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Enriching egg yolk fractions-tris-based extenders with cow skimmed milk improved the cryopreservation of bull sperm

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Abstract

The effects of substituting whole egg yolk (WEY) in a tris-citrate-based extender with two derived fractions, including a buffer-soluble fraction (BSF) and an ammonium sulfate insoluble yolk fraction, on the freezability of bull sperm were investigated. The BSF and ammonium sulfate insoluble yolk fraction levels of egg yolk in the respective diluents were consistent with their extracted values from an equivalent volume (20.00%) of egg yolk in the control diluent. The extenders were then enriched with 0.00, 5.00, and 10.00% (v/v) of pasteurized skim milk. Semen samples were collected weekly over five consecutive weeks from six adult bulls, and the standard ejaculates were pooled. The pooled semen was subsequently divided into seven experimental extenders and frozen in 0.50 mL French straws. Various sperm quality parameters, including kinematics, acrosome integrity, capacitation, and DNA fragmentation, were evaluated post-thawing. Results indicated that sperm kinematics and the percentage of acrosome-reacted sperm in experimental extenders were not significantly different from those in the control group. The milk-free ammonium sulfate insoluble yolk fraction extender exhibited higher percentages of capacitated sperms, but lower percentages of spermatozoa with intact DNA compared to the WEY extender sperm. The addition of 10.00% milk into the BSF diluent resulted in a significant increase in the proportion of sperm with intact DNA and a notable decrease in the percentage of sperm with partially fragmented DNA compared to the control. In conclusion, a BSF extender enriched with 10.00% cow's skimmed milk is recommended as a substitute for WEY in the cryopreservation of bull semen.

Introduction

Egg yolk is essential in sperm cryopreservation, serving as a cryoprotectant to prevent cold shock and as a storage factor to maintain sperm viability.[1] However, its granules, high-density lipoproteins, certain granular components, and specific minerals can be cytotoxic,[1] impede sperm respiration,[2] hinder sperm metabolism,[3] and interfere with laboratory assays and computer-assisted sperm analysis.[4] Moreover, it has been reported that progesterone[5] and high-density lipoproteins[6] of egg yolk can promote sperm capacitation while compromising the viability of cryopreserved sperm cells. Other draw-backs of including the whole egg yolk (WEY) in tris-based diluents are the variable composition and possible contamination by some microbial agents, which can result in reduced fertilization capacity.[7] To mitigate these dis-advantages, there is an urgent need to replace WEY with its cryoprotective fractions or alternative substitutes.[8]

According to the literature review, plant-derived egg yolk substitutes in sperm-freezing media have yielded mixed results. In this context, the ability of Andromed®, an egg yolk-free diluent, to preserve the quality of frozen-thawed goat sperm and maintain fertility was found to be comparable to that of Optidyl®, an egg yolk-based diluent.[9] However, European red deer spermatozoa demonstrated superior viability and functionality when preserved in a yolk-based diluent (Bovidyle®) compared with egg yolk-free alternatives (BioXcell® and Bovifree®).[10]

Contamination of semen diluents can be minimized while preserving animal-derived components by extracting the low-density lipoprotein (LDL)-rich fraction of egg yolk or employing filtration techniques.[11] Research indicates that straightforward methods, like syringe filtration, can reduce yolk turbidity and improve sperm cold tolerance when integrated into the sperm expanders designed for dogs and cats.[11] Additionally, the clarified egg yolk-tris-glycerol, the water-soluble fraction of the tris- glycerol-egg

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yolk diluent obtained through centrifugation, has been shown to yield results comparable to those of the egg yolk-tris-glycerol extender while facilitating metabolic assays.[2]

Chicken egg yolk plasma primarily consists of LDLs, which account for approximately 85.00% of its weight, while the remaining 15.00% comprises protein components.[12] It is prepared by separating the large granules from the WEY through centrifugation. The protein component of egg yolk plasma, known as livetin, precipitates when treated with 40.00% ammonium sulfate, resulting in a liquid that is rich in LDLs.[13] Purifying LDLs from egg yolk helps eliminate harmful substances that inhibit sperm respiration and motility.[14] Thus, removing these harmful substances through LDL purification may enhance the kinematic indices of frozen-thawed sperm cells.[15] According to the literature review, incorporating milk components with egg yolk enhances sperm cryopreservation across various species. For instance, adding 5.00% egg yolk to a milk-based extender improved the quality of ram spermatozoa stored at 5.00 ˚C.[16] Furthermore, incorporating skim milk from cows or sheep into tris-LDL or tris-egg yolk plasma diluents significantly enhances the viability, motility, and membrane integrity of ovine sperm after freezing and thawing.[17] This combination enables a reduced concentration of LDLs in tris-based diluents for buffalo semen, thereby optimizing sperm cryopreservation and minimizing the reliance on animal-derived compounds in the extender formulation.[7,14] However, the interaction between LDLs and other extra-cellular cryoprotectants, such as skimmed milk, remains poorly understood. The concentration of various lipoproteins in the diluent and their interaction with other components, including those derived from milk, may influence the functional and structural aspects of sperm cells during cryo-preservation.[13] Therefore, this study investigated the effects of combining different concentrations of cow's skim milk with egg yolk fractions in a tris-based extender on the quality of the frozen-thawed bull sperm.

Materials and Methods

Extenders preparations. To prepare a buffer-soluble fraction (BSF) of egg yolk, an equal volume of egg yolk collected from four fresh eggs (20.00 mL) was mixed with tris buffer as 3.785 g of Tris aminomethane (Merck, Darmstadt, Germany), 2.11 g of citric acid (Merck Millipore, Burlington, USA), 1.60 g of fructose (Merck), 100,000 units of penicillin G (Meiji Seika Co., Tokyo, Japan), and 100 mg of streptomycin (Shafa Darou Co., Tehran, Iran) in 100 mL of distilled water, and homogenized using a magnetic stirrer for 1 hr at 4.00 ˚C. After two cycles of centrifugation (9,000 *g* for 45 min at 4.00 ˚C), the supernatant was collected as a BSF and stored at 4.00 ˚C. The ammonium sulfate insoluble yolk fraction (ASIF) was prepared by incorporating 40.00% w/v ammonium sulfate (Merck) into the BSF and homogenizing the mixture at 4.00 ˚ C for 1 hr. Following two rounds of centrifugation, the supernatant was transferred to a sterile, semi-permeable cellulose tube with a molecular weight cutoff of 12,000 - 14,000. The tubes were then placed into cylinders filled with a buffer solution for 36 hr. The dialysates were exchanged three times at 8-hr intervals. Subsequently, the fluid inside the dialysis tubes was centrifuged at 9,000 *g* for 45 min at 4.00 ˚ C. The supernatant was collected as the ASIF.[17] The final osmotic pressure and pH of the egg yolk fractions were adjusted to 320 - 325 mOsm kg^{-1} and 7.00, respectively, by adding distilled water and tris solution. The prepared BSF and ASIF were combined with varying amounts of pasteurized skimmed cow's milk (0.00, 5.00, and 10.00 mL; depending on the treatment) and glycerol (7.00 mL), and then brought to a final volume of 100 mL with buffer solution.

Semen preparation. During each day of semen collection, an equal portion of the mixture was obtained from six adult Holstein bulls (5 years old) using an artificial vagina. Sperm concentration and motility were assessed using an Accucell photometer (IMV, L'Aigle, France) and light microscopy (Scientific Instrument Co., Sunnyvale, USA). The concentration of ejaculates should exceed 1.00×10^9 *per* mL, and sperm motility must surpass 75.00%. The prepared semen pool was divided into seven aliquots and diluted with various experimental diluents to achieve a final concentration of 4.00×10^7 sperm *per* mL. After cooling for 3 hr at 4.00 ˚C, the diluted semen samples were packed into 0.50 mL straws using an automated filling and sealing machine (MPP Uno; Minitube, Tiefenbach, Germany). The straws were refrigerated for 3.50 hr before being frozen with a semi-automatic sperm-freezing machine (Minitube). The Medical Ethics Committee of Yasuj University of Medical Sciences, Yasouj, Iran, approved the experimental procedure for animal experiments (Approval ID: IR.YUMS.REC.1404.073).

Semen evaluation post-thaw. Four straws from each treatment group were thawed and pooled at 37.00 ˚ C for 35 sec. The viability of the sperm was assessed using the supra-vital Eosin-Nigrosin staining method. Live sperm cells appeared unstained, while those that stained red were considered dead.[18] The hypo-osmotic solution test was conducted to evaluate the plasma membrane integrity. The percentage of sperm cells exhibiting swollen and coiled tails, which indicates they are alive and normal, was recorded as a positive response rate to the hypo-osmotic solution.[19] The computer-assisted sperm analyzer (Test Sperm 3.2; Video Test, St. Petersburg, Russia) was utilized to evaluate sperm kinematics and motility characteristics. Calcium-dependent changes associated with capacitation and the status of the sperm acrosome were assessed using a chlortetracycline fluorescence assay and a fluorescent light microscope (Zeiss, Oberkochen, Germany).[20] Sperm DNA fragmentation, specifically double-strand breaks, was evaluated using the sperm chromatin dispersion test with a commercial kit (Houshmand Fanavar Co., Tehran, Iran). The sperm chromatin dispersion assay was employed to assess sperm DNA fragmentation.[21] The procedure involved mixing the sperm samples with low-melting agarose and placing them on a prepared glass slide. After cooling and removing the coverslip, the slides were treated with an acid solution to denature the DNA, followed by a neutralizing and lysing solution. The slides were then dehydrated with ethanol and stained. Under a microscope, sperms exhibiting a large halo around the head were considered to have intact DNA, whereas those with a smaller halo or no halo were classified as having partially and completely fragmented DNA, respectively (Fig. 1).

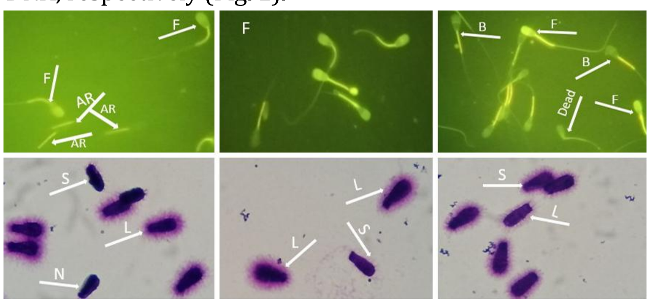

Fig. 1. The images illustrate acrosome integrity (top row with a green background) and DNA fragmentation (bottom row with a purple background) tests in bull sperm (400× magnification). In the top row, F represents intact acrosome, B indicates capacitated sperm, and AR denotes acrosome-reactive sperm. In the bottom row, L signifies a large halo (indicating intact DNA), S represents a small halo (indicating partially fragmented DNA), and N indicates the absence of a halo (indicating completely fragmented DNA).

Statistical analysis. The experimental protocol was replicated five times over five consecutive weeks, and the data were analyzed using one-way ANOVA in R Software (version 4.0. 2; R Foundation for Statistical Computing, Vienna, Austria). Means were compared using Tukey's multiple comparison test and expressed as mean ± standard error of the mean. Statistical significance was set at a probability level of 0.05.

Results

Sperm motility parameters. Total motility, progressive motility, viability, and the percentage of positive hypo-osmotic solution test (sperm did not differ significantly among the treatment groups ($p > 0.05$; Table 1). In the BSF - 0.00% milk, ASIF - 5.00% milk, and ASIF - 10.00% milk treatments, there was a significant reduction in the percentage of low motile spermatozoa compared to the control group ($p < 0.05$). Furthermore, the beat cross frequency in the BSF - 10.00% milk extender was superior to that in the ASIF - 0.00% milk and ASIF - 5.00% milk treatments ($p < 0.05$).

Sperm acrosome integrity and capacitation status. The results for acrosome integrity, capacitation status, and DNA fragmentation of cryopreserved sperm cells in different experimental extenders are shown in Table 2. The uncapacitated sperms with intact acrosomes did not show a significant difference between the treatments and control ($p > 0.05$). However, after freeze-thawing, this sperm quality attribute in ASIF - 5.00% milk and ASIF - 10.00% milk extenders was superior to that in the BSF - 0.00% milk diluent ($p < 0.05$). The percentage of capacitated sperms displayed a significant difference

Table 1. Viability and kinematic parameters of bull spermatozoa after freeze-thawing with different experimental extenders.

Parameters	Control	ASIF-milk (%)			BSF-milk (%)			*p*-value	SEM
		0.00	5.00	10.00	0.00	5.00	10.00		
Live sperm	74.93	71.96	75.66	73.98	72.89	74.35	75.42	0.34	1.23
HOST	56.37	45.87	51.45	50.04	42.45	52.47	51.91	0.10	3.29
TMS (%)	65.40	67.46	67.30	65.70	69.86	70.50	64.12	0.90	3.69
PMS (%)	36.80	39.48	41.68	39.16	43.80	45.30	39.66	0.69	3.51
NPMS (%)	7.64	10.26	9.40	9.88	8.92	7.80	6.80	0.04	0.09
LMS (%)	20.94^{a}	17.72^{ab}	16.22^{b}	16.68^{b}	17.16^{b}	17.40^{ab}	17.68^{ab}	0.01	0.99
VCL (μm sec^{-1})	68.16	77.54	77.33	75.97	77.48	77.99	69.80	0.70	4.87
VSL (μm sec^{-1})	24.58	24.01	24.17	23.30	27.73	29.97	26.11	0.34	2.25
VAP (μm sec^{-1})	27.82	28.06	29.12	27.32	31.50	33.57	29.17	0.53	2.46
STR	0.80	0.79	0.76	0.76	0.79	0.79	0.79	0.26	0.01
LIN	0.31	0.31	0.27	0.27	0.31	0.32	0.32	0.24	0.02
ALH (μm)	1.39	1.72	1.70	1.67	1.56	1.49	1.35	0.14	0.12
WOB	0.37	0.36	0.33	0.33	0.37	0.38	0.37	0.32	0.02
BCF (Hz)	15.32^{ab}	15.07^{b}	15.10^{b}	15.27^{ab}	15.75^{ab}	15.82^{ab}	16.01^{a}	0.004	0.23
MAD (°)	55.04	54.73	54.77	55.04	54.92	54.45	54.62	0.89	0.28

TMS: Total motile sperm; PMS: Progressive motile sperm; NPMS: Non-progressive motile sperm; LMS: Low motile sperm; VCL: Curvilinear velocity; VSL: Straight-line velocity; VAP: Average path velocity; ALH: Amplitude of lateral head displacement; BCF: Beat cross frequency; LIN: Linearity of the curvilinear path (LIN = VSL/VCL); STR: Straightness of the average path (STR = VSL/VAL); MAD: Mean angular displacement; WOB: Wobble of the actual path about the average path (WOB = VAP/VCL); HOST: Hypo-osmotic solution test; WEY: Whole egg yolk; BSF: Buffer-soluble fraction; ASIF: Ammonium sulfate insoluble yolk fraction; SEM: Standard error of the mean.
^{ab} Means in the same row with different superscripts are significantly different ($p < 0.05$).

Table 2. Bull sperm functional parameters after freezing-thawing in different experimental diluents.

Parameters	Control	ASIF-milk (%)			BSF-milk (%)			*p*-value
		0.00	5.00	10.00	0.00	5.00	10.00	
UC - IA (%)	55.80±4.34[ab]	53.40 ± 4.81[ab]	73.75 ± 1.75[a]	66.25 ± 3.06[a]	42.00 ± 4.14[b]	63.80 ± 6.78[ab]	63.40 ± 6.04[ab]	< 0.01
CS (%)	1.80 ± 0.38[cd]	7.60 ± 0.51[a]	1.25 ± 0.25[d]	4.00 ± 0.40[b]	4.25 ± 0.47[b]	3.40 ± 0.61[bc]	1.00 ± 0.32[d]	< 0.01
AR (%)	43.40 ± 3.94[ab]	40.60 ± 6.78[ab]	25.75 ± 1.65[b]	29.50 ± 4.21[ab]	55.75 ± 3.90[a]	36.20 ± 8.48[ab]	35.40 ± 5.36[ab]	0.04
LH (%)	69.40 ± 1.46[bc]	56.60 ± 2.11[d]	79.50 ± 1.55[ab]	80.50 ± 2.90[a]	66.00 ± 3.24[c]	79.60 ± 2.44[a]	84.20 ± 2.37[a]	< 0.01
SH (%)	24.40 ± 1.17[ab]	34.80 ± 3.29[a]	16.50 ± 2.10[bc]	17.25 ± 2.17[bc]	27.25 ± 3.85[ab]	15.60 ± 2.62[bc]	12.60 ± 1.91[c]	0.02
NH (%)	6.20 ± 1.20[ab]	9.00 ± 1.70[a]	4.00 ± 0.71[ab]	2.25 ± 1.31[b]	6.75 ± 1.70[ab]	4.80 ± 0.97[ab]	3.20 ± 1.16[b]	< 0.01

UC - IA: Un-capacitated sperms with intact acrosome; CS: Capacitated sperms; AR: Acrosome reacted sperms; LH: Large halo or the percentage of spermatozoa with intact DNA; SH: Small halo or the percentage of spermatozoa with partially fragmented DNA; NH: No halo or the percentage of spermatozoa with fragmented DNA; WEY: Whole egg yolk; BSF: Buffer-soluble fraction; ASIF: Ammonium sulfate insoluble yolk fraction.

[abc] Means in the same row with different superscripts are significantly different ($p < 0.05$).

between the treatments ($p < 0.01$). The proportion of capacitated sperms in the ASIF - 0.00% milk, BSF - 0.00% milk, and ASIF - 10.00% milk treatments was higher compared to the WEY extender ($p < 0.05$). In contrast, the BSF - 10.00% milk and ASIF - 5.00% milk treatments did not show a significant difference regarding the percentage of capacitated spermatozoa compared to the control ($p > 0.05$). The percentage of acrosome-reacted cells in the experimental diluents did not significantly differ from that in the control group ($p > 0.05$). However, the proportion of capacitated spermatozoa in the ASIF - 5.00% milk diluent was lower than that in the BSF - 0.00% milk diluent ($p < 0.05$).

Sperm DNA integrity. The percentage of sperms with partially fragmented DNA decreased in the BSF - 10.00% milk diluent compared to the control group ($p < 0.05$). Additionally, the proportion of sperms with intact DNA in the BSF - 10.00% milk, BSF - 5.00% milk, and ASIF - 10.00% milk treatments was higher than that in the control group ($p < 0.05$). Furthermore, processing bull semen with the ASIF - 0.00% milk diluent harmed the percentage of sperms with intact DNA after freeze-thawing compared to the control group ($p < 0.05$). However, the proportion of sperms with intact DNA improved when the diluent was enriched with 10.00% milk ($p < 0.05$).

Discussion

Our results indicate that the motility and progressive motility of sperm in diluents containing LDLs did not exceed those in the control diluent. Additionally, DNA integrity was compromised in the frozen-thawed spermatozoa treated with ASIF diluent compared to the control. This finding contrasts with a previous report indicating that ASIF and egg yolk plasma outperformed WEY in preserving the quality of frozen-thawed bull sperm.[13] Research indicates that replacing egg yolk with LDLs can improve sperm quality during cold storage[22] and after the freeze-thawing processes.[23] However, LDLs do not consistently outperform egg yolk, and their effectiveness depends on factors, such as the dosage in the diluent,[24] variations in sperm membrane composition across different species,[25] and the species-specific composition of LDL itself.[26]

The cryoprotective properties of milk and egg yolk are attributed to their ability to remove bovine seminal plasma (BSP) proteins (BSP-A1/A2, BSP-A3, and BSP-30-kDa) from the seminal plasma.[27] The BPS proteins damage the spermatozoa membrane, increasing sensitivity during frozen storage.[28] However, LDL and milk casein micelles may improve sperm membrane integrity during cryopreservation by interacting with BPS proteins.[27]

Our results indicated that the proportion of low-motile spermatozoa stored in a diluent containing WEY (control) was significantly higher than that in several alternatives, including ASIF - 5.00% milk, ASIF - 10.00% milk, and BSF - 10.00% milk (Table 1). This finding aligns with a previous study that observed a slight reduction in the percentage of motile sperm in roosters when the levels of egg yolk in skim milk-based extenders were increased.[29] The researchers attributed this decline to the denser consistency of the yolk, which increased the viscosity of the medium and impeded sperm motility.

The incorporation of milk into diluents, particularly at a 10.00% concentration, showed beneficial effects on the non-kinematic quality attributes of sperm in the current study. Milk casein micelles are thought to act as a primary cryoprotectant, protecting bovine sperm during the freezing process with glycerol.[27]

Although the protective mechanism of casein micelles is not yet fully comprehended,[27] it has been suggested that milk caseins may serve a function similar to that of LDLs.[30] Skim milk contains casein micelles, which can segregate BSP proteins like egg yolk.[27] Incorporating milk into tris-egg yolk diluents may provide an advantage by compensating for the egg yolk's limited ability to separate BSP proteins during semen dilution, especially in ejaculates with low sperm counts, particularly when egg yolk is present in low concentrations in the extender.[27] Despite their similar effects, milk and egg yolk may exhibit synergistic properties due to the distinct nature of the molecules involved in their cold shock protective mechanisms,[27] a finding that was also confirmed in the present study. Additionally, synergistic effects were observed between the plant-derived compounds and LDLs. For instance, combining LDLs (prepared from egg yolk plasma) with a commercial diluent based on plant phospholipids (Andromed®, Minitube) has led to improvements in the straight-line velocity, average path velocity, and curvilinear velocity parameters of bovine spermatozoa following freezing and thawing.[31] Researchers have indicated that even low doses of LDLs added to an extender can enhance sperm motility indicators due to their synergistic effect with plant phospholipids.[31] Centrifuging egg yolks provides no additional benefits compared to using WEYs. Previous research has also produced conflicting findings regarding the cryoprotective effects of egg yolk plasma compared to the WEY. It was reported that sperm survival and motility characteristics improved in diluents containing dialyzed egg yolk compared to the non-dialyzed tris-egg yolk controls, likely due to a decrease in the concentration of cations, such as sodium and potassium.[32] The LDLs extracted from egg yolk, which do not contain levitin, have been shown to enhance the quality of frozen-thawed ram spermatozoa more effectively than diluents containing the BSF of egg yolk.[16] In contrast, García *et al.* found that substituting clarified egg yolk for whole fresh egg yolk in a tris-glycerol-based diluent resulted in a decrease in the percentage of sperms exhibiting progressive motility and an increase in sperms with low mitochondrial activity.[33] A preliminary study indicated that dialysis of egg yolk against the same buffer used in the extender formula did not enhance the motility of frozen-thawed bovine sperm.[34] Variations in the initial cholesterol-to-phospholipid ratio in sperm cell membranes across species, the percentage of egg yolk in extenders, methods of egg yolk preparation, refrigeration duration, and freezing techniques may account for the inconsistencies observed in previous studies.[33,35] In this context, it has been reported that dialyzed skimmed milk alone is inadequate for protecting bovine spermatozoa during the freezing and thawing processes, underscoring the essential role of the non-dialyzable components of skimmed milk in providing this protection.[34] Nevertheless, the inclusion of 10.00% milk in the experimental diluent composition improved the rate of sperms with intact DNA and decreased the proportion of sperms with partially fragmented DNA compared to the control (Table 2). In a previous study, in contrast with our findings, using various concentrations of LDLs (2.00, 4.00, 8.00, and 14.00%) instead of WEY (20.00%; v/v) in the formulation of a diluent composed of two solutions including solution A: TES-tris-fructose; 50.00% (v/v) and solution B: Skim milk; 50.00% (v/v), significantly enhanced buffalo sperm kinetic parameters, although it did not affect sperm DNA fragmentation and membrane integrity post-thawing.[15] The researchers interpreted their results to indicate that LDLs improved sperm motility parameters even at low concentrations by enhancing interactions with milk casein micelles.[15] However, Del Valle *et al.* found that adding casein to Salamon's diluent or a modified swimming-up medium, whether alone or in combination with egg yolk or palm oil, did not impact the quality of frozen-thawed ram sperm.[36] The researchers also noted that the cryoprotective effect of egg yolk may vary based on other components, such as sucrose, in the diluent medium and their concentrations.[36] Skim milk contains carbohydrates, while egg yolk contains lecithin, which may help maintain the integrity of the spermatozoa's lipoprotein envelope.[37] Therefore, synergistic effects may be anticipated. Moreover, lactose in milk is non-permeating and prevents intra-cellular crystallization by creating osmotic pressure outside the cell.[38] The incorporation of milk into the experimental diluents may also provide benefits, especially on sperm DNA fragmentation, by optimizing the lipid ratio in the diluent composition,[39] or enhancing the anti-oxidant properties of the diluents.[40]

In conclusion, substituting egg yolk fractions with WEY in an extender formulation increased the percentage of capacitated sperms, which may be beneficial for *in vitro* fertilization. Notably, the incidence of capacitated sperms with intact acrosomes, a key parameter for assessing *in vitro* fertility, did not differ significantly among the diluents. Unfortunately, this study did not include analyses of components, such as progesterone, calcium, high-density lipoprotein, LDL, or anti-oxidant enzymes in the prepared diluents. Furthermore, incorporating fertility tests and increasing the number of repetitions for DNA and acrosome integrity tests, currently set at five, would have strengthened the conclusions drawn from the study. However, unlike the ASIF diluent, the BSF diluent did not negatively affect the rate of sperms with intact DNA. When the ASIF-based diluent was enriched with 5.00% milk and the BSF diluent was supplemented with either 5.00 or 10.00% milk, the percentage of capacitated spermatozoa remained low after the freezing-thawing process, similar to that of the control group. The enrichment of the BSF diluent with 10.00% milk resulted in a significant increase in the percentage of sperms with intact DNA and a notable decrease in the proportion of sperms with partially fragmented DNA compared to the control. Consequently, replacing WEY with BSF in a tris diluent formulation is recommended only when the BSF is fortified with 10.00% cow's skimmed milk.

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Conflict of interest

The authors declare no conflict of interest.

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