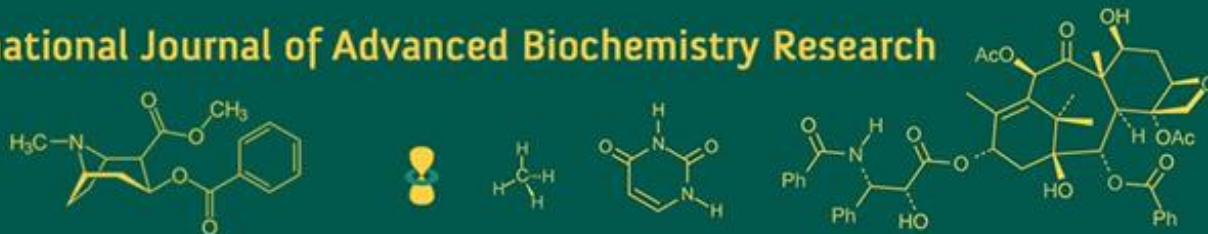


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Aloe vera-based semen extenders as a bio secure alternative to egg yolk for cryopreservation of buffalo bull semen: Evaluation of sperm kinematics and functional integrity

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Abstract

Egg yolk (EY) is routinely incorporated into semen extenders for bovine sperm cryopreservation because of its effective cryoprotective properties; however, concerns regarding microbial contamination, compositional variability, and biosecurity have prompted the search for plant-based alternatives. The present study evaluated the efficacy of *Aloe vera* gel as a substitute for egg yolk in a tris-based extender for the cryopreservation of Murrah Buffalo bull semen. Sixteen ejaculates collected from four healthy Murrah Buffalo bulls were divided into four treatment groups comprising a conventional extender containing 20% egg yolk (control) and extenders supplemented with 10%, 20%, or 30% *Aloe vera* gel. Pre-freeze and post-thaw sperm kinematics were evaluated using computer-assisted sperm analysis, while post-thaw sperm functional characteristics, including high mitochondrial membrane potential, mitochondrial superoxide production, and intact acrosome integrity, were assessed by flow cytometry. The EY extender maintained significantly ($p < 0.05$) higher pre-freeze and post-thaw total motility, progressive motility, curvilinear velocity, average path velocity, straight-line velocity, amplitude of lateral head displacement, and beat cross frequency than all AV-based extenders, whereas linearity and straightness were unaffected. Flow cytometric analysis demonstrated significantly higher HMMP and intact acrosome integrity together with lower mitochondrial superoxide production in the EY group. Increasing *Aloe vera* concentration resulted in a progressive decline in sperm kinematics, mitochondrial function, and acrosome integrity, accompanied by increased mitochondrial oxidative stress. Among the AV formulations, the 10% AV extender consistently performed better than the 20% and 30% AV extenders but remained inferior to the conventional EY extender. In conclusion, *Aloe vera* gel, at the concentrations evaluated, did not provide cryoprotection comparable to egg yolk and therefore cannot be considered a complete replacement for egg yolk in the cryopreservation of Murrah Buffalo bull semen.

Keywords: *Aloe vera*, egg yolk, semen extender, cryopreservation, Murrah buffalo bull, sperm kinematics, sperm functional integrity, mitochondrial membrane potential, acrosome integrity, flow cytometry

1. Introduction

Artificial insemination (AI) is one of the most important reproductive biotechnologies for accelerating genetic improvement in livestock through the widespread dissemination of superior germplasm while minimizing the transmission of venereal diseases associated with natural mating (Januskauskas and Zilinskas, 2002; Holt, 1997)^[18, 14]. The success of AI programs largely depends on the availability of high-quality cryopreserved semen capable of maintaining its structural and functional integrity after thawing. However, despite continuous improvements in cryopreservation protocols, the freeze-thaw process remains a major source of sperm injury, resulting in reduced post-thaw motility, membrane integrity, mitochondrial activity, and fertilizing ability.

During cooling and freezing, spermatozoa are subjected to multiple physical and biochemical stresses, including osmotic imbalance, protein denaturation, membrane destabilization, intracellular ice crystal formation, cellular acidosis, cytoskeletal disruption, and depletion of intracellular energy reserves (Urgur *et al.*, 2019). These alterations are accompanied by excessive production of reactive oxygen species (ROS), which are considered one of the principal causes of cryoinjury. Although physiological concentrations of ROS are essential for sperm capacitation and acrosome reaction, excessive ROS generation overwhelms the endogenous antioxidant defense system, resulting in oxidative stress (Agarwal *et al.*, 2005)^[1]. Consequently, lipid peroxidation of the sperm plasma membrane, mitochondrial dysfunction, DNA damage, enzyme inactivation, and apoptosis-like changes occur, ultimately compromising sperm viability and

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fertilizing potential (Bilodeau *et al.*, 2000; Jedrzejowska *et al.*, 2013; Pinto *et al.*, 2019) ^[8, 42]. Buffalo spermatozoa are particularly vulnerable to oxidative stress because their plasma membrane is rich in polyunsaturated fatty acids and contains relatively low concentrations of endogenous antioxidants, making them highly susceptible to lipid peroxidation during cryopreservation (Hu *et al.*, 2009; Hu *et al.*, 2010; Qadeer *et al.*, 2016) ^[16, 17].

To minimize cryoinjury, semen extenders containing permeating and non-permeating cryoprotectants are routinely employed during semen preservation. Among these, egg yolk (EY) has remained the gold standard non-permeating cryoprotective component for semen cryopreservation in several domestic species, including bulls, buffaloes, rams, bucks, stallions, boars, and even humans (Roca *et al.*, 1997; Wall and Foote, 1999; Hammadeh *et al.*, 2001; Pagl *et al.*, 2006; Stradaoli *et al.*, 2007; Malo *et al.*, 2010) ^[31, 43, 13, 25, 38, 21]. The cryoprotective action of egg yolk is mainly attributed to its phospholipids, cholesterol, and low-density lipoproteins (LDL), which stabilize the sperm plasma membrane, reduce cold shock, preserve membrane fluidity, and protect the acrosome during the freeze-thaw process. Consequently, egg yolk-based extenders consistently maintain satisfactory post-thaw sperm quality and fertility.

Despite its proven efficacy, the use of egg yolk is associated with several biological and practical limitations. Being an animal-derived product, egg yolk exhibits considerable batch-to-batch variation and carries the risk of xenobiotic and microbial contamination (Bousseau *et al.*, 1998; Aires *et al.*, 2003; Gil *et al.*, 2003b) ^[2]. Contamination may occur either through vertical (transovarian) transmission or by horizontal penetration of microorganisms through the eggshell after oviposition (Board and Tranter, 1995; Gantois *et al.*, 2009) ^[9, 12]. Pathogens including *Escherichia coli*, *Pseudomonas* spp., *Salmonella* spp., *Staphylococcus* spp., *Bacillus* spp., *Mycoplasma* spp., viruses, and fungal spores may survive within the nutrient-rich yolk matrix and remain viable even after cryopreservation (Setlow, 2006; Bielanski, 2007; Gantois *et al.*, 2009) ^[33, 7, 12]. Furthermore, egg yolk particles often interfere with microscopic observations, computer-assisted sperm analysis (CASA), and biochemical assays because of the presence of particulate material resembling spermatozoa. Increasing international regulations governing the movement of animal genetic resources have also emphasized the need for semen extenders free from animal-derived ingredients to improve biosecurity and facilitate international trade (WOAH, 2023; European Commission, 2020; DAFF, 2023; USDA, 2025) ^[10, 45, 41].

These limitations have stimulated intensive research toward the development of chemically defined and plant-derived semen extenders. Commercial soybean lecithin-based extenders such as AndroMed®, Bioxcell®, and Biociphos® have demonstrated encouraging results in several livestock species by eliminating the biosecurity concerns associated with egg yolk while maintaining acceptable post-thaw sperm quality (Aires *et al.*, 2003; Amirat *et al.*, 2005) ^[2, 3]. However, these commercial formulations remain relatively expensive, their exact compositions are proprietary, and their cryoprotective efficacy has not consistently equaled that of egg yolk under all experimental conditions. Therefore, the search for inexpensive, natural, and readily available plant-derived cryoprotectants continues.

Among natural plant products, *Aloe vera* (*Aloe barbadensis* Miller) has attracted considerable scientific interest because of

its diverse pharmacological and biological properties. For centuries, *Aloe vera* has been used in traditional medicine for its therapeutic, antimicrobial, antiinflammatory, antioxidant, wound-healing, hepatoprotective, and immunomodulatory activities (Marshall, 1990; Tiwari and Rao, 2002; Kosif *et al.*, 2008; Fakhrildin and Sodani, 2014) ^[22, 39, 19, 11]. The leaf gel contains numerous bioactive constituents, including polysaccharides (particularly acemannan), glycoproteins, amino acids, vitamins (A, C, B₂, and B₁₂), minerals, enzymes, flavonoids, and phenolic compounds (Quispe *et al.*, 2018) ^[30]. Use of *Aloe vera*-based extender for chilling and freezing collared peccary (*Pecari tajacu*) semen. These constituents possess potent antioxidant activity capable of scavenging excessive reactive oxygen species generated during cryopreservation, thereby reducing lipid peroxidation, preserving mitochondrial function, and maintaining sperm membrane integrity (Pinto *et al.*, 2020 and Patel *et al.*, 2022) ^[27, 28]. In addition, *Aloe vera* exhibits antimicrobial and antifungal activities that may further improve the hygienic quality of semen extenders while eliminating the biosecurity concerns associated with animal-derived products (Ros-santaella *et al.*, 2021 and Singh *et al.*, 2020) ^[32]. Owing to these properties, *Aloe vera* has recently been investigated as a natural cryoprotective alternative or antioxidant additive during livestock semen preservation.

Recent studies have reported encouraging outcomes following the incorporation of *Aloe vera* into semen extenders. Supplementation of *Aloe vera* improved post-thaw sperm motility, membrane integrity, acrosomal integrity, and antioxidant status in bull semen, suggesting that its bioactive constituents can alleviate cryopreservation-induced oxidative damage (Singh *et al.*, 2020b) ^[34, 35]. More importantly, a recent study in collared peccary (*Pecari tajacu*) demonstrated that 20% *Aloe vera* extract successfully replaced egg yolk in a Tris-based extender, maintaining satisfactory sperm quality after both chilling and freezing. The authors concluded that 20% *Aloe vera* could be used as an effective alternative substitute for egg yolk in the formulation of Tris extenders for semen preservation (Souza *et al.*, 2016) ^[37]. These findings indicate that *Aloe vera* possesses genuine cryoprotective potential and support its evaluation as a plant-derived substitute for conventional egg yolk extenders rather than merely as an antioxidant supplement. Despite these promising reports, information regarding the use of *Aloe vera* as a complete replacement for egg yolk in buffalo bull semen cryopreservation remains extremely limited. Moreover, the concentration-dependent effects of *Aloe vera* on buffalo spermatozoa have not been systematically investigated using advanced functional techniques. Most previous studies have evaluated only conventional semen quality parameters, whereas objective assessment of sperm kinematics by computer-assisted sperm analysis (CASA), together with flow cytometric evaluation of mitochondrial membrane potential, mitochondrial superoxide production, and acrosomal integrity, has received little attention. Since buffalo spermatozoa exhibit species-specific membrane characteristics and greater susceptibility to cryoinjury than many other domestic species, results obtained in other animals cannot be directly extrapolated to buffaloes. Therefore, further investigation is required to determine whether the promising cryoprotective effects of *Aloe vera* observed in other species can be reproduced in buffalo semen.

Accordingly, the present study was undertaken to evaluate different concentrations (10%, 20%, and 30%) of *Aloe vera* gel as a plant-based alternative to egg yolk in a Tris-based extender

for the cryopreservation of Murrah buffalo bull semen. Pre-freeze and post-thaw sperm kinematics were assessed using computer-assisted sperm analysis (CASA), while post-thaw sperm functional competence was evaluated by flow cytometric assessment of mitochondrial membrane potential, mitochondrial superoxide production, and acrosome integrity. The study was designed to determine whether *Aloe vera* could serve as a practical, economical, and biosecure alternative to conventional egg yolk-based extenders and to identify the concentration that provides optimal cryoprotection for buffalo spermatozoa.

2. Materials and Methods

2.1 Ethical approval

Animal experiments were conducted in accordance with the guidelines of the Institute Animal Ethics Committee (IAEC), ICAR-Central Institute for Research on Buffaloes. The experimental protocol was approved by the Institute Animal Ethics Committee under approval number IAEC-CIRB/2021/105. All experimental procedures involving animals were performed in accordance with the guidelines of the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Government of India.

2.2 Experimental Animals and Semen collection

Semen was collected from four healthy Murrah buffalo bulls (3-5 years old) maintained under uniform management conditions. A total of 16 ejaculates (four per bull) were obtained using an artificial vagina. Each ejaculate was evaluated for volume, colour, sperm concentration (Accucell bovine photometer, IMV Technologies, L'Aigle, France), and motility. Only ejaculates with $\geq 70\%$ sperm motility were included in the study.

2.3 Preparation of Aloe vera gel

Fresh mature leaves of *Aloe barbadensis* Miller were collected, thoroughly washed under running tap water, and rinsed with sterile distilled water. The outer rind was aseptically removed, and the inner gel was collected. The gel was homogenized using a sterile blender and filtered through sterile muslin cloth followed by Whatman No. 1 filter paper to remove fibrous material. The filtrate was centrifuged at $3,260 \times g$ for 30 min at 4°C , and the clear supernatant was collected and stored at 4°C until use. Fresh *Aloe vera* gel extract was prepared on the day of semen processing for extender preparation.

2.4 Preparation of semen extenders

A Tris-based extender comprising Tris (3.02%, w/v), citric acid (1.67%, w/v), fructose (1.0%, w / v), glycerol (6.4%, v/v), penicillin (1000 IU/mL; Sigma-Aldrich, St. Louis, MO, USA), and streptomycin (1000 $\mu\text{g/mL}$; Sigma-Aldrich, St. Louis, MO, USA) was used as the basal extender. Four experimental extenders were prepared: (i) EY (control) containing 20% (v/v) egg yolk, (ii) 10% AV containing 10% (v/v) *Aloe vera* gel, (iii) 20% AV containing 20% (v/v) *Aloe vera* gel, and (iv) 30% AV containing 30% (v/v) *Aloe vera* gel. All extenders were identical in composition except that egg yolk was replaced with the respective concentration of *Aloe vera* gel.

2.5 Semen processing and cryopreservation

Each ejaculate was divided into four equal aliquots and diluted with Tris-based extenders containing either 20% egg yolk (EY; control) or 10%, 20%, or 30% *Aloe vera* (AV) gel to achieve a final concentration of 80×10^6 spermatozoa/mL. Diluted semen was filled into 0.25-mL French straws (IMV Technologies,

France), sealed, cooled to 4°C , and equilibrated for 4 h. The straws were frozen in liquid nitrogen vapour at 4 cm above the liquid nitrogen surface for 12 min before being plunged into liquid nitrogen (-196°C) for storage. After 2-3 days, the straws were thawed at 37°C for 30 s and evaluated for pre-freeze and post-thaw sperm quality using computer-assisted sperm analysis (CASA), while post-thaw mitochondrial membrane potential, mitochondrial superoxide production, and acrosome integrity were assessed by flow cytometry.

2.6 Sperm quality assessment

2.6.1 Computer-assisted sperm analysis (CASA)

Sperm motility and kinematic parameters were evaluated in both pre-freeze and post-thaw semen samples using a Computer-Assisted Sperm Analysis (CASA) system according to a previously published method (Singh *et al.*, 2013) [36]. Prior to analysis, semen samples were diluted with prewarmed Tris buffer to a final concentration of approximately 40×10^6 spermatozoa/mL. Subsequently, 1 μL of diluted semen was loaded onto a pre-warmed (38°C) 20- μm Leja chamber slide, and sperm motion was assessed in five microscopic fields located near the centre of the chamber. The CASA system was operated at 37°C with a frame acquisition rate of 60 Hz and 30 captured frames. Instrument settings included a minimum contrast of 35, minimum cell size of 5 pixels, cell size of 9 pixels, and cell intensity of 110 pixels. Threshold values were established at 50 $\mu\text{m/s}$ for average path velocity (VAP) and 70% for straightness (STR), with VAP and straightline velocity (VSL) cut-off values of 30 $\mu\text{m/s}$ and 15 $\mu\text{m/s}$, respectively. The recorded kinematic parameters included total motility (TM), progressive motility (PM), curvilinear velocity (VCL), average path velocity (VAP), straight-line velocity (VSL), linearity (LIN), straightness (STR), amplitude of lateral head displacement (ALH), and beat-cross frequency (BCF).

2.6.2 Flow cytometric analysis

Post-thaw sperm functional parameters were determined using a CytoFLEX flow cytometer (Beckman Coulter Life Sciences) equipped with 488- and 633-nm excitation lasers. Green and orange fluorescence signals were detected through the FL-1 (525/40 nm) and FL-2 (585/42 nm) channels, respectively, while forward scatter was measured using a 488/8 nm band-pass filter. Samples were analyzed at a flow rate of 30 $\mu\text{L/min}$, and 10,000 sperm events were recorded for each sample. Data acquisition and analysis were performed using CytExpert software.

2.6.2.1 High mitochondrial membrane potential

High mitochondrial membrane potential (HMMP) was assessed using the fluorescent probe JC-1 (Invitrogen, Catalogue No. T3168) following the procedure described by (Kumar *et al.*, 2021) [20]. Washed spermatozoa were resuspended in 950 μL phosphate-buffered saline (PBS) and stained with JC-1 to obtain a final concentration of 2 μM . After incubation at 37°C for 15 min, excess stain was removed by washing with PBS. Samples were then analyzed by flow cytometry, where debris was excluded by appropriate gating and the proportion of sperm exhibiting JC-1 aggregate fluorescence was recorded as the percentage of sperm with high mitochondrial membrane potential.

2.6.2.2 Acrosome integrity

Acrosomal integrity was evaluated using *Pisum sativum* agglutinin (PSA) in combination with propidium iodide (PI)

according to the method described previously (Yadav *et al.*, 2023) [46]. Following washing, sperm pellets were resuspended in 950 μ L PBS, mixed with 50 μ L PSA stock solution (1.5 mM), and incubated at 37 °C for 10 min. Subsequently, 0.6 μ L PI was added, and the samples were incubated for an additional 5 min before flow cytometric analysis. The percentage of sperm with intact acrosomes was calculated using CytExpert software.

2.6.2.3 Mitochondrial superoxide production

Mitochondrial superoxide generation was determined using *MitoSOXTM Red* (Invitrogen, Catalogue No. M36008) following the method described by (Arjun *et al.*, 2022) [5]. Washed sperm pellets were suspended in 950 μ L PBS, and *MitoSOXTM Red* was added to achieve a final concentration of 0.125 μ M. After incubation in the dark at 37 °C for 10 min, excess dye was removed by washing with PBS. The stained spermatozoa were analyzed by flow cytometry, and the percentage of *MitoSOXTM Red*-positive sperm was recorded as an indicator of mitochondrial superoxide production.

2.7 Statistical analysis

All data were analyzed using IBM SPSS Statistics (Version 23.0; IBM Corp., Armonk, NY, USA). Data were initially examined for normality and homogeneity of variance before statistical analysis. Differences among the experimental groups were assessed using one-way analysis of variance (ANOVA). When significant treatment effects were detected, Duncan's Multiple Range Test (DMRT) was applied for post hoc comparisons to identify differences among treatment means. Statistical significance was established at $P \leq 0.05$. The results are presented as mean $\pm$ standard error of the mean (SEM).

3. Results

3.1 Effect of Aloe vera-based extenders on pre-freeze sperm kinematics

The pre-freeze sperm kinematic parameters obtained by CASA are presented in Table 1. Significant differences ($P \geq 0.05$) were observed among the experimental extenders for total motility (TM), progressive motility (PM), curvilinear velocity (VCL), average path velocity (VAP), straight-line velocity (VSL), amplitude of lateral head displacement (ALH), and beat-cross frequency (BCF), whereas linearity (LIN) and straightness (STR) did not differ significantly among the treatment groups.

The egg yolk (EY) extender exhibited the highest TM (81.83 $\pm$ 2.43%) and PM (27.37 $\pm$ 2.35%), followed by the 10% AV, 20% AV, and 30% AV extenders. Similarly, sperm velocity parameters, including VCL (298.67 $\pm$ 16.99 μ m / s), VAP (169.36 $\pm$ 4.24 μ m / s), and VSL (106.88 $\pm$ 4.23 μ m / s), were significantly higher ($P < 0.05$) in the EY group than in all *Aloe vera*-treated groups. Among the *Aloe vera* extenders, the 10% AV treatment showed significantly greater TM, PM, VCL, VAP, and VSL than the 30% AV group, while values for the 20% AV group were generally intermediate. Amplitude of lateral head displacement was greatest in the EY group (20.95 $\pm$ 1.34 μ m), followed by 10% AV, 20% AV, and 30% AV. Beat-cross frequency also declined progressively with increasing *Aloe vera* concentration, although the value observed in the 10% AV group was statistically comparable to that of the EY group. No significant differences ($P > 0.05$) were observed for linearity or straightness among the treatment groups.

3.2 Effect of Aloe vera-based extenders on post-thaw sperm kinematics

Post-thaw CASA parameters are summarized in Table 2. Cryopreservation resulted in a reduction in all sperm kinematic

parameters irrespective of extender composition; however, significant differences ($P < 0.05$) were observed among the experimental groups. The EY extender maintained significantly higher TM (70.50 $\pm$ 2.25%) and PM (24.80 $\pm$ 1.95%) than all *Aloe vera*-based extenders. Total motility decreased to 45.20 $\pm$ 1.65%, 38.60 $\pm$ 1.45%, and 33.20 $\pm$ 1.25% in the 10%, 20%, and 30% AV groups, respectively. Likewise, progressive motility declined significantly with increasing *Aloe vera* concentration. The highest post-thaw sperm velocity parameters were recorded in the EY group, including VCL (275.60 $\pm$ 15.80 μ m / s), VAP (155.40 $\pm$ 4.65 μ m / s), and VSL (97.80 $\pm$ 4.10 μ m / s), which were significantly greater ($P < 0.05$) than those observed in all *Aloe vera* treatments. Among the *Aloe vera* groups, the 10% AV extender consistently yielded higher velocity parameters than the 20% and 30% AV extenders. Post-thaw ALH and BCF also followed a similar trend, with significantly higher values in the EY group and progressive reductions as the *Aloe vera* concentration increased. In contrast, linearity (LIN) and straightness (STR) remained statistically similar ($P > 0.05$) among all treatment groups.

3.3 Effect of Aloe vera-based extenders on post-thaw sperm functional attributes

The post-thaw sperm functional characteristics assessed by flow cytometry are presented in Table 3. Significant differences ($P < 0.05$) were observed among the treatment groups for high mitochondrial membrane potential (HMMP), mitochondrial superoxide production, and intact acrosome integrity. The percentage of sperm exhibiting high mitochondrial membrane potential was highest in the EY group (49.63 $\pm$ 1.34%) and declined significantly with increasing concentrations of *Aloe vera*, reaching the lowest value in the 30% AV group (20.30 $\pm$ 0.59%). Conversely, mitochondrial superoxide production increased significantly ($P < 0.05$) with increasing *Aloe vera* concentration. The EY extender showed the lowest percentage of mitochondrial superoxide-positive sperm (47.35 $\pm$ 1.23%), whereas the highest value was recorded in the 30% AV group (78.20 $\pm$ 1.80%). Similarly, intact acrosome integrity was significantly greater in the EY group (53.25 $\pm$ 1.48%) than in all *Aloe vera* treatments. Acrosome integrity progressively decreased from 30.67 $\pm$ 1.77% in the 10% AV group to 20.08 $\pm$ 1.00% in the 30% AV group, indicating a concentration-dependent decline in acrosomal preservation following cryopreservation.

4. Discussion

The present study investigated the suitability of *Aloe vera* (AV) gel as a plant-derived alternative to egg yolk (EY) in a *Tris*-based extender for cryopreservation of Murrah buffalo bull semen. Overall, the conventional EY extender consistently preserved sperm quality better than all AV-based extenders throughout cryopreservation. Pre-freeze and post-thaw CASA analyses demonstrated significantly higher sperm motility and velocity parameters in the EY group, while flow cytometric evaluation further confirmed superior mitochondrial membrane potential, lower mitochondrial superoxide production, and greater acrosomal integrity. A progressive deterioration in sperm function was observed with increasing concentrations of AV, indicating that AV, under the present conditions, could not adequately replace the membrane-protective properties of egg yolk.

Cryopreservation is associated with multiple physicochemical stresses that compromise sperm survival. During cooling and freezing, spermatozoa experience osmotic imbalance, membrane phase transitions, intracellular ice crystal formation,

protein denaturation, electrolyte disturbances, and oxidative stress. These events collectively disrupt plasma membrane organization, reduce mitochondrial activity, impair ATP production, and ultimately decrease fertilizing ability (Watson, 2000; Aitken and Baker, 2004; Agarwal *et al.*, 2014) [44]. Buffalo spermatozoa are particularly susceptible to cryoinjury because their plasma membrane contains a high proportion of polyunsaturated fatty acids and relatively limited endogenous antioxidant defenses, making them highly vulnerable to lipid peroxidation during freeze-thawing (Andrabi, 2009; Kumar *et al.*, 2015) [4]. Therefore, effective membrane stabilization remains one of the principal requirements of semen extenders for buffalo semen cryopreservation. The superior performance of the EY extender observed in the present study agrees with extensive evidence supporting egg yolk as one of the most effective non-permeating cryoprotectants for mammalian semen preservation (Aires *et al.*, 2003; Amirat *et al.*, 2005) [3]. Egg yolk contains abundant low-density lipoproteins (LDL), phospholipids, and cholesterol, which adsorb onto the sperm plasma membrane, maintain membrane fluidity, reduce cold shock, and protect membrane proteins against cryodamage (Moussa *et al.*, 2002) [24]. These components also reduce the interaction between seminal plasma proteins and membrane phospholipids, thereby minimizing irreversible membrane destabilization during freezing and thawing. Consequently, spermatozoa cryopreserved in the EY extender maintained significantly greater structural and functional integrity than those preserved in AV-based extenders. Computer-assisted sperm analysis demonstrated that total motility, progressive motility, curvilinear velocity, average path velocity, and straight-line velocity declined significantly in all AV-treated groups, with the greatest reduction occurring at 30% AV. In contrast, linearity and straightness remained statistically unchanged among treatments, indicating that AV primarily affected the proportion of motile sperm and their swimming speed rather than altering the trajectory of sperm movement. Similarly, reduced amplitude of lateral head displacement and beat-cross frequency in AV groups suggest impaired flagellar activity resulting from insufficient ATP production. Since sperm motility depends largely on mitochondrial ATP synthesis and membrane integrity, deterioration of these parameters reflects inadequate cryoprotection during the freeze-thaw process (Mortimer, 2000; Versteegen *et al.*, 2002) [47]. Flow cytometric analysis further supported the CASA findings. Mitochondrial membrane potential (HMMP) was significantly higher in sperm preserved with EY than in all AV treatments, while HMMP progressively declined as AV concentration increased. Mitochondria are the principal source of ATP required for sperm motility, and maintenance of mitochondrial membrane potential is considered a reliable indicator of sperm functionality and fertilizing competence (Garner and Johnson, 1995; Peña *et al.*, 2009). Loss of mitochondrial membrane potential reflects disruption of the mitochondrial electron transport chain and reduced oxidative phosphorylation, ultimately leading to decreased ATP synthesis and impaired sperm movement. The reduced HMMP observed in AV-treated sperm therefore provides a mechanistic explanation for the lower motility and velocity parameters recorded following cryopreservation. Mitochondrial dysfunction was further evidenced by significantly greater mitochondrial superoxide production in AV-treated spermatozoa, particularly in the 30% AV group. Excessive generation of reactive oxygen species during cryopreservation is widely recognized as one of the major causes of sperm cryodamage because ROS initiate lipid

peroxidation of the plasma membrane, oxidize proteins, damage mitochondrial DNA, and trigger apoptotic pathways (Aitken and Baker, 2004; Agarwal *et al.*, 2014). Although *Aloe vera* contains numerous antioxidant constituents, including acemannan, polysaccharides, vitamins, flavonoids, and phenolic compounds (Marshall, 1990; Hamman, 2008; Surjushe *et al.*, 2008) [22], the present findings suggest that these antioxidants alone were insufficient to counteract oxidative damage during buffalo semen cryopreservation. Increasing AV concentration did not improve antioxidant protection but instead was associated with progressively higher mitochondrial superoxide production. This observation may indicate that higher concentrations of AV alter extender viscosity or osmolarity, interfere with membrane permeability, or reduce the availability of essential membrane-protective molecules required during freezing.

Preservation of acrosomal integrity is essential for successful fertilization because the acrosome contains hydrolytic enzymes required for zona pellucida penetration. In the present study, intact acrosome percentages were significantly higher in the EY group than in AV-treated groups, with increasing acrosomal damage occurring as AV concentration increased. These observations suggest that AV could not adequately stabilize the acrosomal membrane during cryopreservation.

Previous studies have demonstrated that LDL and phospholipids present in egg yolk effectively protect both plasma and acrosomal membranes against cold shock and osmotic injury (Moussa *et al.*, 2002; Watson, 2000; Amirat *et al.*, 2005) [24, 44, 3]. Consequently, superior acrosomal preservation in the EY group was expected and is consistent with the higher post-thaw sperm quality observed in the present investigation.

Although the present findings demonstrate inferior cryoprotective efficacy of AV compared with EY, they should be interpreted alongside previous studies conducted in other species. Souza *et al.* (2016) [37], working with collared peccary (*Pecari tajacu*), reported that a *Tris* extender containing 20% *Aloe vera* successfully replaced egg yolk during semen chilling and freezing, producing postthaw sperm motility, membrane integrity, viability, and morphology comparable to the conventional EY extender. Likewise, several investigations have reported beneficial effects of AV as an antioxidant supplement during semen preservation in cattle, sheep, goats, rabbits, and poultry. These studies attributed the protective effects of AV to its antioxidant, antimicrobial, antiinflammatory, and membrane-healing properties. However, the results of the present study indicate that these beneficial properties alone are insufficient to replace the complex membrane-stabilizing functions provided by egg yolk during buffalo semen cryopreservation.

The discrepancy between the present study and previous reports may primarily reflect species-specific differences in sperm membrane composition and cryotolerance. Buffalo spermatozoa possess a membrane lipid profile that differs substantially from many domestic and wild species, making them particularly susceptible to membrane destabilization and oxidative damage during freezing (Andrabi, 2009) [4]. Furthermore, differences in the source of *Aloe vera*, extraction procedures, gel processing, extender formulation, glycerol concentration, freezing protocol, and semen evaluation methods may also contribute to variable outcomes among studies. In the present investigation, fresh *Aloe vera* gel was directly incorporated into the extender. Although rich in bioactive compounds, the gel lacks the low-density lipoproteins,

phospholipids, and cholesterol that represent the principal cryoprotective constituents of egg yolk. Consequently, antioxidant activity alone was unable to compensate for the absence of these membrane-stabilizing molecules. An additional consideration is the concentration-dependent response observed in the present study. Increasing the concentration of *Aloe vera* from 10% to 30% progressively reduced sperm motility, mitochondrial membrane potential, and acrosomal integrity, while increasing mitochondrial superoxide production. These findings indicate that excessive concentrations of *Aloe vera* may adversely alter the physicochemical characteristics of the extender, particularly its viscosity due to the high content of mucilaginous polysaccharides, thereby impairing sperm movement and limiting effective interaction between the extender and the sperm plasma membrane. Similar observations were reported by Barbosa *et al.* (2020) [6], who found that supplementation of 20% *Aloe vera* extract did not improve post-thaw sperm quality compared with 10% *Aloe vera* and suggested that further standardization of the physicochemical properties of *Aloe vera*-based extenders is necessary for successful cryopreservation. Likewise, Moreira *et al.* (2021) [23] reported that extenders containing *Aloe vera* were less effective than conventional egg yolk-based extenders in preserving epididymal sperm quality, indicating that increasing the proportion of *Aloe vera* does not necessarily enhance cryoprotection. In agreement with these reports, the 10% *Aloe vera* extender consistently yielded superior sperm quality compared with the 20% and 30% formulations, suggesting that lower concentrations exert fewer detrimental effects. Nevertheless, even the 10% *Aloe vera* extender remained significantly inferior to the conventional egg yolk extender, demonstrating that reducing *Aloe vera* concentration alone was insufficient to achieve cryoprotection comparable with that provided by egg yolk. Overall, the findings of the present study demonstrate that *Aloe vera* gel, when used as the sole replacement for egg yolk, cannot provide sufficient cryoprotection for Murrah buffalo bull semen. The superior performance of the EY extender confirms the critical importance of membrane-stabilizing lipoproteins during cryopreservation. Nevertheless, the recognized antioxidant and antimicrobial properties of AV suggest that it may still possess value as a supplementary additive rather than a complete substitute for egg yolk. Future studies should therefore focus on purification of *Aloe vera* bioactive fractions, optimization of extraction techniques, standardization of gel composition, or its combination with membrane-protective compounds such as purified LDL, soy lecithin, liposomes, or other natural antioxidants. Such approaches may facilitate the development of a pathogen-free, economical, and plant-based extender capable of maintaining buffalo sperm function while reducing the biosecurity concerns associated with animal-derived ingredients.

5. Conclusion

The present study demonstrated that replacing egg yolk with *Aloe vera* gel in a Tris-based extender significantly compromised the cryosurvival of Murrah buffalo bull spermatozoa. Egg yolk-based extender consistently produced superior pre-freeze and post-thaw sperm kinematics, mitochondrial membrane potential, and acrosome integrity, while maintaining lower mitochondrial superoxide production than all *Aloe vera* treatments. Among the *Aloe vera* formulations, the 10% concentration performed better than the 20% and 30% extenders but remained significantly inferior to

the conventional egg yolk extender. Therefore, under the conditions of the present study, *Aloe vera* gel cannot be recommended as a complete replacement for egg yolk in buffalo semen cryopreservation, although further optimization and combination with membrane-protective components may improve its future applicability.

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Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

CRediT authorship contribution statement

Vinay Kumar: Writing - original draft, Visualization, Validation, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Pradeep Kumar:** Writing - review & editing, Resources, Investigation, Funding acquisition, Conceptualization. **Jasmer Dalal:** Writing - review & editing, Supervision, Software, Formal analysis. **R K Sharma:** Validation, Supervision, Formal analysis, Funding acquisition. **Meeti punetha:** Visualization, Supervision, Formal analysis. **Dharmendra Kumar:** Visualization, Validation, Supervision, Formal analysis. **Renu Bala:** Visualization, Validation, Supervision, Formal analysis, Data curation. **Priyanka Ghanghas:** Visualization, Validation, Supervision, Formal analysis.

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