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RESEARCH ARTICLE

Effect of Egg Yolk and Carboxylated Poly-l Lysine (CPLL) Supplementation to Tris-Citrate Extender on Post-Thaw Semen Quality of Beetal Male Goats

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A B S T R A C T

The current study was planned to investigate the effects of supplementation of different concentrations of egg yolk (EY) and Carboxylated Poly-l Lysine (CPLL) on the post-thawed sperm quality of male Beetal goats. For this purpose, pooled semen of bucks (n=6) was diluted with Tris-citric acid egg yolk (TCEY) extender containing different concentrations of EY (0, 5 and 15%) and CPLL (0, 0.1, 0.15, 0.20 and 0.25%). Data were presented as means with pooled SEM. The main effects, EY and CPLL concentrations, and their interaction were analyzed using multifactor ANOVA. Minimum values of progressive motility (PM) and motion kinematics of spermatozoa were observed in extenders having 0% EY with different CPLL concentrations. The extender containing 5% EY and 0.25% CPLL resulted in better ($P<0.05$) PM than 0 and 15% EY extenders with different CPLL concentrations. The ratio ($P<0.05$) of sperm with intact plasma membrane was higher in 5% EY extender with 0.20 and 0.25% CPLL than in 15% EY extender with corresponding CPLL concentrations The EY concentration significantly influenced sperm acrosomal integrity, as 0% EY extenders showed lower ($P<0.05$) sperm acrosome integrity than 5 and 15% EY extenders. At various concentrations of CPLL, both 5 and 15% EY extenders had similar ($P>0.05$) ratios of acrosomal membrane-intact sperm. In comparison to 0 and 15% EY extenders with various CPLL concentrations, 5% EY extenders had better ($P<0.05$) sperm mitochondrial integrity (MI), especially with 0.25% CPLL concentration. In conclusion, PM, PMI, and MI of Beetal goat spermatozoa were significantly improved in Tris-citrate extender containing 5% EY and supplemented with 0.25% CPLL.

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INTRODUCTION

Cryopreservation of male goat semen using egg yolk-based extenders is challenging because seminal phospholipase (PLA_2) interacts with the egg yolk (EY) and plasma membrane of spermatozoa to generate lysophospholipids, which are toxic to spermatozoa (Leboeuf *et al.,* 2000). The PLA_2 is found in male goats (Kamal *et al.,* 2022), rams (Roldan and Mollinedo, 1991), and bull semen (Marques *et al.,* 2000). Due to the dynamic nature of PLA_2 in male goat semen, the viability, motility, and velocity of spermatozoa decrease after dilution with EY extender (Riesco *et al.,* 2020). Therefore, researchers often suggest removing PLA_2 from goat seminal plasma by centrifugation or diluting its concentration with a buffer (Leboeuf *et al.,* 2000). However, this can adversely affect the quality of the spermatozoa and their ability to survive after thawing (Azeredo *et al.,* 2001). Alternatively, milk-based diluents without lipids or triglycerides or those containing lipase inhibitors, such as bulbourethral secreted monomeric glycoprotein 60-kDa (BUSgp60), are recommended to minimize sperm-lipase interaction (Purdy, 2006). In addition, extenders without EY or milk are appraised through supplementation of plant-based

proteins such as soy lecithin (Kundu *et al.,* 2000; Salmani *et al.,* 2014) for the stabilization of sperm plasma membrane during cryopreservation.

In an earlier study, carboxylated poly-l-lysine (CPLL), a polyampholytic substance, has been proposed as a cryoprotectant for mammalian cells (Matsumura and Hyon, 2009). It contains a specific ratio of amino acids and carboxyl groups and has antifreeze protein-like properties (Maehara *et al.,* 2013). This compound is impermeable to cell membranes (Maehara *et al.,* 2013) and acts as a membrane stabilizer like egg yolk (Matsumura and Hyon, 2009). Carboxylated poly-l-lysine at lower concentrations improves cryo-tolerance by binding to cell membranes and inhibiting ice recrystallization (Biggs *et al.,* 2017) during the freeze-thaw process (Breton *et al.,* 2000). These properties presumably render CPLL a suitable replacement for EY for stabilizing the sperm plasma membrane during cryo-survival.

It has been demonstrated that CPLL supplementation in semen extenders improves post-thaw quality and fertility of bovine spermatozoa (Fujikawa *et al.,* 2018; Küçük *et al.,* 2021). In buffalo bull semen extender, CPLL supplementation partially replaces glycerol as a cryoprotectant (Tariq *et al.,* 2020) and EY as a sperm plasma membrane stabilizer (Akhter *et al.,* 2020b). A recent study supports the notion that CPLL supplementation to Tris EY (15%)-based extender improves post-thaw quality of male goat spermatozoa (Zhang *et al.,* 2023). However, the scope of the above study seems quite limited, as only 15% of EY was evaluated in combination with various concentrations of CPLL; thus, providing little insight into the ability of CPLL to stabilize membranes under reduced concentrations of EY for the cryo-survival of goat spermatozoa. Therefore, the current study was designed to evaluate the impact of different combinations of EY and CPLL concentrations on the post-thaw quality of Beetal male goat spermatozoa.

MATERIALS AND METHODS

Animals and management: Six adult Beetal male goats, aged 1.8±0.20 years and weighing 35.3±0.96kg, were housed in the animal shed at the University of Veterinary and Animal Sciences, Lahore, Pakistan. The experiment was conducted in the year 2021 during the breeding season of goats (September and October). Each animal was fed green fodder at 10% of the body weight and 0.5kg of concentrate daily, with free access to water. In addition, they were allowed to graze for one hour daily.

Preparation of Carboxylated poly-l-lysine (CPLL): The CPLL solution was prepared by dissolving 1.3g succinic anhydride (Sigma Aldrich, St. Louis, MO, USA) in 10mL stock solution of 25% epsilon-poly-1-lysine (PLL, Macklin Biochemical Co., Shanghai, China), followed by incubation at 50°C for 1 hour to induce carboxylation (Matsumura and Hyon, 2009). Four different concentrations of CPLL were prepared by adding 0.1, 0.15, 0.20 and 0.25 mL of CPLL solution to make it 100mL of Tris-based extender containing 0, 5, and 15% EY, with 15% EY served as control (Salmani *et al.,* 2014).

Semen collection, processing, and thawing: The study involved collecting semen from six male goats twice a week for three weeks, using an artificial vagina. Initially, each ejaculate was evaluated for volume and sperm motility. Ejaculates having >60% motile sperm were pooled on each collection day to avoid individual variations. After determining the total volume and concentration of spermatozoa in each pooled ejaculate, Tris-Citric acid extenders containing three concentrations of EY (0, 5 and 15%), 7% glycerol and various CPLL concentrations (0, 0.1, 0.15, 0.20 and 0.25%) were used for dilution to achieve a final concentration of 50 million spermatozoa per mL. Diluted semen samples were packaged into 0.5 ml French straws, cooled, and equilibrated at 4°C for 4 hours before freezing at -196°C, using the conventional freezing method (Abbas *et al.,* 2021). Three straws from each combination of EY and CPLL per replicate (n=6) were pooled and thawed at 37°C for 10 seconds to assess spermatozoa quality.

Post-thawed semen evaluation using CASA: Post-thaw progressive motility (PM) and motion kinematics of spermatozoa were analyzed using a computer-assisted semen analyzer (CASA; AndroVision®, Minitube, Germany). Endpoints for sperm motility kinematics were adjusted, as described previously (Abbas *et al.,* 2021). At least 400 spermatozoa were assessed for each motility parameter in CASA using Mekler's chamber.

Post-thawed semen evaluation using phase contrast microscope: Plasma membrane integrity (PMI) and acrosomal membrane integrity (AMI) of spermatozoa were evaluated under a phase contrast microscope. For PMI, 50µL semen sample and 500µL hypoosmotic solution were incubated at 37°C for 1hr and examined under a phase contrast microscope (Olympus BX51, UK). A total of 100 spermatozoa were examined for characteristic swollen or coiled tails to confirm intact plasma membrane (Khan and Ijaz, 2008). For AMI, a 10µL semen sample was fixed with 4% paraformaldehyde, washed with buffer saline, and stained with 0.22% Coomassie brilliant blue stain. A total of 100 spermatozoa were counted under a phase contrast microscope to assess the acrosome membrane integrity characterized by its solid blue appearance (Larson and Miller, 1999).

Post-thawed semen evaluation using Flow cytometry: A flow cytometer (Attune™ NxT Acoustic, ThermoFisher® USA) was used to assess the DNA integrity, mitochondrial integrity, and necrosis of spermatozoa. Assays were standardized by analyzing unknown stained spermatozoa, positive heat-treated spermatozoa and negative controls (unstained). Spermatozoa with intact DNA, intact mitochondria, and necrosis were recorded in each sample, as described below. The target population of spermatozoa was identified using forward and side scatter voltage gating and 10,000 cells/events were recorded to generate a histogram based on the stained spermatozoa population, as shown in Fig. 1 (Molina *et al.,* 2023).

Sperm DNA integrity: To confirm the DNA integrity of spermatozoa, an established fluorescent dye Acridine orange (AO) was used, which emits specific fluorescence when intercalated to intact ds-DNA. An AttuneX® excitation/emission filter (500/526nm) was used to quantify spermatozoa with intact DNA fluorescing after AO staining.

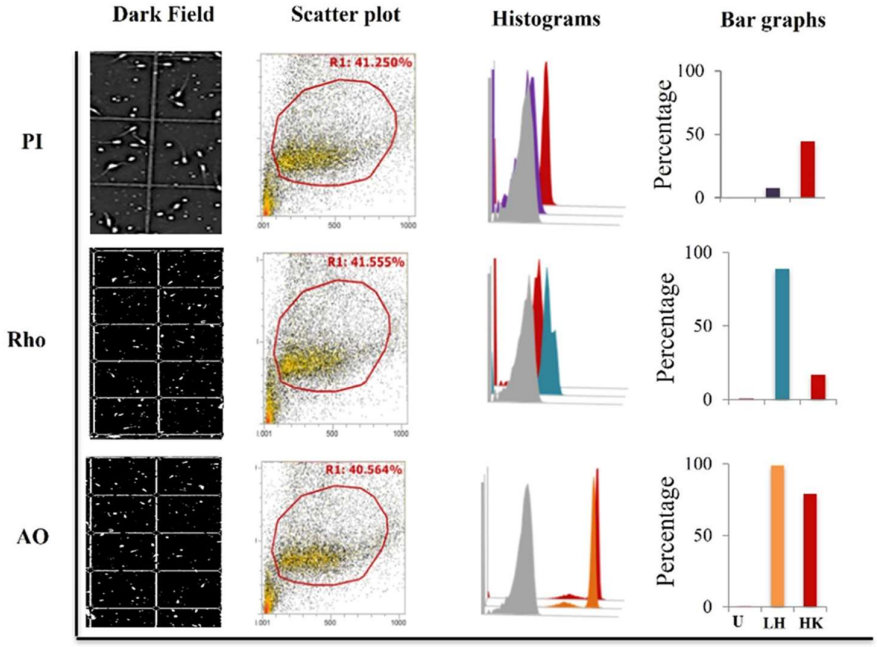

Fig. 1: Darkfield image acquired with the CASA motility assay shows intact sperm after thawing. Scatter plot shows forward scatter and side scatter gating of buck sperm. Histograms show the overlay of unstained (U), live healthy (LH) and heat-killed (HK) sperm. Bar graphs show %ages of Propidium Iodide, Rhodamine and Acridine Orange-positive sperm.

Briefly, 50µL semen was mixed with phosphate buffer solution (PBS) to make 1000µL volume and 5µL AO (10µg/mL) dye was added to the mixture. Later, the mixture was incubated in the dark for 10 minutes at 37°C, centrifuged at 3000 rpm for 5 minutes and the sperm pellet was resuspended in PBS (1000µL) before running on the flow cytometer (Mohammed *et al.,* 2015).

Sperm mitochondrial integrity (MI): To evaluate the mitochondrial integrity of spermatozoa, samples were stained with Rhodamine 123 dye (Rho123), which is sequestered inside active mitochondria having intact membrane. The spermatozoa with damaged mitochondria are not stained with Rho123; therefore, the AttuneX® excitation/emission filter (508/528nm) was used to quantify the Rho123-positive spermatozoa. A 50µL semen sample was diluted in 50µL of PBS and 5µL Rho123 (10µg/mL) was added. After incubating in the dark at 37°C for 10 minutes, the mixture was diluted with PBS to 1000µL, centrifuged at 3000 rpm for 5 minutes and the sperm pellet was suspended in PBS (1000µL) before running on the flow cytometer (Zou *et al.,* 2010).

Sperm necrosis: To assess necrotic spermatozoa, Propidium Iodide (PI) dye was used to detect the dead spermatozoa, as PI penetrates inside them and binds with DNA, whereas PI is impermeable to live spermatozoa. The AttuneX® excitation/emission filter (535/617nm) was deployed to quantify the PI-positive spermatozoa in the sample. The semen sample (50µL) was mixed with PBS to make a 1000µL volume, followed by the addition of 5µL of PI (10µg//ml). The sample was incubated in the dark (37°C for 10 minutes), followed by centrifugation (3000 rpm for 5 min). The supernatant was discarded and the sperm pellet was suspended again with PBS (1000µL) and immediately analyzed using a flow cytometer (Molina *et al.,* 2023).

Statistical analysis: Data were presented as mean with Pooled SEM and normalized using Shapiro-Wilk test. Main effects, EY and CPLL concentrations, and their interactions were analyzed using multifactor analysis of variance (ANOVA). Tukey's test was applied for pairwise comparisons. Data were analyzed using SPSS® (version 20, IBM, NY, USA) with a significance level of $P<0.05$.

RESULTS

Post-thawed semen evaluation using CASA: The EY as a main factor showed a significant ($P<0.05$) effect on progressive motility (PM) and motion kinematics of spermatozoa. Minimum values of PM and motion kinematics were observed in extenders having 0% EY with different CPLL concentrations. The extender containing 5% EY and 0.25% CPLL resulted in better ($P<0.05$) PM than the 0% and 15% EY extenders with different CPLL concentrations. However, motion kinematics (VCL, VSL, VAP and ALH) were similar ($P>0.05$) between 5 and 15% EY extenders with different CPLL concentrations. The CPLL as the main factor had a non-significant effect on PM and motion kinematics. Moreover, no interaction was found between EY and CPLL concentrations for PM and motion kinematics except for ALH (Table 1).

Post-thawed semen evaluation using Phase Contrast Microscope

Plasma membrane integrity: The main effects of EY and CPLL concentrations separately, as well as their interactions, on PMI of spermatozoa, were significant ($P<0.05$). In 0% EY extenders with different CPLL concentrations, the PMI decreased ($P<0.05$) compared to that for 5 and 15% EY extenders. The PMI was highest

Table 1: Comparison of post-thawed sperm progressive motility and kinematics after extending with 0, 5, and 15% EY supplemented with CPLL concentrations of 0.0, 0.1, 0.15, 0.2 and 0.25%. Data are presented as means with pooled SEM.

Parameters		CPLL (%)					Pooled SEM	P-value		
		0	0.1	0.15	0.20	0.25		EY	CPLL	EY*CPLL
	Egg yolk (%)									
PM (%)	0	5.5^{d}	5.1^{d}	4.1^{d}	5.8^{d}	5.7^{d}	0.7	0.00	0.40	0.11
	5	39.9^{abc}	47.4^{ab}	39.4^{abc}	43.0^{abc}	53.6^{a}	4.4			
	15	38.9^{abc}	36.5^{abc}	41.7^{abc}	35.5^{bc}	29.5^{bc}	2.0			
VCL (um/s)	0	19.1^{e}	15.9^{e}	17.8^{e}	20.9^{e}	18.5^{e}	1.8	0.00	0.71	0.17
	5	67.7^{abcd}	82.7^{abcd}	79.1^{abcd}	87.5^{abc}	89.0^{ab}	7.8			
	15	89.4^{ab}	95.8^{a}	88.7^{abc}	84.3^{abcd}	79.0^{abcd}	4.8			
VSL (um/s)	0	13.6^{cd}	12.9^{d}	9.8^{d}	11.7^{d}	11.6^{d}	1.6	0.00	0.69	0.50
	5	35.7^{ab}	37.0^{ab}	34.4^{ab}	41.5^{ab}	46.5^{a}	3.9			
	15	39.8^{ab}	41.5^{ab}	44.5^{ab}	41.8^{ab}	41.4^{ab}	3.9			
VAP (um/s)	0	14.7^{d}	13.8^{d}	11.3^{d}	14.9^{d}	12.3^{d}	1.7	0.00	0.57	0.07
	5	38.1^{abc}	42.5^{abc}	39.5^{abc}	49.9^{a}	51.3^{a}	3.3			
	15	47.6^{abc}	49.3^{ab}	46.9^{abc}	45.1^{abc}	41.6^{abc}	3.1			
ALH (um/s)	0	0.1^{e}	0.1^{e}	0.2^{e}	0.2^{e}	0.1^{e}	0.02	0.00	0.88	0.00
	5	0.6^{abcd}	0.7^{abcd}	0.7^{abcd}	0.7^{abcd}	0.8^{ab}	0.06			
	15	0.8^{a}	0.8^{a}	0.8^{abc}	0.7^{abcd}	0.6^{abcd}	0.02			

PM = Progressive motility, VCL= Curvilinear velocity, VSL = Straight linear velocity, VAP = Average path velocity, ALH = Lateral head displacement. For each semen quality parameter, different superscript letters between various concentrations of egg yolk (EY) and Carboxylated poly-l lysine (CPLL) concentrations represent a significant difference (P<0.05).

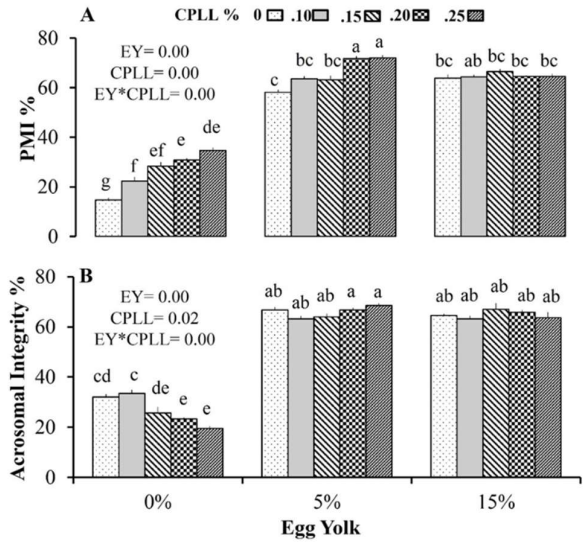

Fig. 2: Comparison of post-thawed sperm plasma membrane integrity (PMI) (A) and acrosomal integrity (B) for samples extended with 0, 5 and 15% EY and supplemented with different concentrations of CPLL (0, 0.10, 0.15, 0.20, and 0.25%). Different superscripts between different concentrations of EY and CPLL represent a significant difference (P<0.05). EY= Egg yolk; CPLL= Carboxylated poly-l lysine.

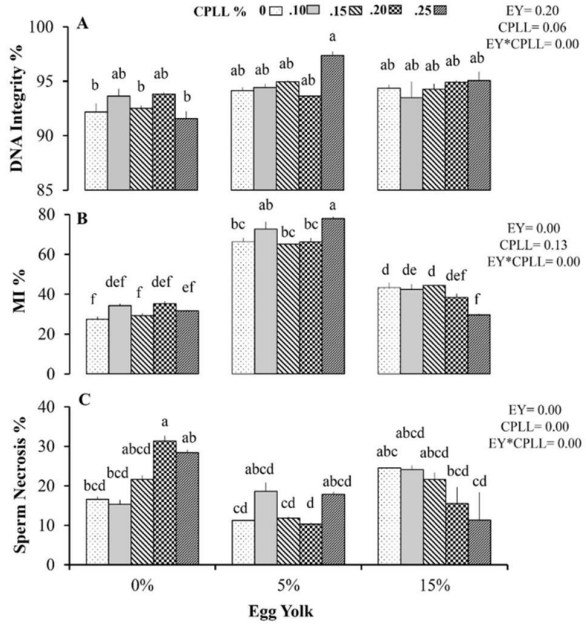

Fig. 3: Comparison of post-thawed sperm DNA integrity (A), mitochondrial integrity (MI) (B), and sperm necrosis (C) for samples extended with 0, 5, and 15% EY and supplemented with different concentrations of CPLL (0, 0.10, 0.15, 0.20, and 0.25%). Superscripts with different letters represent the significant difference (P<0.05) between CPLL and EY concentrations. EY= Egg yolk; CPLL= Carboxylated poly-l lysine.

(P<0.05) in 5% EY extenders with 0.20 and 0.25% CPLL concentrations compared to all other combinations of EY and CPLL (Fig. 2A).

Acrosomal membrane integrity: The main effects of EY and CPLL concentrations separately, as well as their interactions, on AMI of spermatozoa were found to be significant (P<0.05). Spermatozoa in 0% EY extenders showed lower (P<0.05) AMI compared to those in 5 and 15% EY extenders with different CPLL concentrations (Fig. 2B).

Post-thawed semen evaluation using Flow cytometry

Sperm DNA integrity: As the main factors, EY and CPLL concentrations separately had a non-significant (P>0.05) effect on DNA integrity, but their interaction showed a significant (P<0.05) effect on DNA integrity of Beetal male goat spermatozoa. DNA integrity of spermatozoa was similar (P>0.05) for extenders containing 0, 5 and 15% EY (Fig. 3A).

Sperm mitochondrial integrity: The EY concentrations had a significant (P<0.05) effect on MI of Beetal goat spermatozoa. The 5% EY extender had higher (P<0.05) sperm MI than 0 and 15% EY extenders with different CPLL concentrations. The CPLL as a main factor showed a non-significant (P>0.05) effect on sperm MI, while the interaction between EY and CPLL concentrations had a significant (P<0.05) effect. The highest MI of spermatozoa was observed in the extender with 5% EY having 0.25% CPLL compared to all other combinations of EY and CPLL (Fig. 3B).

Sperm necrosis: Both the main effects of EY and CPLL concentrations separately and their interaction were significant ($P<0.05$) for sperm necrosis. The ratio of necrotic spermatozoa was higher ($P<0.05$) in the 0% EY extender having 0.2% CPLL than in the 5% EY + CPLL 0, 0.15, and 0.2% and 15% EY extender + CPLL 0.2 and 0.25% (Fig. 3C).

DISCUSSION

The current study was designed to determine the effects of supplementation of different concentrations of CPLL with the decreased concentrations of EY in the Tris-citrate extender on the quality of post-thawed spermatozoa of male goats. Conventionally, EY protects plasma membrane of spermatozoa from cold shock; however, its higher concentration in extenders is not recommended due to its interaction with phospholipase A2 (PLA_2) present in the seminal plasma of goats (Monteiro *et al.,* 2022). Based on the low spermatozoa quality in extenders with a combination of 0% EY and 0.25% CPLL, it seemed that CPLL had little impact in the absence of EY, however, its supplementation in 5 or 15% EY extenders had a positive impact on the quality of post-thawed Beetal male goat spermatozoa. In addition, the combination of 5% EY with CPLL showed better results in terms of PM and PMI of spermatozoa than 15% EY with various CPLL concentrations. Similar results from a previous report on buffalo bull semen indicate an improvement in DNA integrity of spermatozoa during cryopreservation when 15% EY was replaced with 5% CPLL in tris-citric acid-based extender (Akhter *et al.,* 2020b).

Results of this study revealed that both EY and CPLL as main factors significantly improved structural and functional attributes of sperm in extenders having 5% EY compared to those having 15% EY supplemented with 0.25% CPLL. A more pronounced effect of CPLL with 5% than that with 15% EY seems to be due to comparatively low production of lysolecithin from EY coagulation by PLA_2 (Beltran *et al.,* 2013). Moreover, higher CPLL concentration at 5% EY can result in better extracellular binding and antifreeze conditions for spermatozoa, resulting in better PM, PMI, and MI values than those with 15% EY. In addition, higher concentrations of lipoproteins in EY have been suggested to mask the influence of CPLL on the plasma membrane of spermatozoa (Layek *et al.,* 2016). In support, similar findings regarding improvement in buffalo bull spermatozoa quality have been documented when a higher concentration of CPLL (0.75%) in Tris-citrate-EY extender was used (Tariq *et al.,* 2020). Another study on the storage of bottlenose dolphin liquid semen also suggested the use of 1% CPLL for improved motility of spermatozoa (Shimizu *et al.,* 2022).

In the current study, there was no difference in DNA integrity between 0% EY (0.1 and 0.20 CPLL), 5 and 15% EY extenders with various CPLL concentrations. This demonstrates that the CPLL preserved the DNA integrity of spermatozoa during cryopreservation. Similar findings have been documented for buffalo bull spermatozoa, where CPLL preserved DNA integrity by reducing lipid peroxidation during cryopreservation in a tris-citric acid-based extender (Akhter *et al.,* 2020a). In the current study, acrosomal integrity of spermatozoa in 5 and 15% EY extenders supplemented with various concentrations of CPLL did not differ. In contrast, CPLL alone (without EY) did not affect sperm acrosomal integrity, suggesting that EY is unavoidable for the cryoprotection of spermatozoa. These results differ from those of a previous study in which certain combinations of CPLL and glycerol improved the acrosomal integrity of buffalo bull spermatozoa (Tariq *et al.,* 2020). The discrepancy between the results of the two studies could be due to different concentrations of cryoprotectants, as the previous study used only 15% EY with various concentrations (0-7%) of glycerol.

Conclusions: Based on the results of the present study, it can be concluded that the addition of 0.25% CPLL in 5% EY extender significantly improved the progressive motility, plasma membrane integrity, and mitochondrial integrity of post-thawed spermatozoa of Beetal male goats.

Authors' contribution: MIRK and IHS conceived the idea, designed the study, analyzed the data, and wrote the manuscript. MS and IHS performed flow cytometry. MSR helped in preparing the CPLL and semen extenders. AIC, AA, and IM took care of housing and feeding of animals and assisted in semen collection. MRY and NH helped with data analyses. All authors critically reviewed the manuscript and approved the final draft for submission.

Ethical approval: All experimental procedures used in this study were approved by the ethical review committee of the University of Veterinary and Animal Sciences, Lahore, Pakistan.

Conflict of interest: Authors declare no conflict of interests and have no financial or personal relationship(s), which may have inappropriately influenced the research and write-up of this manuscript.

Data availability: The data that support the findings of this study are available from the corresponding author upon reasonable request.

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