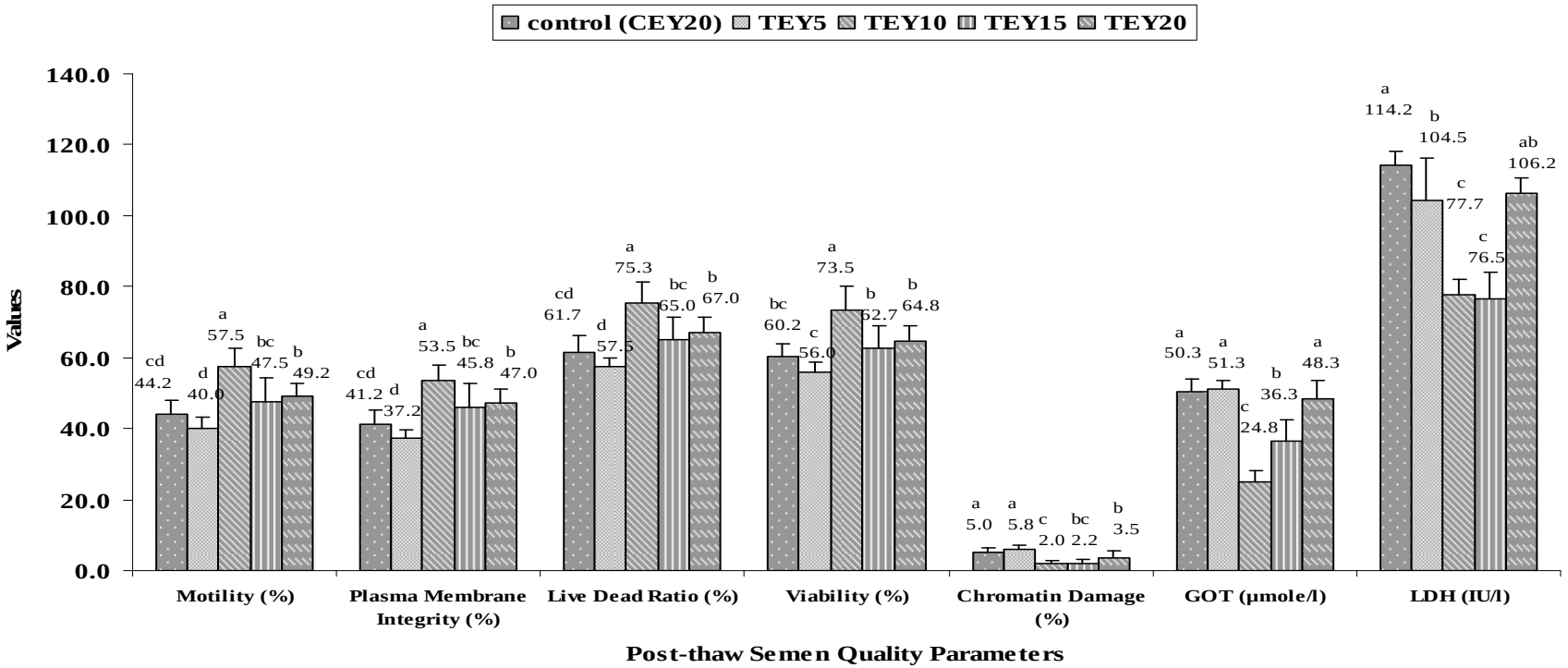

Figure 2. Post-thaw quality (Motility, Plasma membrane integrity, Live dead ratio and Viability) and enzyme release (GOT and LDH) of buffalo bull spermatozoa frozen with different concentrations of Turkey egg yolk in extender. Total numbers of ejaculates were 36 (2 ejaculates/each of 6 bull /collection).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

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.