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Nano-depletion of morbid spermatozoa up-regulate Ca2+ channel, depolarization of membrane potential and fertility in buffalo

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A B S T R A C T

Despite recent advances in technique of spermatozoa cryopreservation, there are still ejaculates present that fail to meet strict quality standard; mainly due to detrimental effect of imbalance of free radicals. The omnipresence of dead/defective spermatozoa in ejaculates of eutherian species is a major source of excessive free radicals. Though sperm-selection techniques, as well as addition of antioxidants addressed the problem to a certain extent, the major source of free radicals in the semen remained, causing much damage. This study attempts to remove dead/damaged spermatozoa using negative fertility-marker. The effect is unraveled by Hypo-osmotic (HOS), and fluorescein-conjugated *Pisum sativum* agglutinin (FITC-PSA) assay, further confirmed by Ca^{2+}-regulating mechanisms and depolarization of sperm membrane potential, reduction in concentration of free radicals and finally by *in vitro* fertility assay. The study involved functionalization of iron oxide nanoparticles (IONPs) with silane followed by bio-conjugation with *anti*-ubiquitin antibodies. The nano-purification of semen using *anti*-ubiquitin conjugated iron oxide nanoparticles (IONPs) (antibody concentrations 0.5, 1.0 and 2.0 μg/ml) was attempted. The efficiency of nano-purification was 18.1%–43.8% in the study. The results revealed greater ($P \leq 0.05$) spermatozoa population with intact plasma membrane, acrosome integrity, high mitochondrial membrane potential and pattern-F (least intracellular Ca^{2+}), evidence of low lipid peroxidation and higher total antioxidant capacity in nano-purified groups. More number of spermatozoa were bound to zona pellucida of matured oocytes from nano-depleted than non-depleted group. The findings demonstrate antibody concentration of 1.0 μg/ml bio-conjugated with IONPs as most efficient in enriching the ejaculate with functional spermatozoa with the highest percentage of zona binding.

1. Introduction

The presence of dead and defective spermatozoa in ejaculates is one of the major causes of poor semen quality. The dead and defective spermatozoa have been pinned as main source of the reactive oxygen species (ROS) generators [17]. Damage to the spermatozoa membrane, either in male reproductive tract or following ejaculation, is responsible for increased activity of aromatic L-amino acid oxidase [36]. This result in oxidative de-amination of amino acids such as L-tryptophan, L-phenylalanine and L-tyrosine followed by generation of oxidants [1, 45]. The produced hydrogen peroxide (H_2O_2) and ammonia are highly toxic to the contemporary normal spermatozoa [4] resulting in loss of the functionality and other cellular damage. All such changes ultimately affect the fertility potential of spermatozoa.

The spermatozoa are particularly susceptible to ROS damage due to low antioxidant capacity and inability to synthesize membrane components [19]. The damage from generated ROS is due to lipid peroxidation (LPO) of plasma membrane involving disturbances in membrane fluidity. Such cascade of events causes increased cholesterol efflux and resulting calcium (Ca^{2+}) influx, followed by capacitation-associated events, priming the acrosome for exocytosis which cumulatively depicts cryo-capacitation. It is known that the spermatozoa of buffalo are more prone to oxidative damage compared to bull because of richness in polyunsaturated fatty acids (PUFAs) [29]. The greater concentration of PUFAs is associated with higher freezing and thawing associated damage, lower post-thaw sperm motility and conception rates of buffalo semen [28]. Thus, dead spermatozoa present a detrimental effect on buffalo semen fertility which is accentuated during cryopreservation.

Moreover, process of cryopreservation introduces stress on spermatozoa which may result in sperm damage those then produce detrimental levels of ROS [22]. The ROS induced damage comprises mitochondrial injury with a marked decrease in mitochondrial membrane potential (MMP) [49]. The mitochondria are known to generate

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energy to sustenance sperm functionality, hence poor MMP could serve as good indicator of the functional impairment. Such cumulative evidences show necessity of removal of dead and damaged spermatozoa from ejaculates immediately after collection.

The earlier literatures have reported various techniques for the removal of dead and damaged spermatozoa from fresh ejaculate [3,7,26,48]. However, such techniques are time-consuming, impractical for routine application and bulk semen processing in semen station [27,32]. Currently, nano-purification of ejaculates has been suggested as a viable technique to improve spermatozoa selection process without impairing the fertility [8,15]. The technique harbors on the fact that dead and defective spermatozoa express unique cell surface constituents that are not found in morphologically normal spermatozoa capable of fertilizing oocytes. Such sperm negative biomarkers can be targeted using specific probes during semen purification [41]. Ubiquitin is one such biomarker that has been found to correlate with poor spermatozoa quality and infertility. Sperm surface ubiquitination was reported in spermatozoa with abnormal morphology, DNA fragmentation [42], and acrosome damage [31] as well as the dysregulation and ectopic accumulation of the fertility associated proteins [23]. This fact supports the utilization of the ubiquitin as appropriate sperm cells marker [43] for nano-purification of semen. The technique offers advantages of simplicity and fastidious process, low cost, high specificity and sensitivity by use of immuno-specific marker [11] and appears suitable for mass production of buffalo semen doses.

In this study, ubiquitin conjugated IONPs were designed for semen nano-purification. We hypothesized that the nano-purification could improve membrane functionality, regulate Ca^{2+} channel and mitochondrial membrane potential, free radical concentration, and *in vitro* fertilizing potential of the buffalo spermatozoa. Hence, the objective of the present study was to evaluate effect of nano-purification on quality and fertility of buffalo semen. To achieve this, IONPs were conjugated with *anti*-ubiquitin antibody and were used in semen following validation.

2. Materials and methods

2.1. Materials

All the chemicals required in the experiment were purchased from Sigma-Aldrich (USA). The bare IONPs (Sigma-Aldrich, Germany) had average size of 20 nm and iron concentration of 5 mg/ml as mentioned by the manufacture. The purchased monoclonal *anti*-ubiquitin Ab (catalogue number SAB2702287) produced in mouse had concentration of 1 mg/ml as indicated by manufacture.

2.2. Preparation and characterization of Ab-IONPs

The IONPs were functionalized with 3-APTES (3-aminopropyl triethoxysilane) using aqueous silanization method as described by Villa et al. [47]. In brief, 10 ml of IONPs (2 g/l) was taken in conical flask and 0.4 ml of 3-APTES (2% v/v) and 10 ml of ethanol (99%) were added. The conical flask was covered and placed in incubator at the temperature 50 °C for 24 h. Following cooling, the IONPs were washed magnetically five times with ethanol followed by Milli-Q-water each. The silanized IONPs was finally dispersed at concentration of 25 mg/ml in Milli-Q-water.

The bio-conjugation of the silanized IONPs with monoclonal *anti*-ubiquitin Ab was done using EDC-NHS as described by Sung et al. [40] with certain modifications. Freshly prepared 2.5 ml of 0.1 M N-hydroxysuccinimide (NHS) was mixed with 665 μl of 75 mM 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC). A 5 μl of *anti*-ubiquitin Ab (@1 μg/μl) was added and reaction mixture was kept at room temperature for 30 min. Then 2.5 ml of the silanized IONPs were added to the reaction mixture and was kept in the rotatory shaker at 30 rpm for 90 min. The obtained bio-conjugated IONPs were washed magnetically five times with PBS and then finally suspended in PBS. The Ab-IONPs were stored at 4 °C till required for further use.

2.2.1. Estimation of iron concentration

The iron concentration in the IONPs was estimated following functionalization and bio-conjugation of NPs by the method described by Wu et al. [50]. In short, 0.5 ml of nanoparticle sample was mixed with equal volume of 2 N HCl in a glass test tube and solution was boiled for 10 min. The acidified solution was then cooled and 120 μl of 5 M KSCN was added. The sample was measured at 480 nm using bio-spectrophotometer (Eppendorf, Germany). The iron concentration of the nanoparticle sample was calculated using the ferric chloride standard curve generated by estimating different concentration of ferric chloride and expressed as mg/l.

2.2.2. Characterization of iron oxide nanoparticles

The determination of size of IONPs was done by Zetasizer (Microtrac, Montgomeryville, USA) using dynamic light scattering (DLS) method. The transmission light micrsocopy (TEM) was used for ascertainment of size and morphology. The samples for TEM were prepared by the method described by Vatasescu-Balcan et al. [46]. The characterization was done on bare IONPs, after functionalization and post Ab-conjugation.

2.3. Animal ethics statement

The Institutional Animal Ethics Committee (IAEC), ICAR-Indian Veterinary Research Institute had approved this investigation; as No invasive technique was used during collection of samples.

2.4. Experimental animals and collection of semen

The semen samples were collected from four Murrah buffalo (*Bubalus bubalis*) bulls of 4–6 years age. The bulls were housed in individual pen in an environmentally controlled building with exposure to natural day light. The bulls were kept under ideal feeding and management conditions during the entire period of investigation.

Semen was collected two times in a day and twice a week during morning hours (8 a.m.–9 a.m.) using artificial vagina. Immediately after collection, the ejaculated semen were placed in a water bath at 34 °C. The Tris-Egg-Yolk-Glycerol (TYG) extender was utilized for semen dilution in the present study.

2.4.1. Study groups and nano-purification of semen

A total 24 ejaculates were utilized for the study. Ejaculates with minimum 2.0 ml volume, 3+ mass motility, 500 million/ml sperm concentration and ≥70% individual progressive motility were selected. Each sample was divided into 4 groups: Group I (control) and Group II, Group III, Group IV (treatments). Each group contained fixed number (150 ± 25 million) of dead/damaged spermatozoa. In the treatment group, Ab-IONPs were added at the ratio of 1:0.5, 1:1 and 1:2 in Group II, Group III, and Group IV, respectively based on concentration of Ab-IONPs. The Ab concentration @1 μg/ml was used to attain the above ratio.

The desired volumes of Ab-IONPs were added to the semen sample in the test tubes. The semen containing Ab-IONPs was placed against an external magnetic field consisting of magnets fixed in polycarbonate stand (Fig. 1a). The tubes were incubated for 5 min in water bath at 34 °C. All the Ab-IONPs including bound with ubiquitinated sperm (dead/damaged) were pulled down to the bottom of the glass tube while supernatant contained free non-ubiquitinated spermatozoa (viable) (Fig. 1b). The nano-purified semen was aspirated and collected in a different pre-warmed tube.

2.4.2. Semen processing

Following nano-purification, the semen from each group was diluted

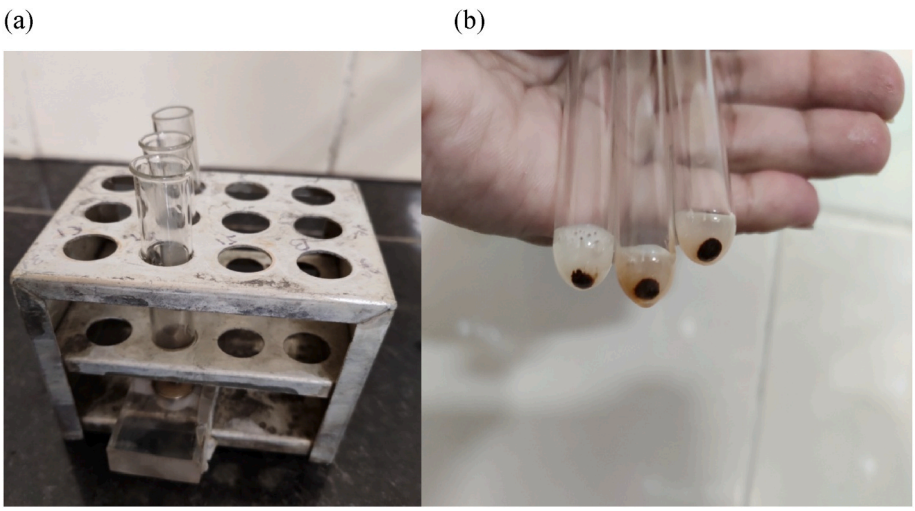

Fig. 1. Nano-purification of semen. (a) Test tube containing suspension of antibody conjugated iron oxide nanoparticle (Ab-IONPs) and semen. The magnet is placed below to pull down free Ab-IONPs or IONPs bound with dead and damaged spermatozoa (b) Pellets of Ab-IONPs bound with dead and damaged spermatozoa.

with the TYG extender to achieve final concentration of 80 million/ml. The filling of semen and sealing of straws (French mini straws) was done using an automatic filling machine (IMV, L'Aigle, France). Thereafter, the straws were positioned in the racks and transferred to cold handling cabinet (IMV, L'Aigle, France) at 5 °C for 4 h for equilibration. Following equilibration, the racks containing straws were transferred to anautomatic freezer (IMV, L'Aigle, France) for freezing. Finally, the straws were plunged directly into the liquid nitrogen (LN2, −196 °C).

2.4.3. Thawing of semen

Straws were placed in water maintained at temperature 37 °C for 30 s and semen was poured into glass tube kept at 37 °C in water bath. The semen was used immediately for evaluation of various semen quality parameters.

2.5. Semen examination at fresh stage

2.5.1. Mass motility

The mass motility was determined by placing a drop of undiluted fresh semen on glass slide without cover slip & mounted on a stage maintained at 37 °C under low power of microscope. It was graded on the scale of 0–5+ [35].

2.5.2. Concentration and individual progressive motility

The spermatozoa concentration and individual progressive motility (IPM) were assessed by an automated sperm quality analyzer-Vision for bulls (SQA-Vb, Medical Electronic Systems, Israel).

2.5.3. Viability

The spermatozoa viability was estimated by vital staining technique using Eosin Nigrosin stain [44]. Briefly, a drop of Eosin Nigrosin stain was taken on glass slide to which one drop of semen was added, and mixed quickly. After 30 s a thin smear was made on the same slide. The smear was air dried immediately and examined under oil immersion objective of bright field microscope (RH-84UXL, Unilab). A total of 200 sperm cells were counted in each slide and per cent value was calculated. The fully and partially stained spermatozoa were considered as non-viable.

2.5.4. Sperm morphological abnormalities

Percentage of sperm morphological abnormalities was determined using same staining procedure as described for the viability of spermatozoa.

2.5.5. Hypo-osmotic swelling (HOS) test

The HOS test was performed to assess the biochemical integrity of plasma membrane of spermatozoa as per the method described by Jeyendran et al. [18]. One ml of hypo-osmotic solution (osmolality 150 mOsm/l) was taken in a 5 ml glass tube and 0.1 ml of semen was added. The sperm suspension was mixed well and incubated in water bath at 37 °C for 60 min. After incubation a drop was placed on glass slide and covered with cover slip. The slides were examined under a high power magnification (400X) of a bright field microscope (RH-84UXL, Unilab).

Different types of tail swelling pattern were recorded as mentioned by Prasad et al. [33]. Pattern A: No swelling, complete loss of membrane integrity, Pattern B: Swelling at the tip of the tail, Pattern C: Different types of hair-pin like swelling, Pattern D: Complete tail swelling. The sperm cells displaying the swelling pattern B, C and D were considered positive for the HOS test.

2.5.6. Efficiency of nano-purification

The efficiency of nano-purification was determined through assessment of total sperm recovery/initial sperm concentration and variation (increment/decrement) in semen quality parameters such as IPM, viability, morphological abnormalities, plasma membrane integrity and acrosome integrity following nano-depletion of dead and damaged spermatozoa.

$$\text{Efficiency}\ (\%) = \frac{\text{Value after nanopurification} - \text{Value before nanopurification}}{\text{Value before nanopurification}}\ \text{X}\ 100$$

2.6. Evaluation of semen at post-thaw stage

The IPM, sperm viability and morphological abnormalities and HOS test were assessed by the methods as described earlier.

2.6.1. Evaluation of acrosome integrity by FITC-PSA

The FITC-PSA-PI (Fluorescein isothiocyanate conjugated with *Pisum sativum* agglutinin lectins-Propidium iodide) was used to evaluate integrity of acrosome and plasma membrane as per the protocol described by De Jonge and Barratt [12]. In brief, 100 μl of semen sample was taken in a micro-centrifuge tube and PBS was added to make volume up to 1 ml. The sample was washed twice using PBS by centrifugation at 170*g* for 10 min. Supernatant was discarded and final volume was made to 100 μl with PBS kept at 37 °C. A 2.0 μl of PI solution (stock 1 mg/ml) was added and kept in dark for 2 min. Then, excess PI was removed by adding 900 μl of PBS followed by centrifugation at 800*g* for 5 min. The supernatant was removed and final volume was made to 100 μl with PBS. A 20 μl of sperm suspension was taken and smear was made on slide and was air dried. The spermatozoa were fixed with 100% methanol for 5 min. Excess methanol was removed by washing the slide with PBS. The slide was then overlaid with FITC-PSA working solution (2.5 μg/ml) & kept in incubator at 37 °C in dark for 30 min. The excess FITC-PSA was removed by washing the slide with PBS. A drop of anti-fade solution (0.22 M 1, 4-diazo-bicyclo (2, 2, 2) octane dissolved in glycerol: Phosphate buffered saline (9:1)) was placed on the stained smear. The slide was examined immediately under the fluorescent microscope (MT6300, Meiji Techno, Japan) with blue and green filter at 40x. A total of 200 spermatozoa were counted and categorize as follow:

PSA positive & PI negative: Acrosome intact live (AIL)
PSA positive & PI positive: Acrosome intact dead (AID)
PSA negative & PI negative: Acrosome reacted live (ARL)
PSA negative & PI positive: Acrosome reacted dead ARD)

2.6.2. Evaluation of mitochondrial membrane potential by JC-1

The MMP of post-thaw semen samples were evaluated by the method described by Kasai et al. [21]. A semen sample was washed twice with HTF-HEPES (IMV, L'Aigle, France) and then diluted with 200 μl HTF-HEPES. A 20 μl of JC-1 working solution (2 μM) was added into sperm suspension and incubated at 37 °C for 15 min. Following incubation, a drop of sperm suspension was placed on slide, covered with cover slip and observed under fluorescent microscope (MT6300, Meiji Techno, Japan).

Spermatozoa having high MMP appeared red to orange (green filter) while spermatozoa with low MMP appeared green (blue filter). Two images (both filters) of the same field were merged to get a final image.

2.6.3. Evaluation of capacitation by chlortetracycline (CTC) assay

The CTC method was used to determine capacitation status of spermatozoa as method explained by Fraser et al. [16]. A 10 μl of semen was mixed with equal volume of freshly prepared CTC solution on a glass slide at room temperature. After 5 s, 1.5 ml of glutaraldehyde (12.5% v/v in 20 mM Tris-HCl) was added. A drop of anti-fade solution was added and the slide was covered with cover slip and examined using fluorescent microscope (MT6300, Meiji Techno, Japan). The spermatozoa were classified as:

Pattern F: Uniform bright fluorescence over a whole head (non-capacitated spermatozoa)

Pattern B: Fluorescence-free band in the post-acrosomal region (capacitated spermatozoa)

Pattern AR: Dull fluorescence over the whole head except a thin punctate band of fluorescence along the equatorial segment (acrosome reacted spermatozoa).

2.7. Assessment of oxidative status

2.7.1. Estimation of LPO of spermatozoa

The LPO of spermatozoa was assessed by determining the malondialdehyde (MDA) concentration in the sperm pellet by thiobarbituric acid-trichloroacetic acid (TBA-TCA) by the method described by Suleiman et al. [39]. The spermatozoa pellet was suspended in PBS to obtain the concentration of 20 million/ml. A 1 ml of spermatozoa suspension was added to into 2 ml of TBA-TCA reagent and kept in boiling water for 1 h. The sample was centrifuged at 3000 rpm for 10 min at room temperature. The supernatant was taken and absorbance was read in bio-spectrophotometer (Eppendorf, Germany) at 535 nm against TBA-TCA reagent as blank. The MDA (μmol/ml per 20 million spermatozoa) was calculated using formula $\text{MDA}(\mu\text{mol}/\text{ml per 20 million spermatozoa}) = \frac{\text{OD x } 10^6 \text{ X total volume of reaction mixture (3ml)}}{1.56 \text{ x } 10^5 \text{ total sample volume (1ml)}}$

2.7.2. Estimation of total antioxidant capacity (TAC)

The TAC in seminal plasma was measured using ferric reducing/antioxidant power (FRAP) assay as described by Benzie and Strain [6]. A 100 μl of seminal plasma (1:10 diluted) was mixed with 3 ml of working FRAP reagent and vortexed at room temperature. The absorbance (593 nm) was measured using bio-spectrophotometer (Eppendorf, Germany) at 0min against blank. The samples were remained in bio-spectrophotometer at 37 °C under simple kinetics and absorption was again measured after 4 min. The ascorbic acid standards (1000 μM) were processed in the same way as seminal plasma sample. The concentration of TAC (μmol) was calculated using formula

$$\text{FRAP value of sample } (\mu\text{mol}) = \frac{\text{Change in absorbance of sample from 0 to 4 minute}}{\text{Change in absorbance of standard from 0 to 4 minute}} \times \text{FRAP value of standard } (1000\mu\text{M})$$

2.8. In vitro fertility test (zona binding assay)

The effect of nano-purification on semen fertility was compared between control and treatment group (Group III) yielding best results. The samples from both the groups were subjected to zona binding assay. The method described by Bag et al. [5] was followed throughout *in vitro* fertility test. The evaluation of zona binding to matured oocytes was observed under inverted microscope to count number spermatozoa bound to zona (IX51, Olympus, Tokyo, Japan).

2.9. Statistical analysis

The data recorded in percent values were subjected to angular transformation before the analysis. One-way analysis of variance (ANOVA) was performed using Graphpad Prism software (version 6). The level of significance $p < 0.05$ was used to define statistical significance in this study. Data are presented as Mean ± SEM.

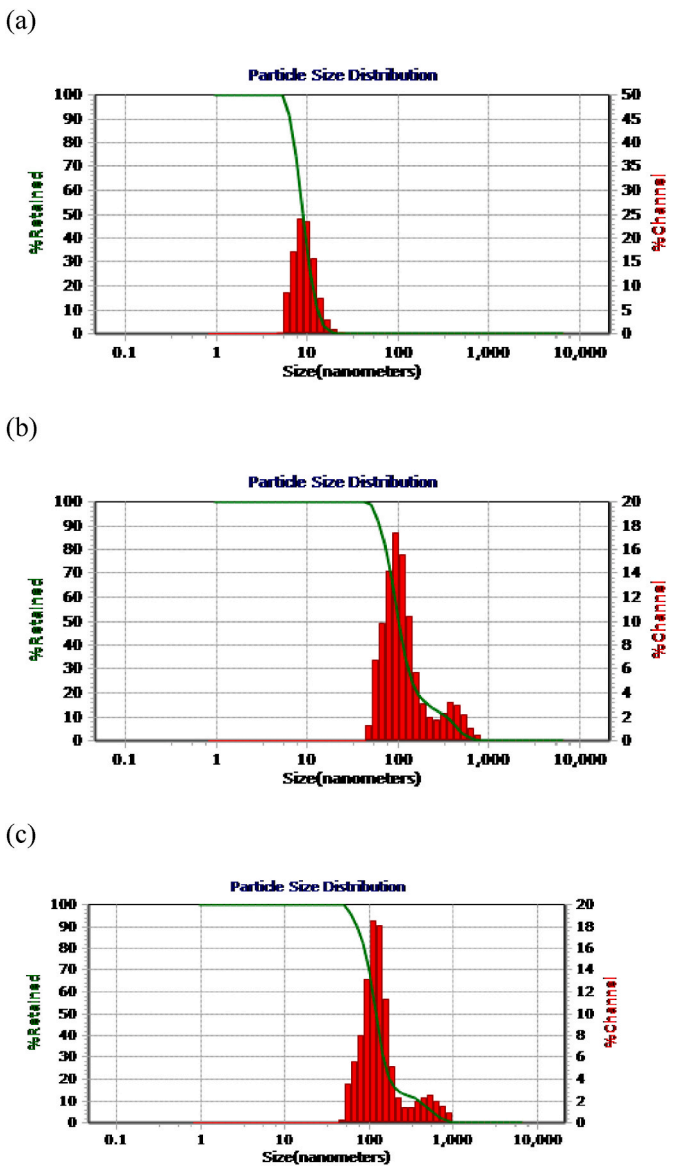

Fig. 2. Size distribution of nanoparticles using DLS technique. The graphs of (a) bare, (b) silanized and (c) antibody-conjugated iron oxide nanoparticles.

3. Results

3.1. Iron concentration of IONPs

The iron concentration was 2.2 mg/ml and 0.9 mg/ml after functionalization and bio-conjugation with *anti*-ubiquitin, respectively.

3.2. Characterization of IONPs

The size distribution of IONPs at bare, post-functionalization and post-conjugation using DLS techniques is presented in Fig. 2a–c. The average size of purchased IONPs was uniformly 9.5 nm (100%). After functionalization with silane, the size was 406 nm and 71.8 nm with population proportion of 9.4% and 90.6%, respectively. Post-conjugation with Ab average size of 13.9% and 86.1% population proportion was 484 nm and 115.1 nm, respectively.

The size and morphology of IONPs observed under TEM has been depicted in Fig. 3a–c. The average size of bare IONPs, silanized and Ab-IONPs were 10.2 nm, 12.8 nm and 20.9 nm, respectively with round shape.

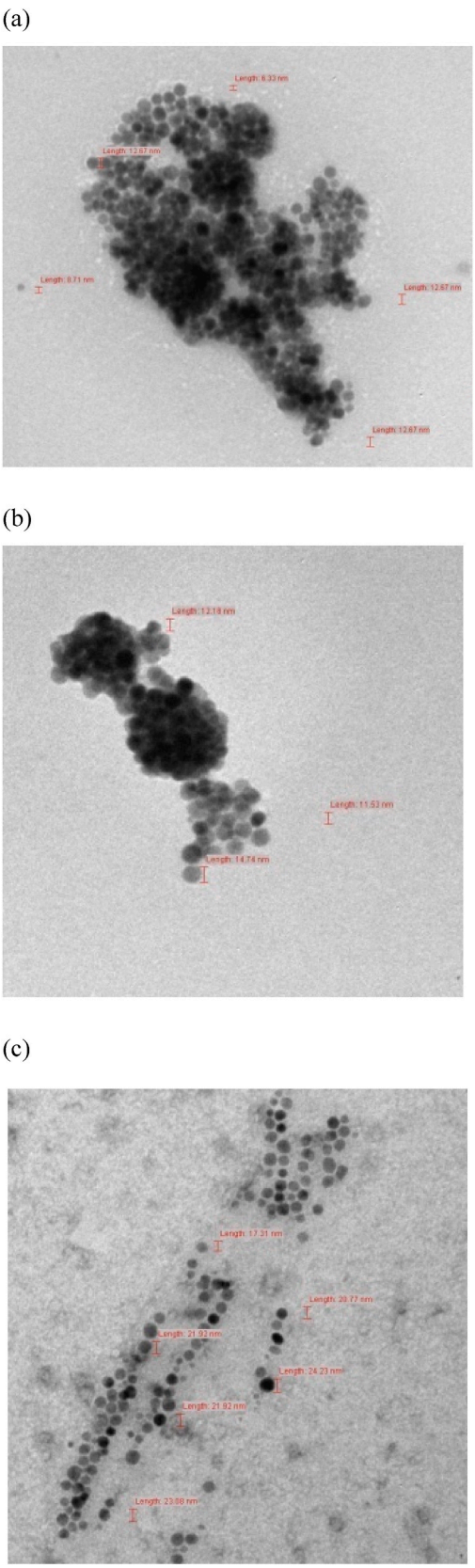

Fig. 3. TEM images depicting size and morphology of nanoparticles at various stages. The images of (a) bare, (b) silanized and (c) antibody-conjugated iron oxide nanoparticles at 12000X.

Table 1
Seminal attributes of experimental Murrah buffalo bulls (n = 24) post-collection.

Parameter	Volume (ml)	Mass motility	Sperm conc. (millionmL^{-1})	Individual progressive motility (%)	Viability (%)	Morphological abnormality (%)	HOS test positive sperm (%)	Acrosome integrity (%)
Mean ± SEM	2.8 ± 0.30	3.7 ± 0.1	1153.9 ± 88.7	75.4 ± 1.1	80.3 ± 1.0	5.5 ± 0.4	71.4 ± 1.5	72.4 ± 1.4

HOS test, Hypo-osmotic swelling test.

Table 2
Effect of nano-purification on semen quality parameters at fresh stage (n = 24; Mean ± SEM).

Parameter	Control	Treatment		
	Group I	Group II	Group III	Group IV
Sperm concentration	1153.9 ± 88.7^{d}	952.8 ± 87.4^{c}	789.4 ± 73.0^{b}	648.3 ± 62.6^{a}
IPM (%)	75.4 ± 1.1^{b}	79.5 ± 1.7^{bc}	83.7 ± 1.2^{ac}	86.2 ± 1.2^{a}
Viability (%)	80.3 ± 1.0^{c}	85.2 ± 1.3^{b}	88.8 ± 0.9^{ab}	90.4 ± 1.2^{a}
Sperm abnormality (%)	5.5 ± 0.4^{c}	3.7 ± 0.3^{b}	2.6 ± 0.3^{ab}	1.9 ± 0.2^{a}
HOS test positive spermatozoa (%)	71.4 ± 1.5^{c}	77.2 ± 1.7^{b}	80.4 ± 1.2^{ab}	82.6 ± 1.3^{a}

IPM, Individual progressive motility; HOS test, Hypo-osmotic swelling test. Mean values bearing different superscripts (a: b: c: d = $p < 0.05$) differ significantly within row.

Table 3
Efficiency of nano-purification on physio-morphological parameters of spermatozoa (n = 24; Mean ± SEM) at fresh stage. Data are presented in %.

Parameter	Treatment		
	Group II	Group III	Group IV
Sperm concentration	18.1 ± 3.1^{c}	31.3 ± 3.2^{b}	43.8 ± 3.6^{a}
IPM	5.4 ± 1.4^{b}	11.3 ± 1.6^{a}	14.6 ± 1.8^{a}
Viability	6.1 ± 1.2^{b}	10.9 ± 1.6^{a}	12.7 ± 1.6^{a}
Sperm abnormality	33.6 ± 4.2^{c}	55.6 ± 4.2^{b}	67.1 ± 3.3^{a}
HOS test positive spermatozoa	8.3 ± 1.5^{b}	13.3 ± 2.1^{ab}	16.3 ± 2.2^{a}

IPM, Individual progressive motility; HOS test, Hypo-osmotic swelling test. Mean values bearing different superscripts (a: b: c = $p < 0.05$) differ significantly within row.

3.3. Semen examination

The mean values of volume (mL), mass motility, sperm concentration (million/mL), IPM (%), viability (%) and membrane integrity (%) is presented in Table 1.

Table 4
Effect of nano-purification on semen quality parameters at post-thaw stage (n = 24; Mean ± SEM).

Parameter	Control	Treatment		
	Group I	Group II	Group III	Group IV
Post-thaw motility	33.7 ± 2.2	37.1 ± 2.6	39.1 ± 2.5	36.2 ± 2.2
Viability (%)	44.4 ± 2.1	47.7 ± 2.8	49.1 ± 2.6	47.2 ± 2.6
Sperm abnormality (%)	6.1 ± 0.2^{b}	4.3 ± 0.3^{a}	3.7 ± 0.4^{a}	3.4 ± 0.4^{a}
HOS test positive spermatozoa (%)	40.0 ± 2.1	42.7 ± 3.0	43.2 ± 3.1	40.5 ± 3.3

HOS test, Hypo-osmotic swelling test. Mean values bearing different superscripts (a: b = $p < 0.001$) differ significantly within row.

3.3.1. Effect of nano-purification on fresh semen quality

The effect of nano-treatment on semen quality is presented in Table 2. The mean values of sperm concentration (million/mL) significantly ($p < 0.05$) reduced between control and treatment groups post nano-purification. The concentration was found lowest in group containing highest concentration of antibody (Group IV). The IPM (%) significantly improved ($p < 0.05$) following semen nano-purification in Group III and IV. Similar observation was made in the viable sperm population. The mean population of abnormal spermatozoa (%) was significantly reduced ($p < 0.05$) in nano-purified groups than control group. The lowest population of abnormal spermatozoa was observed in Group IV. The mean value of HOS test positive spermatozoa improved significantly ($p < 0.05$) in treatment groups than control group. However, the result between Group I and II was non-significant ($p > 0.05$).

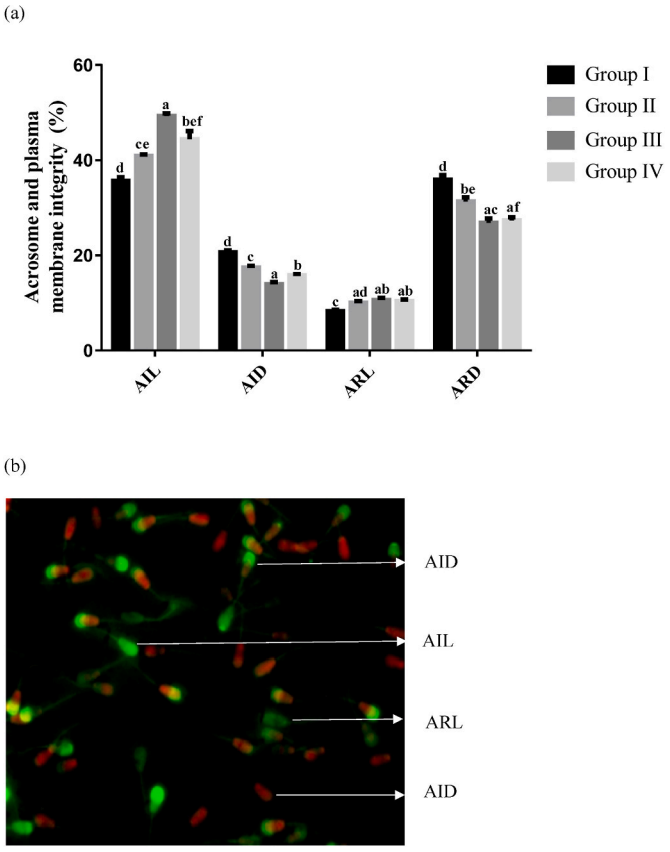

Fig. 4. (a) Effect of nano-purification on acrosome using FITC-PSA-PI (fluorescein-conjugated Pisum sativum agglutinin-propidium iodide) assay at post-thaw stage. Data is presented as Mean ± SEM. Mean values bearing different superscripts [(AIL, a: b = p < 0.05; a: c: d = p < 0.001; f: d = p < 0.001), (AID, a: b = p < 0.05; a: c: d = p < 0.001; b: c = p < 0.01; b: d = p < 0.001), (ARL, b: c = p < 0.001; c: d = p < 0.05), (ARD, b: c = p < 0.01; a: d = p < 0.001; e: f = p < 0.001; e: d = p < 0.05)] differ significantly between groups. AIL, acrosome intact live spermatozoa; AID, acrosome intact dead; ARL, acrosome reacted live; ARD, acrosome reacted dead. Photograph of FITC-PSA-PI stained smear at 400X depicting AIL, AID, ARL and ARD spermatozoa.

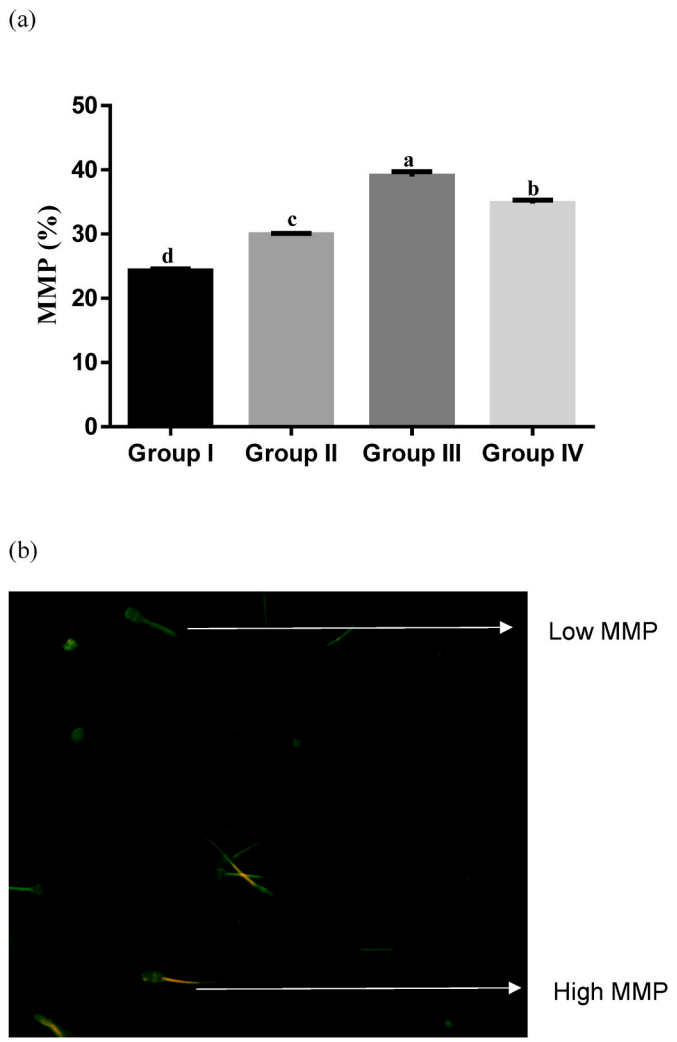

Fig. 5. (a) Effect of removal of nano-purification on MMP (%) using JC-1 probe at post-thaw stage. Data is presented as Mean ± SEM. Mean values bearing different superscripts (a: b: c: d = p < 0.001) differ significantly between groups. MMP, mitochondrial membrane potential. (b): Photograph of JC-1 assay stained smear at 400X depicting spermatozoa with high and low mitochondrial membrane potential (MMP).

3.3.2. *Efficiency of nano-purification on semen quality at fresh stage*

The efficiency of various Ab concentration conjugated with IONPs on the semen quality is presented in Table 3. The mean efficiency (%) of the removal of dead and damaged spermatozoa was significantly higher in Group IV followed by Group III and Group II. Similarly, the mean efficiency (%) of nano-purification on IPM and viability and HOS test positive sperm were found higher in the Group IV. The effectiveness for the removal of morphological abnormal spermatozoa was significantly greater in the Group IV.

3.3.3. *Evaluation of post-thaw semen quality*

The effect of nano-treatment on semen quality at post-thaw stage is presented in Table 4. The effect of nano-purification on post-thaw motility, viability and membrane functionality was non-significant between the groups. However, higher motility and viable population of spermatozoa were observed in the Group III. The morphological abnormality was significantly lower ($p < 0.001$) in treatment as compared to control group. However, the mean difference between the various treatment groups was non-significant.

The mean effect of nano-purification plasma membrane and acrosome integrity (%) at post-thaw stage is presented in Fig. 4. The mean

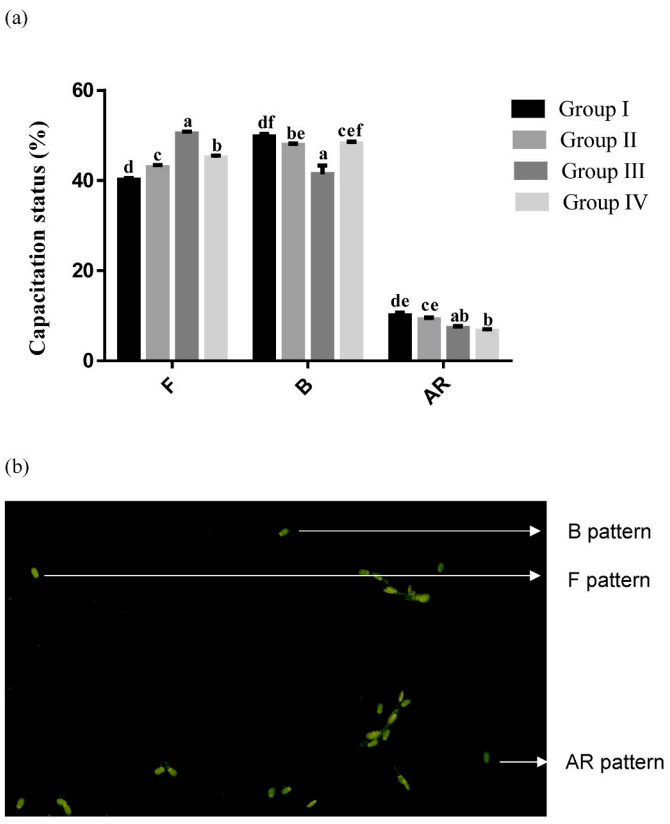

Fig. 6. (a) Effect of nano-purification on capacitation status using CTC assay at post-thaw stage. Data is presented as Mean ± SEM. Mean values bearing different superscripts [(F pattern, a: b: d = p < 0.001; a: c: d = p < 0.001; b: c = p < 0.01), (B pattern, a: b = p < 0.05; a: c = p < 0.01; a: d = p < 0.001; e: f = p < 0.05), (AR pattern, a: c = p < 0.05; a: d = p < 0.01; b: c = p < 0.001; b: d = p < 0.001)] differ significantly between groups. Photograph of CTC assay stained smear at 400X depicting F pattern (non-capacitated spermatozoa), B pattern (capacitated spermatozoa) and AR pattern (acrosome reacted spermatozoa).

Table 5
Effect of nano-purification on status of oxidative stress (n = 24; Mean ± SEM).

	Fresh stage	Post-thaw stage			
		Control	Treatment		
		Group I	Group II	Group III	Group IV
LPO (MDA μmol/ml per 20million spermatozoa	0.3 ± 0.01^{a}	0.7 ± 0.01^{e}	0.5 ± 0.01^{d}	0.4 ± 0.01^{bf}	0.4 ± 0.00^{cg}
TAC (μmol)	1.5 ± 0.09^{a}	0.5 ± 0.01^{e}	0.6 ± 0.01^{d}	0.8 ± 0.01^{b}	0.7 ± 0.01^{cd}

LPO, Lipid peroxidation; TAC, Total antioxidant capacity. Mean values bearing different superscripts (a: b: c: d: e = $p < 0.001$; f: g = $p < 0.01$) differ significantly within row.

value of AIL sperm (%) in Group I, II, III and IV was 35.6 ± 0.7, 40.8 ± 0.2, 49.2 ± 0.5 and 44.3 ± 1.7, respectively. The mean value of AID sperm (%) was 20.6 ± 0.4, 17.4 ± 0.3, 13.9 ± 0.4 and 15.7 ± 0.2 in Group I, II, III and IV, respectively. The mean population of ARL sperm (%) was 8.2 ± 0.3 in Group I, 10.0 ± 0.4 in Group II, 10.6 ± 0.4 in Group III and 10.4 ± 0.2 in Group IV. While, the mean value of ARD sperm (%) was 35.8 ± 0.9 in Group I, 31.2 ± 0.8 in Group II, 26.8 ± 0.9 in Group III and 27.3 ± 0.6 in Group IV.

The mean effect of nano-depletion on depolarization of sperm membrane potential and Ca^{2+-}regulating mechanisms is presented in

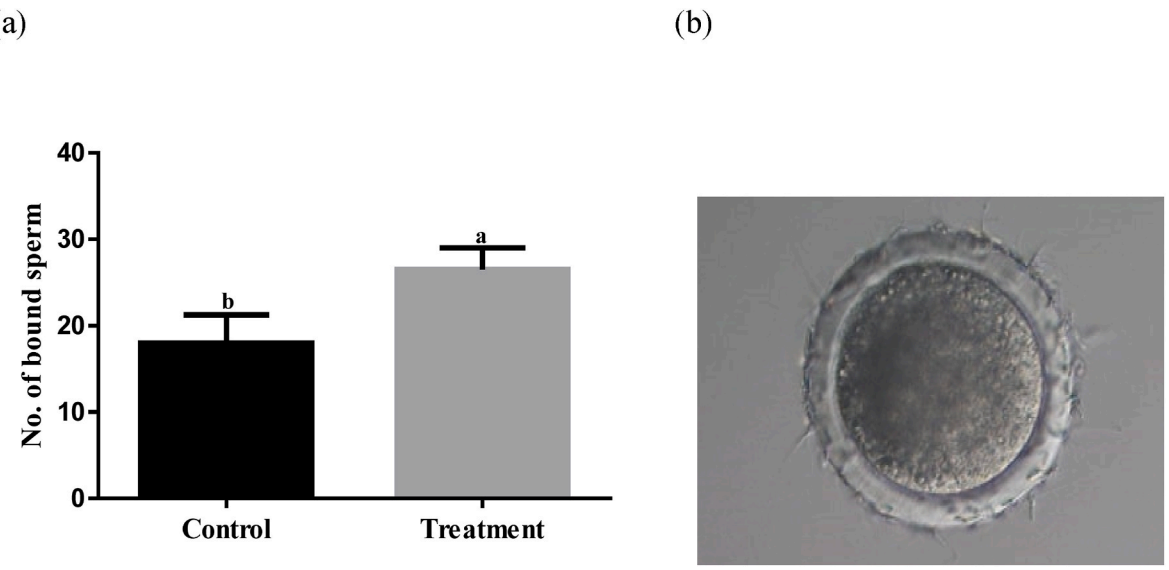

Fig. 7. (a) Effect of removal of dead and damaged spermatozoa on zona binding assay using frozen-thawed semen. Data is presented as Mean ± SEM. Mean values bearing different superscripts (a: b = $p < 0.01$) differ significantly between groups. (b) Photograph under 400× magnification of spermatozoa bound with oocyte in zona binding assay.

Figs. 5 and 6, respectively. The mean value with high MMP (%) in Group I, II, III and IV was 24.2 ± 0.2, 29.9 ± 0.1, 39.0 ± 0.6 and 34.7 ± 0.5, respectively. The mean value of F pattern (%) in Group I, II, III and IV was 40.1 ± 0.4, 42.9 ± 0.4, 50.4 ± 0.4 and 45.1 ± 0.4, respectively. The mean spermatozoa population depicting B pattern (%) was 49.7 ± 0.5 in Group I, 47.8 ± 0.2 in Group II, 41.4 ± 1.8 in Group III and 48.2 ± 0.2 in Group IV. The mean value of AR pattern (%) was 10.1 ± 0.6 in Group I, 9.1 ± 0.3 in Group II, 7.2 ± 0.4 in Group III and 6.6 ± 0.3 in Group IV.

3.3.4. Assessment of oxidative status

The mean level of MDA and TAC were found lowest and highest, respectively at fresh stage. The mean value of MDA (μmolmL^{-1} per 20 million spermatozoa) differs significantly between all the groups with lowest in Group III followed by Group IV, II and I. The TAC differs significantly between all the groups with highest in Group III followed by Group IV, II and I (Table 5).

3.4. In vitro fertility test (evaluation of zona binding assay)

The mean number of spermatozoa bound with zona pellucida was 18.0 ± 3.2 in control and 26.5 ± 2.5 in treatment groups. The effect of removal of dead and damaged spermatozoa on number of bound spermatozoa in zona binding assay is depicted in Fig. 7.

4. Discussion

The removal of dead and damaged spermatozoa is essential to minimize negative effect on contemporary live spermatozoa. The dead and damaged spermatozoa release free radicals excessively that affect motility and membrane integrity of live spermatozoa cells, thereby reducing quality of an ejaculate. The magnetic and bio-functionalization properties of IONPs make them suitable for semen nano-purification. The attribute that defective spermatozoa are being ubiquitinated can be utilized to easily detect surface defects in spermatozoa. The present study attempted designing of Ab-IONPs and demonstrates beneficial effects of nano-purification on functionality and fertility of buffalo semen.

The IONPs used in this study were similar in size (9.5 nm) as obtained by Ali et al. [2] and was smaller than reports of Chen et al. [10] and Bisla et al. [8]. The variation might be due to the sensitivity of the instruments used for size assessment, category of chemicals and methods followed during synthesis. The overall increase in size of IONPs is attributed to multiple surface coating of IONPs following functionalization and bio-conjugation [40,51]. Further, the size of functionalized and bio-conjugated IONPs was found larger using DLS than TEM technique. The bigger size might be due to the surrounding water (solvent) and swelling effect of surface molecules of NPs [20]. A reduction in iron concentration was observed following silane coating and conjugation with *anti*-ubiquitin antibody. The reduced concentration might be due to continued loss of IONPs during repeated magnetic washing of NPs while processing.

It is well known that ubiquitin is expressed on the surface of dead and damaged spermatozoa [8,31,43]. The reduction in dead/damaged spermatozoa population and abnormality percentage in the study is due to application of *anti*-ubiquitin antibody against morphologically defective spermatozoa. The enhancement in IPM, sperm viability and membrane functionality has been observed following separation of dead and moribund spermatozoa from the live ones in the study. The findings concurred with the results of Bisla et al. [8] in buffalo, Caldeira et al. [9] in bull and Durfey et al. [14] in boar semen following nano-separation of dead or defective spermatozoa from the ejaculates. The improvement in semen quality in present study was found to be directly associated with Ab-IONPs concentration. This could be attributed to that fact that greater number of Ab-IONPs were available to bind with dead and damaged spermatozoa in Group IV than Group III and II.

The higher live spermatozoa population (acrosome intact or reacted) was observed in nano-purified groups than control. In contrast, the percentage of dead spermatozoa with acrosome intact or reacted was lower in nano-depleted groups. The higher value of AIL and ARL than AID and ARD in the study suggests better efficacy of Ab-IONPs in selection of spermatozoa with intact plasma membrane. Also, it is possible that some viable spermatozoa might have undergone acrosome reaction during semen processing [38].

The deleterious effect of ROS is more extensive during semen processing and cryopreservation than in fresh semen [34]. Along with structural damages, the ROS is known to decline MMP of spermatozoa [37] which can also be evidenced by reduction in spermatozoa motility [13]. The ROS causes loss of cholesterol from spermatozoa membrane resulting in altered cholesterol/phospholipids ratio and premature capacitation [25]. The higher MMP and spermatozoa population exhibiting F pattern ensuing nano-purification warrant the removal of source of ROS generation (dead and damaged spermatozoa).

The higher oxidative stress level in untreated group might be due to elevated level of LPO. It is universally known that the dead or damaged spermatozoa generate free radicals resulting in self-propagating LPO [24]. The nano-separation of dead and damaged spermatozoa reduced LPO level and increased level of TAC in the current study. The observation is further supported by previous report [8], where higher level of TAC was observed following removal of dead and defective spermatozoa using filtration technique. In the current study, the reduced oxidative stress level has resulted in the much greater values in viability, and acrosome integrity and ultimately increased fertility of spermatozoa. This can be evident by higher binding efficiency of buffalo spermatozoa with zona pellucida in treatment group. Similar to the present study, greater results of IVF trial using nano-purified semen in bull was reported by Odhiambo et al. [30].

5. Conclusion

The study describes the improvement in viability, MMP, capacitation status and fertility of buffalo semen following nano-purification of the fresh ejaculates using IONPs bio-conjugated with *anti*-ubiquitin antibody. The study highlights the importance of depletion of dead and defective spermatozoa, a primary source of free radicals from the ejaculates to minimize the detrimental effect on the contemporary live and healthy cells. The resultant effects were enhanced plasma membrane integrity, intact acrosome and high MMP thus, such spermatozoa had greater potential to bind with zona pellucida of buffalo oocytes *in vitro*. The nano-purification with 1.0 μg/ml antibody concentration (Group III) reflects improvement in semen quality and fertility. Though the results of the current findings advocate maximizing the production of good quality semen in livestock without discarding poor quality ejaculates using nano-purification technique, we suggest undertaking much wider and greater number of fertility trials for a conclusive recommendation. The technique could also be advantageous in increasing chances of conception in human males with suboptimal sperm parameters.

Declaration of conflicting interest

The authors declare no conflicts of interest.

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