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Research Article

Novel Antioxidant Humanin Analogue Restores Bull Semen Cryosurvivability

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The current study was intended to determine the effects of humanin analogue (HNG) supplementation on semen freezability and oxidative stress parameters of cryopreserved bull spermatozoa. A total of 24 semen ejaculates, from Frieswal breeding bulls, were selected for the experiment. Samples with ≥70% progressive motility, ≥500 million spermatozoa/mL, and >2 mL volume were utilized for further processing, supplementation, and cryopreservation. Each sample was divided into four equal groups and the experiment consisted of three treatment groups and one control group. In the control group, the spermatozoa were frozen in an extender without HNG, while in the other three groups, HNG supplementation was done at a concentration of 2, 10, and 20 μM, respectively. The fortification of HNG at a concentration of 10 and 20 μM significantly ($p < 0.05$) improved the percent post-thaw motility and kinetic variables as assessed by CASA, viability, acrosome- and plasma membrane-integrity as compared to 2 μM and control group. The supplementation showed significant ($p < 0.05$) controlled oxidative stress as exhibited by a reduction in lipid peroxidation and reactive oxygen species levels. The supplemented groups also exhibited significant ($p < 0.05$) improvements in total antioxidant levels, increased mean percentage of spermatozoa exhibiting high mitochondrial membrane potential, DNA integrity, acrosome-intact noncapacitated spermatozoa, and zona binding ability. In conclusion, the supplementation of humanin analogue at 10 and 20 μM significantly improved the post-thaw semen quality, functional parameters, and zona binding ability and controlled the oxidative stress in cryopreserved spermatozoa. Thus, the supplementation of its analogue may potentially prove to be a valuable approach for the improvement of semen freezability in crossbred Frieswal bulls.

1. Introduction

Humanin (HN), a mitochondrial-derived cytoprotective 24-amino acid polypeptide has emerged as a new metabolic regulator of the human body and is detected in most of the animal tissues. It has been quantified in different body fluids such as blood plasma, cerebrospinal fluid, ovarian follicular fluid, and in the seminal plasma specifying that it may be a secreted protein [1–6]. It has been targeted and researched recently because of its ubiquitous presence and verified positive outcomes on cell livability and physiological functions to counter varied types of stresses and induced trauma/insults both *in vivo* and *in vitro* [7].

It is worth mentioning that many of these models have oxidative stress as a common component, explaining the answers that the said protection against cell stresses is more general cytoprotective dynamics against oxidative stress [8]. The analogue of humanin (HNG) is now available with a changed amino acid sequence wherein the serine amino acid is replaced by glycine amino acid at the 14th position (Serine:14: glycine mutation), a replacement known to increase the biological activity of the polypeptide to thousand times [9].

Despite considerable progress in the field of sperm cryopreservation, the recovery rate of extended semen after thawing remains low and varies considerably between breeding

bulls. The post-thaw motility/viability is further poor in the case of crossbred bulls. Existing reports suggest that about 45% of ejaculates from crossbred bulls are nonfreezable [10]. Although there are many factors leading to damaged sperm function which results in poor recovery rates following freezing–thawing, the production of reactive oxygen species (ROS) such as hydrogen peroxide (H_2O_2), superoxide anions (O^{2-}), and hydroxyl radicals (OH^-) are known to cause severe damage [11]. It has been known that ROS directly damages the plasma membrane [12] causing leakage of ions and enzymes. This in turn distresses mitochondrial function and integrity of sperm DNA [13]. Thus results in loss of post-thaw sperm motility and dropped potency due to poor fertilization rates [14] and unfavorable variations in early embryonic development [15]. ROS also elicits premature capacitation in spermatozoa wherein the sperm show hyperactivation and exhibit capacitation-like changes. So, to get better post-thaw recovery rates following freezing–thawing, we require certain strong defensive strategies which can work against ROS and avert oxidative stress.

It has been established that mitochondrial-derived peptides, such as HN, have tremendous therapeutic potential against intracellular and extracellular insults particularly oxidative stress [16]. In men, HNG supplementation in the freezing medium has also been shown to protect sperm cells from freezing–thawing-induced sperm damage [17]. Furthermore, HN is known to be involved in the regulation of germ cell homeostasis and spermatogenesis at the cellular level [18]. In rats, it governed the fate of germ cell survival and death during proapoptotic stress to the testis [19], while in mice, intraperitoneal HN injections significantly reduced cyclophosphamide-induced germ cell apoptosis [18]. However, to the best of our knowledge, there is no information regarding the cytoprotective role of novel antioxidant HNG in cryopreservation-induced sperm cell injury in bull semen. Therefore, the aim of the present study was to determine the effects of HNG supplementation on semen quality and oxidative stress parameters of cryopreserved bull spermatozoa.

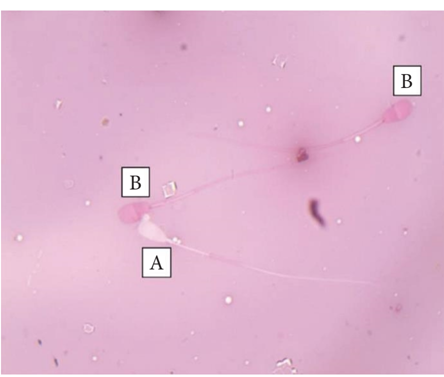

Figure 1: Photomicrograph showing live (colorless; A) and dead (pink colored; B) spermatozoa stained with Eosin–Nigrosin stain (1,000x).

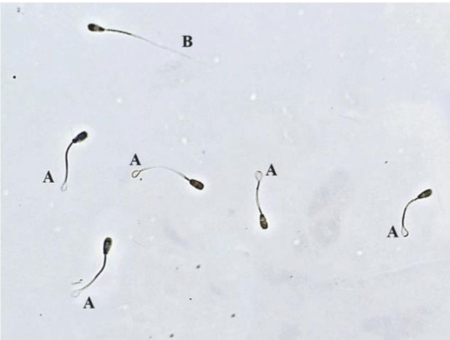

Figure 2: Hypoosmotic swelling test positive (tail coiled; A) and negative (uncoiled; B) spermatozoa 400x.

2. Materials and Methods

The proposed work was conducted at the Division of Cattle Physiology and Reproduction, ICAR-Central Institute for Research on Cattle, Meerut Cantt-250001 (UP) India. The Institute is located at 247 m elevation, a latitude of 29°N, and a longitude of 78°E. It has a monsoon-influenced humid subtropical climate characterized by hot summers and cold winters. Ethical approval was granted by the Institute's Animal Ethics Committee.

2.1. Experimental Animals, Semen Collection, and Selection of Ejaculates. Three healthy Frieswal (Holstein Friesian and Sahiwal cross; having 5/8 Holstein Friesian inheritance) breeding bulls maintained under uniform feeding and housing conditions with an average weight of 500–600 kg were utilized for the study. The ejaculates were collected early morning between 8 and 9 am in graduated glass tubes attached to the artificial vagina as per regular standard practice. A total of 24 semen samples, eight each from three Frieswal breeding bulls, were selected for the experiment. The part of fresh ejaculates was initially diluted (1 : 1) in Tris-fructose-egg yolk-glycerol extender (TEYG) and initial percent progressive motility was scored at 200x magnification with phase contrast microscope (Olympus BX 40) equipped with a thermostage (Tokai Hit, Thermo-control unit). Samples with ≥70% progressive motility, ≥500 million spermatozoa/mL, and >2 mL volume were utilized for further processing, supplementation, and cryopreservation. If any of the ejaculate failed above criteria, it was rejected to avoid any incongruity in the experiment. The volume (mL), pH, and sperm concentration (million/mL) were recorded and tubes containing semen were placed in water bath maintained at 34°C till further assessment and supplementation. At fresh stage, the supravital stain was used to differentiate viable and nonviable spermatozoa (Figure 1). The sperm plasma membrane integrity (Figure 2) was assessed using hypoosmotic swelling test [20] and the percent intact acrosome was assessed using the Giemsa stain (Figure 3), as per the standard protocol [21].

2.2. Semen Dilution and In Vitro Supplementation of Humanin Analogue (HNG) in Extender. The extender used for semen dilution was TEYG with Tris (3.02 g), citric acid (1.67 g), fructose (1.25 g), penicillin (100,000 IU), streptomycin (100 mg), glycerol (7 mL), and egg yolk (20 mL) in Millipore water. The concentration of sperm cells was kept at approximately 80 million progressive motile sperm cells/mL in all the groups. Each sample was divided into four equal groups. In the control group (CG), spermatozoa were frozen

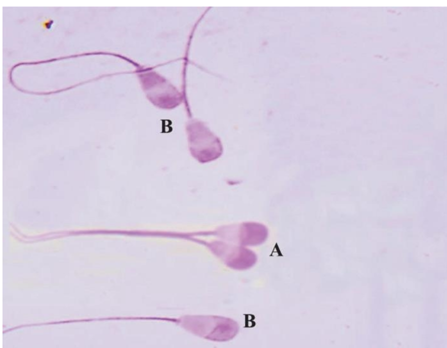

Figure 3: Giemsa stained smears of Frieswal semen. Acrosome intact (A) and acrosome denuded/damaged (B) spermatozoa (1,000x).

in TEYG media without adding HNG, while in the treatment groups (TGs), namely TG-I, -II, and -III, the final concentration of HNG was kept as 2, 10, and 20 μM, respectively. The humanin analogue (Gly14)-Humanin G human was procured from Sigma–Aldrich Chemicals Pvt. Ltd. (part of Merck KGaA Darmstadt, Cat. No. H6161, lot no. 0000098190).

2.3. Processing and Equilibration of Semen Samples. The extended control and TGs were filled and sealed in 0.25 mL French ministraws. The filling of the straws was done manually, and sealing was performed with poly vinyl alcohol powder. After filling and sealing, the printing of the straws was done (IMV Technologies—IS 4), and thereafter the straws were kept for equilibration at 5°C for 4 hr in the horizontal cold handling cabinet (Minitube).

2.4. Semen Evaluation at Prefreeze Stage. Semen quality parameters, viz., percent progressive motility, sperm viability, plasma membrane integrity, and acrosome intactness, were evaluated at prefreeze stage as described previously. For estimation of the oxidative stress status, the straw filled with extended semen from each group was taken into Eppendorf microcentrifuge tubes and was centrifuged at 1,000× g for 20 min at 4°C (Sigma, 3K30). The supernatant was separated, and both the pellet and seminal plasma were stored (−20°C) until assaying, and the storage period did not exceed 3 months. Total antioxidant capacity and ROS were estimated in the seminal plasma whereas lipid peroxidation was estimated in the sperm pellet.

2.5. Estimation of Lipid Peroxidation (LPO). The levels of LPO were determined by measuring the levels of malondialdehyde (MDA) which is a product of lipid peroxidation. MDA was calculated using a modified TBA assay [22]. Briefly, 20×10^6 sperm cells were suspended in 1 mL PBS in 5 mL microcentrifuge tubes. To it, 2 mL TBA-TCA was added. After swirling, the mixture was boiled for 45 min. The combination turned from pale yellow to dark yellow/brown and precipitated. It was cooled and centrifuged at 3,000 rpm for 10 min at room temperature. Without disturbing the precipitate, the supernatant was separated and absorbance was taken at 535 nm in UV–visible spectrophotometer (UV-1900, Shimadzu, Japan). The MDA concentration in sperm cells was determined employing the specific absorbance coefficient with the following formula:

$$\text{MDA } (\mu\text{mol/mL}) = \frac{\text{OD} \times 10^6 \times \text{volume of assay mixture (3 mL)}}{1.56 \times 10^5 \times \text{volume of sample (1 mL)}} = \frac{\text{OD} \times 30}{1.56}. \quad (1)$$

2.6. Estimation of Total Antioxidant Capacity (TAC). The TAC in the supernatant was estimated by the procedure, as described by Benzie and Strain [23]. The assay is based on the principle of reduction of the ferric-tripyridyl-triazine to the ferrous-tripyridyl-triazine by the antioxidants present in the solution at reduced pH. Postreaction a deep blue color is observed the intensity of which is directly proportional to the antioxidant capacity of the sample. Briefly, 100 μL of seminal plasma was vortexed with FRAP (ferric ion reducing antioxidant power) reagent. Immediately after mixing, the OD was assayed at 593 nm (0 min) in UV–visible spectrophotometer (UV-1900, Shimadzu, Japan). Thereafter, the mixture was maintained at 37°C and the next absorbance was recorded after 4 min. The standard was prepared using 1M ascorbic acid solution (the FRAP value of 1M ascorbic acid (standard) solution is 2,000). The FRAP value of the sample in μM was calculated using the undermentioned formula:

$$\text{FRAP value } (\mu\text{mol}) = \frac{\text{Absorbance of sample at 4 min} - \text{Absorbance of sample at 0 min}}{\text{Absorbance of std. at 4 min} - \text{Absorbance of std. at 0 min}} \times 2{,}000. \quad (2)$$

2.7. Estimation of Reactive Oxygen Species (ROS). The ROS assessment in seminal plasma was done vide procedure described by Hayashi et al. [24]. Briefly, 5 μL of seminal plasma and 5 μL of standard (10 different concentrations of H_2O_2, viz., 100, 200, 300, 400, 500, 600, 700, 800, 900, and 1,000 mg/L served as standard) were put in duplicate in a clear microtitre plate (colorimetric version). To it, 140 μL of 0.1 M sodium acetate buffer was dispensed. Thereafter, 100 μL of reaction mixture (R-1 : R-2@1 : 25) was dispensed (Solution R-1 contained 100 μg/mL N, N-diethylphenylenediamine sulfate (DEPPD) in 0.1 M sodium acetate buffer, while solution R-2 was prepared by dissolving ferrous sulfate in 0.1 M

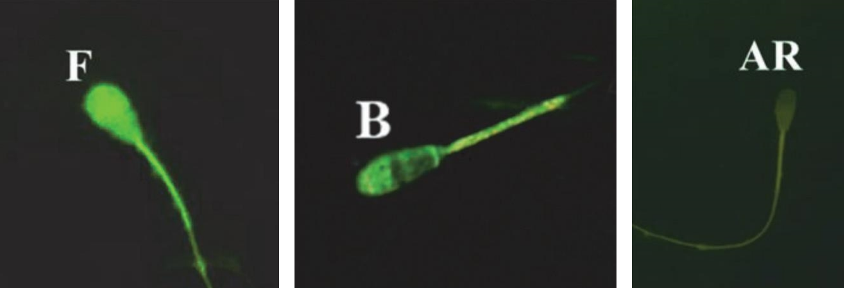

Figure 4: F-pattern (or full form): fluorescence covers the full head of the spermatozoa, which indicates noncapacitated spermatozoa with intact acrosome. B-pattern (or banded): the brilliant distribution of fluorescence is present in the acrosome and the postacrosome region lacks brightness. It indicates capacitated sperm with an intact acrosome. AR-pattern (or acrosome reaction): lack of a fluorescent glow in the head and indicates spermatozoa with a reacted acrosome.

sodium acetate buffer to obtain a final concentration of 4.37 μM). The absorbance was read at 492 nm at 0 and 2 min. A standard curve was plotted relating the intensity of the color (OD value) to the concentration of standards. A standard curve was plotted relating the intensity of the color (OD value) to the concentration of standards, and the values of tests were extrapolated. Increase in absorbance was related to elevated ROS concentrations.

2.8. Semen Freezing. The freezing of samples was done using a programable automated freezing system (IMV, France). Following freezing, the straws were stored in cryogenic containers plunged in liquid nitrogen (−196°C) for storage until assayed.

2.9. Semen Evaluation at Post-Thaw Stage. Frozen samples were thawed in a thawing unit (CITO thawer 12/220V, IMV) at 37°C for 30 s. Following thawing, the samples were evaluated for semen quality parameters, viz., sperm motility, viability, acrosome intactness, plasma membrane integrity, and oxidative stress assessment, as described earlier. Other additional tests performed were objective semen analysis by CASA, incubation test, assessment of sperm mitochondrial transmembrane potential, DNA integrity, cryocapacitation, and heterologous zona-binding assay.

2.10. Computer-Assisted Sperm Motion Analysis (CASA). Frozen-thawed bull semen motility was determined by CASA (Hamilton-Thorne, IVOS) using standard count four chamber slide (Leja®). Briefly, the frozen-thawed semen sample was diluted (v/v, 1 : 3) with prewarmed Tris buffer. The chambers were loaded, and the sperm motility characteristics were recorded in 12 different fields. The parameters recorded were percent total motile sperms (TM), progressively motile sperms (PM), rapid sperms (%), slow sperms (%), and static sperms (%). The average path velocity (VAP), straight line velocity (VSL), curvilinear velocity (VCL), amplitude of lateral head displacement (ALH), BCF and straightness (STR), and linearity (LIN) were also recorded. The software was set as per the standard recommendations for bull semen.

2.11. Incubation Test. The thawed semen samples from each group were placed in a water bath at 37°C. The forward progressive motility of the sperm cells was subjectively assessed every half an hour for a minimum of 2 hr (0, 30, 60, and 120 min) under a light microscope (Olympus BX40) at a magnification of ×200, and at least 10 different fields were observed.

2.12. Evaluation of Capacitation by Chlortetracycline (CTC) Assay. The CTC (Sigma, Cat No. C4881, Lot no. 0000139715) assay was applied to determine the capacitation status of the semen samples as per the procedure described by Fraser et al. [25]. Briefly, equal volume (10 μL) of CTC solution and sperm suspension were mixed in an Eppendorf tube. Thereafter, the mixture was kept at 37°C for 5 s in an opaque box to provide dark conditions. Subsequently, 10 μL of glutaraldehyde solution (12.5%) was added to it. A drop was taken on a clean grease-free slide and a thin smear was made and air-dried. A drop of DABCO (antifade reagent) was put on the slide before covering it with coverslip and the nail varnish was applied along the perimeter of the coverslip. The slide was observed under a fluorescent microscope (80i Nikon, Japan) under oil immersion. A minimum of 200 sperm cells were counted in three different patterns (Figure 4): noncapacitated (F, fluorescence over entire head), capacitated, (B, fluorescence in anterior part of the head), and acrosome-reacted sperms (AR, no/dull fluorescence observed over sperm head).

2.13. DNA Integrity Test. The integrity of sperm cell DNA was assessed following a previously described procedure [26]. In brief, spermatozoa were mixed with 1 mL of noncapacitated medium (2.7 mM KCl, 1.5 mM KH_2PO_4, 8.1 mM Na_2HPO_4, 137 mM NaCl, 5.55 mM glucose, and 1 mM pyruvate in 1,000 mL DW) and then centrifuged at 1,000 rpm for 5 min. The supernatant was discarded, and 100 μL of noncapacitated medium was added to resuspend the pellet. A thin smear was prepared and allowed to air-dry. Subsequently, the slide was fixed in a solution of ethanol and acetone (1 : 1) at 4°C for 2–3 hr. Following fixation, the slide was stained with an acridine orange working solution for 10 min. The slides were gently washed and air-dried. Observation of the slides was conducted under a fluorescent microscope (80i Nikon, Japan) using a blue filter at 1,000x magnification. Sperm with intact DNA emitted green fluorescence, while those with nonintact DNA emitted orange fluorescence, as shown in Figure 5.

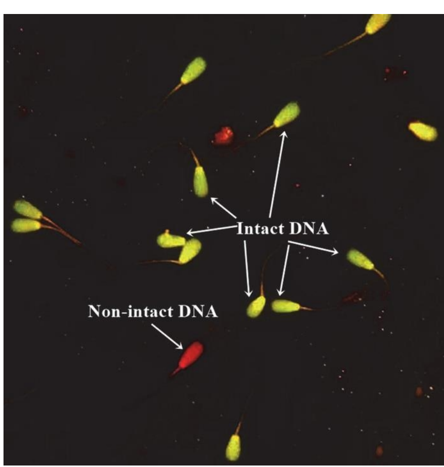

FIGURE 5: Spermatozoa with intact (green fluorescence on head) and nonintact (orange/red fluorescence on head) DNA (1,000x).

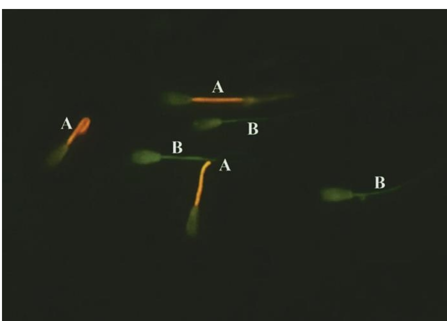

FIGURE 6: Spermatozoa with high (red/orange midpiece; A) and low (green midpiece; B) mitochondrial membrane potential (1,000x).

2.14. Mitochondrial Membrane Potential (MMP) Assessment. The sperm MMP was assessed using a membrane-permeable fluorometric cationic dye JC-1 (Cayman, USA). The procedure followed was described by Srivastava and Pande [27] with little modifications. Briefly, 10 μL of sperm suspension was mixed with 10 μL of working JC-1 solution. The mixture was kept in incubator (5% CO_2 for 20 min at 37°C) in humidified chamber. Thereafter, a drop was put on a slide and a smear was made. A drop of DABCO (antifade agent) was added and a coverslip was placed over it. The observations were performed immediately under an epifluorescence microscope (80i Nikon, Japan) under 1,000x in green filter. This stain selectively enters mitochondria and changes fluorescence characteristics with alteration in MMP. In live and active sperm cells, JC-1 forms complexes (multimeres known as J-aggregates), which fluoresce red/orange (high-MMP). A decrease in MMP, which is an exceedingly early event in apoptosis, results in JC-1 monomers, which fluoresce green, low MMP (Figure 6).

2.15. Heterologous Zona Binding Assay. The post-thaw semen quality was examined with the ability of sperm to bind to zona pellucida between control and best TG, with slight modifications [28]. The ovaries of buffaloes were collected from a local abattoir and were transported to the laboratory within 2 hr in PBS (30–34°C) fortified with gentamicin (200 units/mL) and streptomycin (200 μg/mL). The surrounding tissue was trimmed, and the ovaries were rinsed three times with PBS. The oocytes were collected by slicing procedure in oocyte collection medium. Morphologically excellent to good cumulus–oocyte complexes (COCs) with more than three to four layers of granulosa–cumulus cells and evenly granulated cytoplasm were selected. The selected COCs were washed four to five times in oocyte maturation medium (OMM). Thereafter, five to six COCs were nurtured in 50 μL of OMM topped with paraffin oil, in a CO_2 incubator (NB-203XL, N-Biotek) at 38.5 ± 0.5°C and 5% CO_2 in moistened air for 27 hr. Following incubation, the IVM status was checked under stereo zoom microscope (Nikon, SMZ645) based on cumulus expansion.

For sperm cell preparation and capacitation, the sperm-TALP media was used. Initially, the cryopreserved semen sample was thawed and washed in TALP media (1 : 3) by centrifugation at $500\times g$ for 10 min. The sperm pellet was resuspended in fertilization-TALP, and the concentration was attuned to 10 million spermatozoa per mL and kept in a CO_2 incubator for 6 hr.

After 27 hr of maturation, the oocytes were retrieved and washed five times in fertilization-TALP media. Following washing, six oocytes were cocultured with 20 μL of capacitated spermatozoa in 50 μL of fertilization-TALP suspension under paraffin oil at 38.5 ± 0.5°C and 5% CO_2 in moistened air for 24 hr.

After incubation, the sperm–oocyte complex was washed five to six times in medium-199 with BSA, to remove excess unbound sperm cells. Thereafter, they were fixed in 2.5% glutaraldehyde in PBS for 10 min and then washed with PBS. The complexes were stained with Hoechst 33342 dye (10 μg/mL) for 10–15 min. The stained complexes were washed two to three times with PBS under stereo zoom microscope and, thereafter, were placed on a clean grease-free slide along with a drop. The coverslip was placed on it, which was sealed by nail varnish. The sperm cells attached to zona pellucida were counted using a fluorescent microscope (ECLIPSE-Ni, Nikon, Japan) in a UV filter under 400x magnification. The spermatozoa emitted blue fluorescence (Figure 7). The zona binding index was calculated for test and compared with that of control semen sample.

$$\text{Zona binding index} = \frac{\text{No.of sperm bound to the test group oocytes}}{\text{No.of sperm bound to the control group oocytes}}. \quad (3)$$

2.16. Data Analyses. The data documented was subjected to angular transformation before the analysis. Data were analyzed by analysis of variance (ANOVA), for comparing the means using SPSS 20.0 (SPSS Inc., Chicago, USA) statistical software. Statistical significance was evaluated at a 5% ($p < 0.05$) probability level and the difference between means

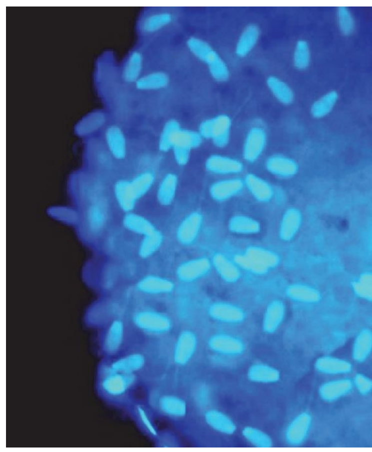

Figure 7: Heterologous sperm–zona binding assay at 40x magnification with Hoechst 33342 staining.

Table 1: Effect of humanin analogue supplementation on individual progressive motility of Frieswal bull spermatozoa ($n = 24$ ejaculates, eight ejaculates per bull) at prefreeze and post-thaw stage of cryopreservation (mean ± SEM).

Stage of processing	Individual progressive motility (%)			
	Control	TG-I (2 μM)	TG-II (10 μM)	TG-III (20 μM)
Prefreeze	68.33 ± 1.27^{cA}	70.42 ± 1.04^{cbA}	72.71 ± 1.04^{bcA}	74.38 ± 1.14^{aA}
Post-thaw	49.58 ± 1.27^{aB}	52.29 ± 1.31^{aB}	61.67 ± 0.98^{bB}	63.75 ± 1.29^{bB}

Mean values bearing different superscripts in lowercase (a, b, c) in a row differ significantly ($p < 0.05$). Mean values bearing different superscripts in uppercase (A, B) in a column differ significantly ($p < 0.001$).

Table 2: Effects of humanin analogue supplementation on motility variables of Frieswal bull spermatozoa ($n = 24$ ejaculates, eight ejaculates per bull) as assessed by CASA after freezing–thawing (mean ± SEM).

Motility variables (%)	Control	TG-I (2 μM)	TG-II (10 μM)	TG-III (20 μM)
Total motility	76.75 ± 1.83^{b}	79.25 ± 1.96^{ab}	83.25 ± 1.96^{a}	84.21 ± 1.70^{a}
Progressive motility	33.42 ± 1.40^{b}	35.29 ± 1.67^{b}	42.75 ± 1.87^{a}	41.33 ± 2.36^{a}
Rapid sperm	49.95 ± 1.93^{b}	54.00 ± 1.89^{ab}	58.54 ± 2.06^{a}	57.45 ± 2.15^{a}
Slow sperm	27.20 ± 1.83	25.25 ± 1.59	24.70 ± 1.69	26.75 ± 1.64
Static sperm	22.83 ± 1.25	20.75 ± 2.16	16.75 ± 1.37	15.79 ± 1.65

Mean values bearing different superscripts (a, b) in a row differ significantly ($p < 0.05$).

was compared using Duncan's multiple range test. Results are expressed as mean ± standard error of mean.

3. Results

3.1. Progressive Motility. The supplementation of HNG in semen extender with 2, 10, and 20 μM in TGs had a significant ($p < 0.05$) positive effect on spermatozoa motility parameters as compared to the control following freezing–thawing when observed subjectively (Table 1).

3.2. Objective Analysis by Computer-Assisted Semen Analysis (CASA)

3.2.1. Sperm Motility Variables. The mean values of spermatozoa motility parameters evaluated by CASA analysis are shown in Table 2. HNG supplementation at different concentrations in semen extender had a variable effect on the motility parameters after cryopreservation.

3.2.2. Sperm Kinetics. The effect of different concentrations of HNG supplementation on sperm kinetic variables is presented in Table 3. The VAP was significantly higher ($p < 0.05$) in TG-II and -III when compared with control. HNG supplementation did not show any significant difference in curvilinear velocity (VCL). However, the amplitude of lateral head displacement (ALH) was significantly ($p < 0.05$) higher in TG-II when compared to control.

The fortification of HNG in semen extender at the concentration of 10 and 20 μM significantly ($p < 0.05$) improved the mean beat cross frequency (BCF) of samples when compared to control. The linearity (LIN) was detected to be significantly higher ($p < 0.05$) in TG-II as compared to control and TG-I. No significant differences were observed in mean straightness (STR) amongst control and TGs.

3.3. Incubation Test. The result of the incubation test showed that the samples from TG-II and TG-III had significantly ($p < 0.05$) more motile spermatozoa after incubation at

Table 3: Effects of humanin analogue supplementation on sperm kinetics Frieswal bull ($n = 24$ ejaculates, eight ejaculates per bull) as assessed by CASA after freezing–thawing (mean ± SEM).

Sperm kinetics	Control	TG-I (2 μM)	TG-II (10 μM)	TG-III (20 μM)
VAP (μm/s)	103.95 ± 3.95^{b}	102.71 ± 2.44^{b}	119.40 ± 4.74^{a}	112.33 ± 4.78^{ab}
VSL (μm/s)	68.05 ± 1.83^{b}	68.25 ± 2.41^{b}	76.98 ± 2.34^{a}	73.40 ± 1.79^{ab}
VCL (μm/s)	172.59 ± 2.70	178.04 ± 4.44	170.81 ± 3.63	178.84 ± 3.15
ALH (μm)	6.98 ± 0.13^{b}	7.25 ± 0.16^{ab}	7.55 ± 0.16^{a}	7.11 ± 0.18^{ab}
BCF (Hz)	22.70 ± 1.15^{c}	23.25 ± 0.79^{ab}	27.71 ± 0.86^{a}	25.56 ± 1.11^{ab}
STR (%)	66.33 ± 1.36	67.21 ± 2.75	66.04 ± 2.56	67.75 ± 3.15
LIN (%)	39.58 ± 1.15^{b}	38.96 ± 1.67^{b}	45.63 ± 1.88^{a}	41.08 ± 1.01^{ab}

Means bearing different superscripts (a, b, c) in a row differ significantly ($p < 0.05$).

Table 4: Effects of humanin analogue supplementation on sperm motility following thawing and incubation at 37°C in Frieswal bull semen ($n = 24$ ejaculates, eight ejaculates per bull) (mean ± SEM).

Incubation time (min)	Motility (%) at different incubation times (mean ± SEM)			
	Control	TG-I (2 μM)	TG-II (10 μM)	TG-III (20 μM)
0	49.58 ± 1.27^{b}	52.29 ± 1.31^{b}	61.67 ± 0.98^{a}	63.75 ± 1.29^{a}
30	44.38 ± 1.29^{b}	46.67 ± 1.11^{b}	55.42 ± 1.08^{a}	54.79 ± 1.29^{a}
60	34.38 ± 1.28^{b}	36.67 ± 1.15^{b}	44.38 ± 1.42^{a}	42.92 ± 1.34^{a}
120	17.71 ± 1.16^{b}	20.00 ± 0.74^{b}	30.00 ± 1.38^{a}	27.70 ± 0.85^{a}

Means bearing different superscripts (a, b) in a row differ significantly ($p < 0.05$).

Table 5: Effect of humanin analogue supplementation on plasma membrane integrity ($n = 24$ ejaculates, eight ejaculates per bull) of Frieswal bull spermatozoa at prefreeze and post-thaw stage of cryopreservation.

Stage of processing	Plasma membrane integrity (%) (mean ± SEM)			
	Control	TG-I (2 μM)	TG-II (10 μM)	TG-III (20 μM)
Prefreeze	58.86 ± 0.95^{bA}	60.24 ± 1.07^{abA}	60.42 ± 0.69^{abA}	62.85 ± 0.95^{aA}
Post-thaw	44.16 ± 1.36^{bB}	45.00 ± 1.30^{bB}	52.46 ± 1.06^{aB}	53.16 ± 1.12^{aB}

Mean values bearing different superscripts in lowercase (a, b) in a row differ significantly ($p < 0.05$). Mean values bearing different superscripts in uppercase (A, B) in a column differ significantly ($p < 0.001$).

Table 6: Effect of humanin analogue supplementation on acrosome integrity ($n = 24$ ejaculates, eight ejaculates per bull) of Frieswal bull spermatozoa at prefreeze and post-thaw stage of cryopreservation.

Stage of processing	Acrosome integrity (%) (mean ± SEM)			
	Control	TG-I (2 μM)	TG-II (10 μM)	TG-III (20 μM)
Prefreeze	80.86 ± 0.47^{A}	80.78 ± 0.41^{A}	81.09 ± 0.31^{A}	81.98 ± 0.61^{A}
Post-thaw	69.15 ± 0.92^{cB}	72.50 ± 0.61^{bB}	77.69 ± 0.51^{aB}	76.85 ± 0.59^{aB}

Mean values bearing different superscripts in lowercase (a, b, c) in a row differ significantly ($p < 0.05$). Mean values bearing different superscripts in uppercase (A, B) in a column differ significantly ($p < 0.001$).

37°C for 0, 30, 60, 90, and 120 min (Table 4) than the control and TG-I.

3.4. Plasma Membrane Integrity. The percentage of HOS-positive spermatozoa at the prefreeze stage had significantly ($p < 0.05$) improved in TG-III as compared to the CG. At post-thaw stage, the mean percentage of spermatozoa with intact plasma membrane in TG-II and -III showed no significant differences between themselves; however, a significant improvement was observed in TG-II and TG-III as compared to TG-I (Table 5).

3.5. Acrosome Integrity. Acrosome integrity of spermatozoa, in control and treatment groups (TG-I, -II, and -III), at prefreeze and post-thaw stage of Frieswal bulls have been presented in Table 6. There were no statistical differences identified in acrosome integrity between the experimental groups and the control at prefreeze stage. At post-thaw stage, mean percentage of spermatozoa with intact acrosome in TG-II and -III showed no significant differences between themselves; however, a significant improvement ($p < 0.05$) was observed in TG-II and TG-III as compared to TG-I.

Table 7: Effect of humanin analogue supplementation on lipid peroxidation ($n = 24$ ejaculates, eight ejaculates per bull) in Frieswal bull spermatozoa at prefreeze and post-thaw stage of cryopreservation (mean ± SEM).

Stage of processing	Malondialdehyde (nM/mL)			
	Control	TG-I (2 μM)	TG-II (10 μM)	TG-III (20 μM)
Prefreeze	614.33 ± 18.07^{aB}	564.90 ± 13.58^{aB}	443.33 ± 17.98^{bB}	454.05 ± 38.03^{bB}
Post-thaw	$1,014.08 \pm 47.10^{aA}$	962.04 ± 43.52^{aA}	690.43 ± 30.70^{bA}	723.12 ± 41.59^{bA}

Mean values bearing different superscripts in lowercase (a, b) in a row differ significantly ($p < 0.05$). Mean values bearing different superscripts in uppercase (A, B) in a column differ significantly ($p < 0.001$).

Table 8: Effect of humanin analogue supplementation on reactive oxygen species (ROS) in the extended seminal fluid of Frieswal bull ($n = 24$ ejaculates, eight ejaculates per bull) at prefreeze and post-thaw stage of cryopreservation (mean ± SEM).

Stage of processing	ROS (units of H_2O_2)			
	Control	TG-I (2 μM)	TG-II (10 μM)	TG-III (20 μM)
Prefreeze	117.12 ± 0.66^{aB}	113.54 ± 1.07^{bB}	108.10 ± 0.41^{cB}	110.06 ± 1.13^{cB}
Post-thaw	222.97 ± 0.91^{aA}	203.97 ± 0.91^{aA}	172.70 ± 1.05^{bA}	161.89 ± 8.74^{bA}

Mean values bearing different superscripts in lowercase (a, b, c) in a row differ significantly ($p < 0.05$). Mean values bearing different superscripts in uppercase (A, B) in a column differ significantly ($p < 0.001$).

Table 9: Effect of humanin analogue supplementation on total antioxidant capacity (TAC) in the extended seminal fluid of Frieswal bull ($n = 24$ ejaculates, eight ejaculates per bull) at prefreeze and post-thaw stage of cryopreservation (mean ± SEM).

Stage of processing	TAC (nM/mL)			
	Control	TG-I (2 μM)	TG-II (10 μM)	TG-III (20 μM)
Prefreeze	997.51 ± 38.83^{bA}	$1,084.10 \pm 21.16^{abA}$	$1,143.80 \pm 39.11^{aA}$	$1,145.31 \pm 40.33^{aA}$
Post-thaw	740.13 ± 9.35^{bB}	781.45 ± 20.21^{bB}	890.77 ± 15.06^{aB}	920.31 ± 28.68^{aB}

Mean values bearing different superscripts in lowercase (a, b) in a row differ significantly ($p < 0.05$). Mean values bearing different superscripts in uppercase (A, B) in a column differ significantly ($p < 0.001$).

Table 10: Effects of humanin analogue supplementation on capacitation status of Frieswal bull spermatozoa ($n = 24$ ejaculates, eight ejaculates per bull) at post-thaw stage.

Capacitation pattern	Mean ± SEM			
	Control	TG-I (2 μM)	TG-II (10 μM)	TG-III (20 μM)
F (full form)	51.88 ± 1.11^{c}	55.38 ± 0.96^{b}	64.29 ± 1.12^{a}	64.08 ± 0.97^{a}
B (banded)	25.25 ± 1.32^{a}	25.33 ± 1.23^{a}	19.92 ± 1.52^{b}	21.38 ± 1.20^{b}
AR (acrosome reacted)	22.88 ± 0.78^{a}	19.29 ± 0.67^{a}	15.79 ± 0.78^{b}	14.54 ± 0.68^{b}

Mean values bearing different superscripts (a, b, c) in a row differ significantly ($p < 0.05$).

3.6. Lipid Peroxidation in Sperm Cells. Overall, the mean MDA levels increased significantly ($p < 0.001$) from prefreeze to the post-thaw stage in all the groups. At prefreeze stage, the control and TG-I showed significantly ($p < 0.05$) higher MDA levels as compared to TG-II and -III. The supplementation of HN analogue in semen extender with 10 and 20 μM showed significant ($p < 0.05$) positive control on lipid peroxidation as compared to the control and TG-I at post-thaw stage (Table 7).

3.7. Reactive Oxygen Species Levels in Seminal Fluid. Overall, the mean ROS levels increased significantly ($p < 0.001$) from prefreeze to the post-thaw stage in control and all the TGs (Table 8). At prefreeze stage, the CG showed significantly ($p < 0.05$) higher ROS production as compared to TG-I, -II, and -III. At post-thaw stage, supplementation of HN analogue in semen extender with 2, 10, and 20 μM in TGs had a positive effect on ROS levels as compared to the CG (Table 8).

3.8. Total Antioxidant Capacity (TAC) in Seminal Plasma. Overall, the mean TAC levels decreased significantly ($p < 0.001$) from prefreeze to the post-thaw stage in control and all the TGs. At prefreeze stage, the CG showed significantly ($p < 0.05$) reduced TAC as compared to TG-II and -III. At the post-thaw stage, the mean TAC level was significantly lower ($p < 0.05$) in the CG and TG-I as compared to TG-II and -III (Table 9).

3.9. Effects of Humanin Analogue Supplementation on Capacitation Status of Frieswal Bull Spermatozoa at Post-Thaw Stage. Humanin analogue supplementation at 10 and 20 μM concentrations in semen extender significantly ($p < 0.05$) improved the F-pattern in comparison to the control and TG-I after cryopreservation (Table 10).

3.10. Effects of Humanin Analogue Supplementation on Mitochondrial Membrane Potential (MMP) and DNA Integrity of Frieswal Bulls' Spermatozoa at Post-Thaw Stage. Overall, the HNG at concentrations of 2, 10, and 20 μM significantly

Table 11: Effects of humanin analogue supplementation on mitochondrial membrane potential (MMP) of Frieswal bull and DNA integrity ($n = 24$ ejaculates, eight ejaculates per bull) spermatozoa at post-thaw stage.

Semen parameter	(Mean ± SEM)			
	Control	TG-I (2 μM)	TG-II (10 μM)	TG-III (20 μM)
High MMP	33.25 ± 1.18^{c}	37.08 ± 1.01^{b}	48.33 ± 0.81^{a}	46.96 ± 0.85^{a}
DNA integrity (%)	88.96 ± 0.80^{b}	90.88 ± 0.79^{ab}	92.25 ± 0.73^{a}	92.88 ± 0.92^{a}

Mean values bearing different superscripts (a, b, c) in a row differ significantly ($p < 0.05$).

Table 12: Effects of humanin analogue supplementation on heterologous zona binding ability (*in vitro* fertility) of Frieswal bull spermatozoa following freezing–thawing (mean ± SEM).

Parameter	Control	TG-II (10 μM)	TG-III (20 μM)
Spermatozoa bound to zona	62.92 ± 2.58^{b}	125.58 ± 5.51^{a}	120.70 ± 5.97^{a}
Binding index	1.00 ± 0.00^{b}	2.05 ± 0.53^{a}	1.97 ± 0.12^{a}

Mean values bearing different superscripts (a, b) in a row differ significantly ($p < 0.05$).

($p < 0.05$) improved spermatozoal MMP compared to the CG. Furthermore, HNG supplementation at 10 and 20 μM in the semen extender also significantly ($p < 0.05$) reduced the DNA damage of sperm cells after cryopreservation compared to the control (Table 11).

3.11. Effects of Humanin Analogue Supplementation on Heterologous Zona Binding Ability (In Vitro Fertility) of Frieswal Bull Spermatozoa Following Freezing–Thawing. The *in vitro* fertility test of TG-II and -III indicated a significantly ($p < 0.05$) increased percentage of spermatozoa bound to zona as compared to the CG (Table 12).

4. Discussion

To our knowledge, this is the first study in which a potent analogue of a proven natural bioprotectant has been added to increase cryosurvival rates in bull semen. The supplementation of HNG at the concentration of 10 and 20 μM in semen extender resulted in significantly higher progressive motility at the post-thaw stage in comparison to the control and TG-I (2 μM). In the present investigation, the favorable effect of HNG on post-thaw sperm motility is obvious due to multiple mechanisms, the most notable of which is the reduced oxidative stress. Oxidative stress is caused by an imbalance between the production of ROS and its neutralization by the semen's own antioxidants. It is a major contributing factor that leads to sperm damage, reduced sperm motility, poor functionality, and eventually cellular death of cryopreserved sperm [29]. It has been reported that these dead and moribund spermatozoa further deteriorate the functionality of their contemporaneous live cells [30]. Thus, the generation of ROS forms a vicious circle of self-generation and spermatozoal death; the greater the number of dead sperm, the greater the oxidative stress, which develops more cryoinjuries [31]. Previous studies from our laboratory have also reported a reduction in post-thaw sperm motility with the elevation of oxidative stress markers like malondialdehyde [32, 33] and ROS [34, 35].

In the present study, the progressive motility assessed by CASA (33.42%–41.33%) is comparatively lower compared to the subjective analysis under a light microscope (49.58%–63.75%). This may be attributed to many factors. Past reports suggested that simple variations introduced in trials created a considerable influence on sperm motility [36]. The underrated or underestimation of motility may also arise due to misperception of nonspermatic substances (debris). At times, a proportionate number of fast-moving cells may also be eliminated because of spermatozoal overlap, cells escaping the analysis area, or ignored because of adjacent-neighbour effects, leading to an underestimation of the motility [37, 38]. Further, the type of semen extender used for processing semen also considerably affects motility parameters when assessed by CASA. In the present study, the semen extender being egg yolk based may be the reason for lower post-thaw motility by CASA as compared to the subjective analysis.

The sperm kinetic parameters, namely VAP, VSL, VCL, ALH, BCF, STR, and LIN, have been correlated with bull fertility [39–42]. While VAP has been found to be a predictive indicator with increased chances of pregnancy in humans [43] and bulls [44], another study found the mean head area to be positively related to fertility [45]. In the present study, a positive effect on sperm kinetic parameters including VAP and VSL, in addition to ALH, BCF, and LIN, was observed, and there were no substantial differences observed amongst distinct groups when VCL and STR were compared. The results showed significantly improved VAP and VSL in the group supplemented with HNG at a concentration of 10 μM as compared to the CG. Earlier, the lower values of VAP and VSL have been demonstrated to predict subfertility of semen samples [44, 46, 47]. Additionally, higher values of BCF, ALH, and LIN in TG-II also suggested that the HNG supplementation may have significantly improved the post-thaw semen quality in Frieswal bulls. A study by Gliozzi et al. [48] has earlier shown that BCF and ALH are positively correlated with the *in vitro* fertility potential of bull spermatozoa. In a previous study, adding HNG to human sperm before freezing improved sperm motility and other postfreezing quality parameters [17]. The positive effect of HNG on the kinematic parameters of sperm could be because it protects spermatozoa by keeping the level of ROS in the sperm microenvironment in check [16].

HNG fortification also resulted in a substantially higher percentage of viable, acrosome- and plasma membrane-intact spermatozoa. This may be attributed to the cytoprotective nature of HN [49], which guards the cells against cryostress or typically against freezing-induced oxidative stress [8]. The supplementation of HNG in the semen extender prior to cryopreservation would have avoided the cellular death as also evident in the other models. HN is well-known for its proactive function as an antiapoptotic molecule [50]. Although the exact mechanism by which HNG might have rescued the spermatozoal death was not studied in the present study, it was speculated that the higher viable sperm in the HNG-supplemented groups may be attributed to the enhanced TAC levels and controlled ROS production in the frozen-thawed semen. HN has previously been shown to protect against oxidative stress in other cellular models such as heart cells [51], retinal cells [8], and myoblasts [52].

The inherent antioxidant defence mechanism decreases in the bull semen when it undergoes the process of cryopreservation [53]. The presence of a considerable amount of polyunsaturated fatty acids in the spermatozoal plasma membrane [54] makes them more susceptible to lipid peroxidation during the freeze–thaw cycle [55]. Once the plasma membrane is breached, it further leads to an influx of potassium ions, enzymes, lipids, etc., resulting in poor mitochondrial function, weak flagellar motor apparatus, and phosphorylation disturbances [56]. In the present study, as compared to the prefreeze stage, lipid peroxidation levels were higher at the post-thaw stage. This was in agreement with the previous studies on Frieswal bulls and other bovines [33, 57] verifying that lipid peroxidation in semen increases with the stages of cryopreservation. The increased lipid peroxidation during freezing–thawing has been attributed to the reduction of antioxidants after semen dilution [58] and higher levels of ROS production from cryoinjured or dying/dead cells [11]. In agreement, we found a progressive escalation in lipid peroxidation as evident by increased malondialdehyde levels in all the groups, including control. However, the supplementation of HNG resulted in controlled lipid peroxidation in crossbred Frieswal bulls.

Cryopreservation-induced impairments in the sperm plasma membrane and oxidation of phospholipids stimulate the production of ROS in spermatozoa [59]. In concurrence, we found a progressive decline in the semen quality that was evident in all the groups, including control. However, controlled ROS levels were observed in the groups supplemented with HNG. The cytoprotective effects of HN are supposed to be due to the promotion of antioxidant enzyme expression that limits ROS generation through various pathways [60]. Guo et al. [61] revealed that intracellularly, when HN is inside a cell, it networks with the proapoptotic signaling peptide BAX. In living cells, the majority of BAX protein is found in the cytosol, but in response to the induction of apoptosis response, BAX undergoes a conformational shift [62]. The activated BAX causes the release of cytochrome C leading to the activation of caspases [63]. BAX has been shown to be activated by a variety of stresses or external factors, including heat, H_2O_2, pH/osmolality changes, and mitochondrial membrane remodeling [63].

Guo et al. [61] showed that HN binds to the inactive conformation of BAX and helps to keep BAX in the inactivated state in the cytosol. Thus, it prevents the translocation of BAX from the cytosol to mitochondria and blocks its association with isolated mitochondria, subduing cytochrome C secretion, which is the first step to cell death/apoptosis and thus prevents staurosporine-induced loss. Mutagenesis findings of HN have so far shown an ideal relationship between attachment to BAX and the competence of HN to subdue BAX-initiated cellular death [64]. It is reasonable to propose that the spermatozoal protection in the present study may be due to the neutralization/reduction in cryopreservation-induced ROS generation. Previous studies have also reported the cytoprotective role of HN against oxidative stress in reproductive tissues [5] and other cells [52]. More research needs to be done to find the target sites of HN in spermatozoal cells that are controlled by oxidative stress.

The measurement of TAC levels has become a crucial index to measure the oxidative stress status of the semen sample [65] and is emerging as a significant analytic and predictive tool in male-related infertility [66]. HN has been known to upregulate various antioxidant proteins [67]. Its supplementation in wide-ranging *in vitro* cellular culture prototypes and oxidative-stress-related diseases-models rescues cell death and reduces cytotoxicity [68]. It mainly functions through extracellular and intracellular pathways, adjusting mitochondrial oxidative phosphorylation and ATP production [69]. Earlier, it had been shown that supplementation of HNG reduced the damage to the mitochondrial membrane of spermatozoa and inhibited the activity of caspase-3 in spermatozoa [17]. Our results suggest that adding HNG to semen extender could protect bull spermatozoa from damage caused by the freezing process.

The assessment of mitochondrial membrane potential is a useful test for predicting sperm fertilization capacity in males [70]. In Frieswal bulls, MMP was found to be strongly correlated with bull fertility [71]. In the present study, HNG supplementation resulted in significantly improved MMP in all the TGs as compared to the control. The study is comparable with the previous studies on other tissues in which HNG protected the cardiac mitochondrial integrity against induced-oxidative stress [52] and attenuated Aβ (25–35)-induced neural cell injury and apoptosis by preventing mitochondrial dysfunction [72]. It is also known to have a progressive impact on therapeutic mitochondrial disorders by inducing the expression of PGC-1α and mitochondrial biogenesis in diabetes [73].

The process of cryopreservation triggers sperm DNA damage [74], which is principally mediated by oxidative stress [29]. DNA fragmentation impacts fertilization and is associated with poor reproductive outcomes [14]. The double strand of DNA is disrupted due to elevated levels of ROS production [75], shrinking of DNA healing enzymes [76], and/or mechanical stress because of cell compaction and freezing–thawing cycle [77, 78]. In the present study, the DNA integrity was significantly improved in the groups with HNG supplementation at 10 and 20 μM concentrations.

In cryopreserved semen, oxidative stress is believed to be the main mediator of freezing-induced DNA disruption [29, 79]. Previous studies in our laboratory [34, 80] and elsewhere [81, 82] have also shown that if the ROS production in the cryopreserved semen is minimized, DNA damage can be controlled. HN is well-known to be a strong antioxidant molecule. It has the potential to counteract oxidative stress via different pathways [83]. It is supposed to be encoded in the mitochondria where it controls ROS production [84]. In the present study, the addition of HNG and subsequently lesser damage to sperm DNA are in agreement with the previous studies indicating that HN can play a key role in checking oxidative stress-induced DNA damage.

Sperm capacitation is known to be an oxidative event [85]. In cryopreserved semen, the overproduction of ROS is believed to be the main mediator of freezing-induced capacitation-like changes [80, 86]. When the ROS levels are superficially increased in semen, the capacitation reaction accelerates, whereas removing them causes a vice-versa effect [87]. In the present study, the addition of HNG and subsequent reduction in cryocapacitation-like changes in spermatozoa are in agreement with the previous studies stating that humanin can play a key role in checking oxidative stress. The result is further supported by the outcomes of oxidative stress in the semen, wherein a significant reduction in ROS levels and enhanced TAC were evident in the HNG-supplemented groups. The changes seen in the HNG-supplemented groups could have been caused by the lower LPO levels and controlled ROS production.

The most commonly used *in vitro* fertility assay is the zona binding test. The total number of spermatozoa bound to the zona pellucida after incubation for a set period of time is counted. A positive correlation between field fertility and the zona binding test has been reported [88]. The higher number of spermatozoa bound to zona pellucida in the HNG-supplemented group may be due to overall improvement in the sperm quality parameters like reduced alterations in the sperm plasma membrane-, acrosome-, and DNA-integrity, and better shielding against cryopreservation-induced-oxidative stress. The higher levels of endogenous HN ovarian follicles have been shown to improve the fertilization rate and promote clinical pregnancies in humans [6].

HN is known to play a crucial role in antioxidant defense through both intracellular and extracellular mechanisms. Intracellularly, it inhibits the activation of proapoptotic BAX and prevents the release of cytochrome C from mitochondria. It also interacts with BID, tBID, IGFBP-3, α-actinin-4, and TRIM11, showcasing diverse regulatory functions [61, 89, 90]. Extracellularly, HN engages receptors like FPRL-1 and IL-6 receptor family subunits, exerting neuroprotective effects and participating in various signaling pathways [7, 91]. Thus, this polypeptide may exhibit the potential to modulate a range of cellular responses and play a pivotal role in defending against apoptosis and oxidative stress. In addition to studies utilizing other cellular models, our investigation, which focused on semen, revealed that this polypeptide possessed the potential to improve semen quality.

5. Conclusion

Our results suggest that supplementation of a novel antioxidant humanin analogue in semen extender may protect bull spermatozoa from cryopreserved-induced-oxidative stress and cell injury. Hence, the supplementation of its analogue may potentially prove to be a valuable approach for the improvement of semen freezability in crossbred Frieswal bulls. It is likely that the cellular protection in the present study may be due to the reduction in ROS-induced lipid peroxidation and a check against oxidative stress during cryopreservation. However, further investigations are required to elucidate the exact mechanism of prevention of cellular death in spermatozoa along with the plausible sites where huamnin regulates oxidative stress in these cells.

Data Availability

The data supporting the results and the detailed protocols used in the study are available from the corresponding author upon request.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

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