RESEARCH ARTICLE

Cryoprotective Effects of Fulvic Acid on Buffalo Bull Spermatozoa: A Concentration-Dependent Response

Man R. Pandya[1*], Dinesh V. Chaudhari[1], Vinayak S. Naiknaware[1], Tushal G. Variya[1], Kiran H. Parmar[1], Dignesh K. Patel[1]

Abstract

The present study was aimed to evaluate the effect of fulvic acid supplementation in Tris-fructose-yolk-glycerol (TFYG) extender on sperm quality attributes of buffalo bulls at successive stages of cryopreservation. Semen ejaculates (n=12) collected from healthy breeding bulls using an artificial vagina were split into four aliquots and extended with TFYG containing 0.00 (control), 0.50, 1.00 and 1.50 % (w/v) fulvic acid. The samples were processed, equilibrated and cryopreserved. Progressive sperm motility was evaluated at the post-dilution, pre-freeze and post-thaw stages, while viability, morphological abnormalities, plasma membrane integrity and acrosome integrity were assessed at the post-dilution and post-thaw stages. The response to fulvic acid was distinctly biphasic. Post-thaw sperm motility and plasma membrane integrity were significantly ($p<0.01$) higher with 1.50 and 1.00 % fulvic acid, and viability with 1.50 %, compared to the control, whereas 0.50 % fulvic acid consistently reduced sperm motility, viability and plasma membrane integrity below the control, the reduction being statistically significant for viability ($p<0.05$). The extender with 1.50 % fulvic acid also maintained significantly better acrosome integrity ($p<0.05$) and lower sperm abnormalities than the lower concentrations and control. The findings indicate that fulvic acid confers cryoprotection above a threshold concentration, below which it exerts the opposite effect, and that 1.50 % (w/v) is the most effective inclusion level for buffalo semen cryopreservation.

Key words: Anti-oxidant, Buffalo bull, Cryopreservation, Fulvic acid, TFYG Extender.

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Introduction

India is the largest milk producing nation in the world, with an output of 239.3 million tonnes during 2023-24 (DAHD, 2023-24), and maintains 109.85 million buffaloes (DAHD; 20th Livestock Census, 2019). The water buffalo (*Bubalus bubalis*) contributes 45 to 49 % of this milk, and its high-fat milk commands a premium price that underpins the profitability of smallholder dairying (Saxena *et al.*, 2020). Genetic improvement of the species rests upon artificial insemination with cryopreserved semen, yet AI coverage in India remains only 28 to 30 % (Panda *et al.*, 2021) and conception rates following insemination with frozen-thawed buffalo semen range between 42.5 and 51.1 % (Bhagat *et al.*, 2020), as buffalo semen freezes less satisfactorily than that of cattle (Kumaresan *et al.*, 2006).

Spermatozoa passing through dilution, cooling, equilibration, freezing and thawing sustain cumulative chemical, osmotic, thermal and mechanical injury, and approximately half the population loses viability and fertilising capacity over a single freeze-thaw cycle (Bucak *et al.*, 2008). Buffalo spermatozoa are particularly vulnerable, owing to a low cholesterol-to-phospholipid ratio, a high content of polyunsaturated fatty acids and a limited endogenous antioxidant reserve in seminal plasma (Longobardi *et al.*, 2021). Reactive oxygen species generated during freezing and thawing consequently overwhelm the antioxidant defence and initiate lipid peroxidation, and fortification of the extender with exogenous antioxidants has been repeatedly advocated to restore the pro-oxidant/antioxidant equilibrium (Bansal and Bilaspuri, 2011).

Attention has lately shifted from synthetic scavengers towards naturally occurring antioxidants of lower cytotoxicity (Ugur *et al*., 2019). Fulvic acid, the fraction of humic substances soluble in both acid and alkaline media, is rich in carboxyl, hydroxyl, phenolic and quinone groups, and exhibits pronounced metal-chelating and free radical scavenging activity (Plaza *et al.*, 2005). Its abundant hydrogen-bonding sites are further believed to improve the water-holding capacity of sperm proteins and thereby retard their denaturation during freezing (Xiao

[1]Department of Veterinary Gynaecology and Obstetrics, College of Veterinary Science and Animal Husbandry, Kamdhenu University, Anand-388 001, Gujarat, India

Corresponding Author: Dr. Man R. Pandya, MVSc Scholar, Department of Veterinary Gynaecology and Obstetrics, College of Veterinary Science and Animal Husbandry, Kamdhenu University, Anand-388001, Gujarat, India. e-mail: manpandya23@gmail.com

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et al., 2018). Literature on fulvic acid in bubaline semen cryopreservation nevertheless remains meagre, being confined to a single caprine trial and isolated investigations conducted outside India, with no systematic evaluation for Indian buffalo breeds. Since the antioxidant activity of quinone-bearing molecules is concentration-dependent and such compounds may act as pro-oxidants when present in excess or in insufficient amount (Watanabe and Forman, 2003; Shadyro *et al.*, 2024), identification of an optimum inclusion level is an essential prerequisite to any practical recommendation. The present study was therefore undertaken to evaluate graded concentrations of fulvic acid in TFYG extender on the sperm quality attributes of buffalo bulls at successive stages of cryopreservation.

Materials and Method

Location, Animals and Experimental Design

The study was carried out at the Central Sperm Station, College of Veterinary Science and Animal Husbandry, Kamdhenu University, Anand, Gujarat (22°35' N, 72°55' E) (India), from December 2025 to February 2026. Sexually mature, clinically healthy, pedigreed breeding buffalo bulls (n=4), maintained under uniform housing and feeding as per the Minimum Standard Protocol (Government of India, 2000), were used. Semen was collected twice weekly during early morning hours using a sterilized artificial vagina (Danish model, IMV Technologies, France) maintained at 42-45°C. Twelve ejaculates were evaluated at weekly intervals under a split-sample design. Ejaculates were held at 34°C until evaluation, and only those exhibiting mass activity ≥3.0 and initial progressive motility ≥75 % were processed further. Sperm concentration was estimated photometrically at 530 nm (Accucell, IMV Technologies, France).

Extender Preparation and Treatment Allocation

Tris buffer was prepared by dissolving 2.42 g tris (hydroxymethyl) aminomethane, 1.36 g citric acid monohydrate and 1.00 g D(-)-fructose in Milli-Q water, adding 6.80 mL anhydrous glycerol and making the volume up to 80 mL. The TFYG extender comprised 80 mL of this buffer, 20 mL fresh egg yolk, 1 lakh IU penicillin G sodium and 100 mg streptomycin sulphate. It was prepared fresh each morning, maintained at 34°C, and divided into four 25-mL aliquots. Fulvic acid (Sigma-Aldrich, USA) was added to three aliquots at 125, 250 and 375 mg to yield final concentrations of 5, 10 and 15 mg/mL (0.50, 1.00 and 1.50 % w/v), the fourth aliquot served as untreated control. Each ejaculate was split into four equal aliquots and diluted at 34°C with the respective extender to a final concentration of 100×10^6 spermatozoa/ mL.

Cryopreservation and Sperm Quality Evaluation

French mini-straws (0.25 mL) were filled and sealed using an automatic filling and sealing machine (IS4 System, IMV Technologies, France), cooled to 4°C over 1-h in a cold-handling cabinet and equilibrated for 4 h at 4°C. Freezing was carried out in a programmable bio-freezer (Digitcool 5300 CE ZH 350, IMV Technologies, France), as per bovine freezing protocol followed by 8-9 min exposure to liquid nitrogen vapour and plunging into liquid nitrogen (-196°C) for 18-24 h. Straws were thawed at 37°C for 30 s.

Progressive sperm motility was assessed subjectively under high power magnification of a Phase contrast microscope fitted with a biotherm stage set at 37°C at the post-dilution, pre-freeze and post-thaw stages. Viability and morphological abnormalities were recorded from eosin-nigrosin smears, acrosomal integrity by Giemsa staining following Hancock's fixation (Kutty *et al.*, 1996), and functional membrane integrity by the hypo-osmotic swelling test in a 150 mOsm/kg sodium citrate-fructose solution incubated at 37°C for 60 min (Jeyendran *et al.*, 1984), at the post-dilution and post-thaw stages. A minimum of 200 spermatozoa were counted per preparation.

Statistical Analysis

Data for various parameters were analysed using one-way ANOVA, followed by Duncan's multiple range test in SPSS (Statistical Package for the Social Sciences), Version 20.0 to determine significant differences among groups at $p<0.05$. Results were expressed as Mean ± SE.

Results and Discussion

The neat ejaculates averaged 4.70 ± 0.26 mL in volume, with a sperm concentration of 1036.82 ± 9.92 million/mL, mass activity 3.79 ± 0.05, progressive motility 82.19 ± 0.44 %, live spermatozoa 87.60 ± 0.37 %, morphological abnormalities 7.71 ± 0.16 %, HOST-reactive spermatozoa 85.89 ± 0.42 % and intact acrosomes 93.79 ± 0.37 %. These values lie within the range established for Indian riverine buffalo bulls maintained under organised farm conditions (Varghese *et al.*, 2016; Chaudhary *et al.*, 2017), confirming that the bulls were reproductively sound and that only ejaculates of acceptable initial quality entered the trial.

Progressive sperm motility declined highly significantly ($p<0.01$) at each successive stage of cryopreservation in all four aliquots (Table 1). The groups were statistically at par on dilution but diverged sharply thereafter. Post-thaw motility was highest with 1.50 % fulvic acid (70.83 ± 1.20 %), followed by 1.00 % (62.92 ± 0.74 %), both significantly ($p<0.05$) exceeding the control (52.08 ± 1.14 %), whereas the 0.50 % group declined below the control at both stages. Ramazani *et al.* (2023) recorded a closely comparable pattern in Azari

buffalo bulls, post-thaw total motility rising to 71.49 % with 1.7 % fulvic acid against 55.47 % in the control, while their two lowest levels of 0.2 and 0.5 % failed to improve upon the control. Xiao *et al.* (2018) obtained a similar gain in goat buck semen at 0.40 % with no further benefit at 0.60 %.

Table 1: Effect of fulvic acid (FA) supplementation in TFYG extender on progressive sperm motility (%) of buffalo bull semen at different stages of cryopreservation (Mean ± SE, n=12)

Treatment Group	On Dilution	Pre-Freeze	Post-Thaw
0.00 % (w/v) FA	81.25±0.89	$65.83±1.03^{A}$	$52.08±1.14^{A}$
0.50 % (w/v) FA	81.67±0.94	$64.17±1.03^{A}$	$49.17±1.03^{A}$
1.00 % (w/v) FA	82.08±0.96	$70.42±1.14^{B}$	$62.92±0.74^{B}$
1.50 % (w/v) FA	82.08±0.74	$75.83±1.20^{C}$	$70.83±1.20^{C}$

Means bearing different superscript within column (A, B, C) differ significantly ($p<0.05$)

Sperm viability declined significantly ($p<0.05$) from dilution to post-thaw stage in all treatments, however it was at par on dilution. Post-thaw sperm viability was significantly highest with 1.50 % fulvic acid (73.33 ± 1.07 %), followed by 1.00 % (64.92 ± 1.17 %), while the 0.50 % group fell significantly below the control (Table 2). Ramazani *et al.* (2023) likewise obtained significantly higher post-thaw viability at 1.1 to 1.7 % fulvic acid, and Abdel-Khalek *et al.* (2019) reported improved semen quality with a humic and fulvic acid mixture in heat-stressed Damascus bucks. Since eosin exclusion depends upon an intact plasmalemma, the higher live counts indicate that fulvic acid restricted peroxidative disruption of the sperm membrane during freezing.

Functional membrane integrity followed the same course, HOST reactivity falling significantly from dilution to post-thaw stage. Post-thaw HOST values were significantly highest at 1.50 % fulvic acid (69.58 ± 1.05 %), followed by 1.00 % (63.83 ± 1.26 %), while the 0.50 % group declined non-significantly below the control. This corresponds closely to the ineffectiveness of the 0.2 and 0.5 % levels recorded by Ramazani *et al.* (2023), whose post-thaw membrane functionality rose to 71.48 % at 1.7 % fulvic acid against 61.47 % in the control. It is worth to note that plasma membrane integrity has proved the attribute most consistently improved by fulvic acid across the available literature (Xiao *et al.*, 2018; Ramazani *et al.*, 2023), a consistency attributable to the chelation of Fe^{2+} and Cu^{2+} by the carboxylate functions of the molecule, which suppresses Fenton-type generation of the hydroxyl radical within an egg yolk-based medium (Winkler and Ghosh, 2018).

Acrosomal integrity also declined significantly from dilution to post-thaw stage. Only the 1.50 % group (83.42 ± 0.68 %) differed significantly from the control (79.33 ± 0.85 %), the 0.00, 0.50 and 1.00 % groups being statistically at par with one another in post-thawed semen (Table 2), indicating that the acrosomal membrane was not susceptible to the sub-optimal behaviour of fulvic acid evident in the other attributes. Panwar *et al.* (2024) similarly found acrosomal integrity unaffected by cysteine in Murrah buffalo semen despite significant gains in motility, viability and membrane integrity, and Tuncer *et al.* (2010) reported no effect of glutathione or cysteine in bull semen, suggesting that the outer acrosomal membrane is protected less readily than the plasmalemma because its damage during freezing arises as much from mechanical and osmotic insult as from lipid peroxidation.

Total morphological abnormalities increased highly significantly ($p<0.01$) from dilution to post-thaw stage, but did not differ significantly among the treatment groups at either stage ($p>0.05$). The numerically lowest values at both stages were nevertheless recorded at 1.50 % fulvic acid and the highest post-thaw value at 0.50 % (Table 2), a directional trend concordant with the other attributes. Morphological defects arise principally during spermatogenesis and epididymal maturation and are therefore not readily modified by an extender additive, and a comparable absence of effect has been reported for caffeine (Chavda *et al*., 2022), silymarin, curcumin and quercetin (Sharma *et al.*, 2024) and for glutathione and cysteine in bull semen (Tuncer *et al.*, 2010).

Considered together, the response to fulvic acid was distinctly biphasic. At 1.00 and 1.50 % every attribute improved over the control, whereas at 0.50 % motility, viability and HOST reactivity each declined below it, the reduction being statistically significant for viability and of consistent direction though not significant for the other two parameters. Polyphenolic and quinone-bearing compounds are redox-amphoteric, and at sub-optimal concentrations the semiquinone radicals generated during scavenging may reduce molecular oxygen or transition metals instead of terminating the peroxidative chain, so that the additive behaves as a pro-oxidant (Watanabe and Forman, 2003; Shadyro *et al.*, 2024). That the two lowest levels tested by Ramazani *et al.* (2023) behaved identically in a different buffalo ecotype lends weight to interpretation. The present findings accordingly place the transition from pro-oxidant to antioxidant behaviour of fulvic acid in buffalo semen between 0.50 and 1.00 % (w/v), with 1.50 % affording the greatest protection across the cryopreservation cycle.

Conclusions

Supplementation of the tris-fructose-yolk-glycerol extender with fulvic acid at 1.50 % (w/v) conferred the greatest protection upon buffalo bull spermatozoa throughout the cryopreservation cycle, improving post-thaw sperm

Table 2: Effect of fulvic acid (FA) supplementation in TFYG extender on sperm quality parameters of buffalo bull semen on dilution and at post-thaw stage (Mean ± SE, n=12)

Treatment Group	Sperm Viability (%)		Plasma Membrane Integrity (HOST) (%)		Acrosomal Integrity (%)		Total Sperm Abnormalities (%)	
	On Dilution	Post-Thaw	On Dilution	Post-Thaw	On Dilution	Post-Thaw	On Dilution	Post-Thaw
0.00 % (w/v) FA	84.00±1.10	62.17±1.05^{B}	81.83±1.04	59.50±1.11^{A}	91.33±0.69	79.33±0.85^{A}	8.91±0.19	11.42±0.37
0.50 % (w/v) FA	82.83±0.98	58.75±0.92^{A}	81.08±1.33	56.58±1.04^{A}	91.33±0.94	79.58±0.99^{A}	8.83±0.24	11.5±0.57
1.00 % (w/v) FA	84.33±1.08	64.92±1.17^{B}	82.91±1.06	63.83±1.26^{B}	91.83±0.74	81.17±0.53^{A}	8.41±0.14	11.33±0.35
1.50 % (w/v) FA	85.17±0.87	73.33±1.07^{C}	83.91±0.98	69.58±1.05^{C}	92.67±0.56	83.42±0.68^{B}	8.00±0.21	10.17±0.44

Means bearing different superscript within column (A, B, C) differ significantly ($p<0.05$)

quality over the untreated control, with 1.00 % conferring intermediate benefit. Inclusion at 0.50 % fulvic acid was not merely ineffective but consistently reduced motility, viability and plasma membrane integrity below control values, indicating that the protective action of fulvic acid emerges only above a threshold concentration. Since the response continued to rise at the highest level examined, 1.50 % represents the most effective concentration among those tested rather than a demonstrated optimum.

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