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LOW FERTILE BULLS COULD BE USED FOR SEMEN CRYOPRESERVATION AFTER SUPPLEMENTATION OF EXTENDER WITH ZINC COMPOUNDS

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Abstract
Zinc plays a significant role in the male reproductive system where it is believed to have protective function in terms of sperm functional quality parameters such as antioxidant activity. We hypothesized that addition of zinc sulphate and zinc oxide in the extender will improve the quality and fertility of cryopreserved low-fertility Nili-Ravi buffalo sperm. Semen was collected from three bulls using an artificial vagina (42 °C). Qualifying ejaculates from each bull were divided into aliquots and diluted (at 37 °C, 50 x 10^6 sperm/mL) in *tris*-citric acid extender containing zinc oxide (at 25, 50, 75 and 100 µg/mL), zinc sulphate (at 50, 100, 150, 200 and 250 µg/mL) and control (without supplementation). After 24 h of storage, straws were thawed at 37 ◦C for 30s to assess post-thaw sperm quality. Post thaw improvement ($P < 0.05$) in sperm progressive motility and plasma membrane integrity was recorded in extender containing zinc oxide (50 µg/mL) and zinc sulphate (100 µg/mL) compared to control. *In vivo* fertility rate was evaluated by inseminating 50 buffaloes (25 inseminations per extender; zinc oxide at 50 µg/mL, zinc sulphate at 100 µg/mL) 12 hours after standing heat. The fertility rate of low fertility buffalo bull sperm in terms of positive pregnancy at 90 days after insemination was clinically higher in extender containing zinc sulphate (54%) and zinc oxide (40%). In conclusion, supplementation of zinc oxide (50 µg/mL), zinc sulphate (100 µg/mL) in the extender improved the sperm post thaw quality parameters and yielded higher *in vivo* fertility rate.
Keywords: Low fertility bull, cryopreservation, Zinc Oxide, Zinc Sulphate, artificial insemination

Introduction
Cryopreservation and artificial insemination of semen have a significant impact on dairy and beef industry. Domestic animals such as goats, cows, buffaloes, and sheep are multi-use animals in the

industry, providing raw materials such as high-quality proteins in the form of meat and milk, as well as excreta as fertilizer, their skin, wool, and drought power are also utilized (Irshad et al., 2011). Even though cryopreservation is a facilitated method for preserving bull semen, there are still significant gaps in understanding and knowledge regarding this technique. Different bulls exhibit varying behavior in terms of sperm viability during the cryopreservation process, which can be influenced by their fertility status (Ugur et al., 2019). If the bull's fertility is already low for any reason, its semen experiences more significant damaging effects during the post-thaw process compared to the semen of a normally fertile bull (Lemma, 2011). Only the sperm of bulls that exhibit high-quality post-thaw motility are accepted for cryopreservation. Low-fertility semen, on the other hand, is discarded and replaced by the semen of other bulls. Low-fertile bulls that do not meet the classification criteria are culled and used for slaughter purposes (Nagata et al., 2019).
Cryopreservation technique is best for preserving high fertility semen but not for low-fertility semen as they have low antioxidant supply and high rate of oxidative stress which causes damaging effects as dead sperm production is the main reason to produce reactive oxygen species (ROS). By decreasing oxidative stress and reducing osmotic pressure, post thaw quality semen of low fertile bulls could be improved (Guthrie and Welch, 2012). Filtration methods can be used to separate normal sperm cells whereas lipid peroxidation and oxidative stress were decreased by the addition of antioxidants to extenders which are associated with cryopreservation (Lemma, 2011). Disturbance in the balance between production of reactive oxygen species and antioxidant defense system not only destroy morphology of sperm but also viability, DNA integrity and motility (Tremellen, 2008).
Zinc compounds (oxide and sulphate) attribute a central part in numerous functioning as an antioxidant. Zinc function as a scavenger for hydroxyl radicals, by inhibiting NADPH oxidase reduces DNA fragmentation mechanism and decreasing lipid peroxidation (Çoyan et al., 2011). Zinc compounds is used as an extender in different breeds of bulls for example holstein (Awan et al., 2017), water buffaloes (Dorostkar et al., 2014), stallion (Ghallab et al., 2017) and Friesian bulls (Fouad and Ashour, 2021), where zinc compounds in combination improved the fertility. Different research experiments have depicted that zinc compounds can clear up radicals brought by different elements, including reducing malondialdehyde (MDA) as well as ionizing radiation, levels, so it is considered as antioxidant trace metal (Goel et al., 2005). Evidence obtained that higher sperm motility, viability and reduce abnormal morphology were observed when seminal plasma contain sufficient amount of zinc (Alavi-Shoushtari et al., 2009). Keeping in view the properties of zinc as antioxidants and the use of glass wool filtration to remove dead and damaged sperm, the following study was designed. According to available information, the supplementation of zinc compounds (sulphate and oxides) alone in the extender has never been evaluated for cryopreservation of low fertile buffalo bull sperm to improve the semen quality. It has been depicted that the filtration (to remove dead sperm; additional source of ROS) plus addition of zinc compounds to the semen extender may improve the quality of semen collected from low fertile bulls which can improve economy by saving a big pool of discarded semen from low fertile bulls.

Materials and Methods Extender preparation

Tris-citric acid buffer (pH =7.0, osmotic pressure 320 mOsmol Kg_{-1}), consisted of 1.56 g citric acid (Fisher Scientific, UK) and 3.0 g tris–(hydroxymethyl)-aminomethane (Research Organics, USA) in 73 mL distilled water, was used for the experimental extenders. Buffer was added with antibiotics combination; streptomycin sulphate @ 1mg/mL, procaine penicillin@300IU/mL, glycerol (Riedel-deHaen, Germany) 7%; Fructose (Scharlau, Spain) 0.2% wt/vol; benzyl penicillin@ 100IU/mL®
available as Sinbiotic (China). In experiment 1, different concentrations of zinc sulphate (Riedel-deHaen) (0µg, 50µg, 100µg, 150µg, 200µg and 250µg) was added and prepared six experimental

extenders. In experiment 2, zinc oxide (Riedel-deHaen) was added in different concentrations (0µg, 25µg, 50µg, 75µg and100µg) for five experimental extenders.

Semen collection
In each three replicates (weekly intervals) semen was collected from six adult Nili-Ravi buffalo bulls using an artificial vagina (42°C) and samples were immediately evaluated for motility (%), concentration (X-200) and volume using phase contrast microscope and spectrophotometer respectively. Furthermore, the qualifying ejaculates were split into six and five aliquots for dilution in 1st and 2nd experimental extenders respectively.

Glass wool filtration
Glass wool column was made by inserting glass wool (30 mg) (microfiber code 112; John Manville, USA) into the barrel of a 3 mL syringe, compressed to a final thick-ness of 3 mm. Sperm washing media (SWM: Modified Ca^{+2} free Sperm-TALP) were used for sample dilution before glass wool filtration (Husna et al*.,* 2016). The column was rinsed and pre-equilibrated with SWM until the filtrate observed under microscope was free of glass wool fibers. The diluted semen suspension was then centrifuged (6 min at 300g) and after that, it was re-suspended in 2 mL of SWM. The suspension was placed on top of the glass wool column, allowed to filter by gravity, discarding the first three drops and remaining filtrate was collected. Progressive motility was observed by picking a drop of semen on an oil-free, dirt-free, clean warmed up glass slide, and a coverslip was used to cover it before placing it on the microscope (biotherm) at 37°C. Same was done for observation of both filtered and unfiltered semen. A maximum of 5 to 7 fields was observed under the final magnification of 400X.

Semen processing
Diluted suspensions from experiment-1 (n=6) and experiment-2 (n=5) were cryopreserved and thawed using standard techniques as previously reported by Husna et al*.,* (2016). The filtered semen suspensions were adjusted to a final concentration of 60×10^6/mL sperm using Tris-citric acid extender at a temperature of 37°C. Next, the sample was cooled (4°C), equilibrated (4°C, 4 hr period) and filled in 0.5 mL french straws with a suction pump in a cold cabinet unit (4°C). The straws were kept on liquid nitrogen vapors (10 min), plunged, stored in liquid nitrogen and transported to the PMAS- Arid Agriculture University-Rawalpindi, for sperm post-thaw quality evaluation. After 24 h, straws were thawed in a water bath (37°C, 30 s) and incubated in a water bath for assessment of post-thaw semen quality.

Post thaw semen quality Sperm progressive motility
Using a phase-contrast microscope (BX20 Olympus, X400, Tokyo, Japan), the progressive motility of sperm was evaluated by preparing a pre-warmed (37◦C) glass slide by placing a semen drop on it.

Sperm plasma membrane integrity
Plasma membrane integrity was evaluated by supravital hypo-osmotic swelling test (HOST) as performed by (Husna et al*.,* 2016). Fresh solution for HOST prepared by 1.35 g fructose (Scharlau) and 0.73 g sodium citrate (Merck) in 100 mL distilled water (osmotic pressure, 190 mOsmol/kg). Semen sample (50 µL) was mixed with 500 µL of the pre-warmed HOST solution and incubated (37°C, 30-40 min). After that, a drop of incubated semen was placed on a slide, cover-slipped and observed microscopically using 400X magnification lens. 200 sperm per experimental extender were observed in five different fields. Sperm having swollen tails are considered intact with biochemically active sperm plasma membranes, while unswollen tails are disrupted and inactive with nonfunctional sperm plasma membrane (Husna et al*.,* 2017).

Sperm viability
Sperm viability (live: dead) was evaluated using dual staining procedure (Qadeer et al., 2016). Briefly, an equal volume of Trypan-blue and semen were placed on a slide (room temperature) and mixed. Smears were air-dried and fixed with formaldehyde-neutral red (5 min). Slides were washed using distilled water and Giemsa stain (7.5%) was applied (4 h). After that, slides were washed, air dried and mounted with mounting media. Using phase contrast microscopy (1000× magnification) 200 sperms were observed in each smear and results were expressed as % of viable sperm (live sperm with intact acrosome) of total cells. Sperm with no staining and purple acrosome were regarded as viable with an intact acrosome, while sperm with blue staining were indicated dead.

Sperm chromatin integrity
Chromatin integrity in sperm was evaluated by toluidine blue (Mello and Beletti, 2002). Diluents sperm were air-dried (30 min, 4°C) before being dipped in 96 percent ethanol-acetone (1:1) for fixing. Acid hydrolysis with 4NHCl was performed (25℃) for 10-30 min. Smear sample was rinsed with the help of distilled water thrice a time every two min. For staining purposes, toluidine blue was used (10 min) and observed using phase contrast microscope (1000X). Sperm with dark stain was believed to have broken chromatin structure, whereas the bright sperm stain was considered to have functioning chromatin. For each experimental extender, around 200 sperm were seen.

Sperm acrosomal integrity
Formaldehyde-citrate solution (2.9 g tri-sodium citrate dihydrate and 1 mL of 37% solution of formaldehyde in 99 mL distilled water) was used for sperm acrosomal integrity evaluation. Briefly, sample (100 µL) was mixed with formaldehyde-citrate solution (500 µL) and 200 sperm were evaluated under a phase contrast microscope (1000×) magnification using oil immersion. Intact acrosome and shining head were considered to have a normal acrosome, while blunt-ended heads were considered to have a disrupted acrosome (Husna et al., 2017).

Artificial insemination and pregnancy rates
After post-thaw analysis, the best concentration from both experiments was picked for *in vivo* fertility trials. 50 buffaloes were inseminated (25 for each group) in Tehsil/District Haripur, Pakistan during the peak breeding season (October to December). The inseminations were done using low-fertile buffalo bull semen supplemented with 100µg zinc sulphate and 50µg zinc oxide after 24 hours of standing heat. After 3 months of insemination, the pregnancy rate was confirmed through rectal palpation.

Statistical analysis
The data on semen quality parameters are presented as means ± SD. Data were analyzed using analysis of variance (ANOVA). Where the F ratio was found significant i.e. ($P < 0.05$), then post-hoc comparisons such as Tukey's significance test were applied. Fertility results are analyzed using chi square test. The data were analyzed using Prism Graph pad (Version-5).

Ethical statement
This study was approved by ethical committee at University of Sialkot for the use of animals.

RESULTS

Zinc Oxide

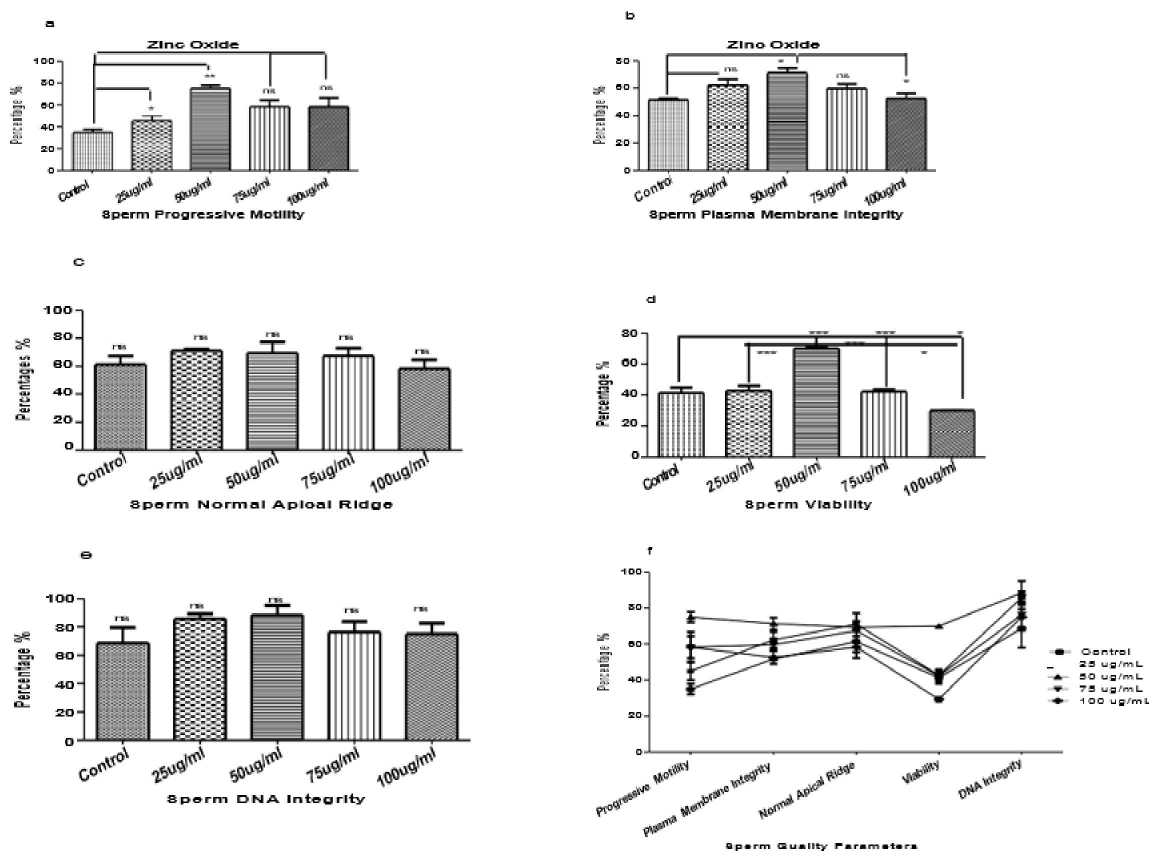

Figure 1. **Effect of zinc oxide on post thaw sperm quality parameters** shown are the bar graphs of effect of zinc oxide on post thaw sperm quality parameters on (a) motility, (b) plasma membrane integrity, (c) normal apical ridge, (d) viability, (e) chromatin integrity and (f) all these in collective form in sub fertile buffalo bulls with different concentrations (Mean±SE). these experiments were replicated in triplicate and all the experimental are given in material and method section. Bars showing *, **, *** differ significantly ($P<0.05$) whereas "ns" shows non-significant ($P>0.05$) difference.

The data on the effect of zinc oxide in the extender on the percentage of post-thaw sperm quality parameters shown in Figure 1. Higher ($P<0.05$) sperm quality Parameters (motility, plasma membrane integrity, normal apical ridge, viability and chromatin integrity) was observed in an extender containing zinc oxide at concentration 50µg compared to all other concentrations: 25µg, 75µg, 100µg, as well as the control.

Zinc Sulphate

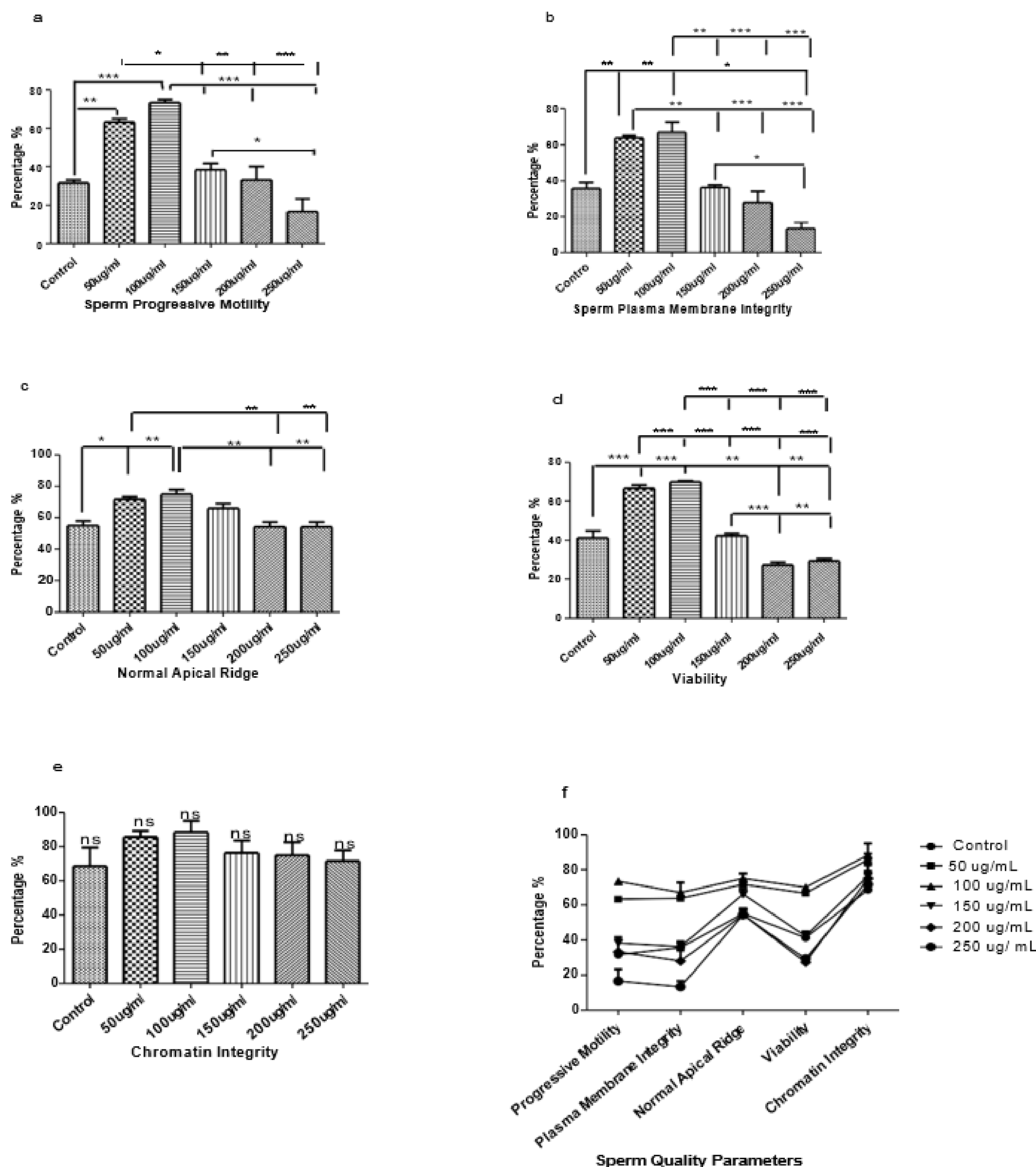

Figure 2. **Effect of zinc sulphate on post thaw sperm quality parameters** shown are the bar graphs of effect of zinc sulphate on post thaw sperm quality parameters on (a) motility, (b) plasma membrane integrity, (c) normal apical ridge, (d) viability, (e) chromatin integrity and (f) all these in collective form in sub fertile buffalo bulls with different concentrations (Mean±SE). These experiments were replicated in triplicate and all the experimental are given in material and method section. Bars showing *, **, *** differ significantly (P<0.05) whereas "ns" shows non-significant (P>0.05) difference.

The data on the effect of zinc sulphate in the extender on the percentage of post-thaw sperm quality parameters shown in Figure 2. Higher (P<0.05) sperm quality Parameters (motility, plasma membrane integrity, normal apical ridge, viability and chromatin integrity) was observed in an extender containing zinc sulphate at concentration 100µg compared to all other concentrations: 150µg, 200µg, 250µg, and the control

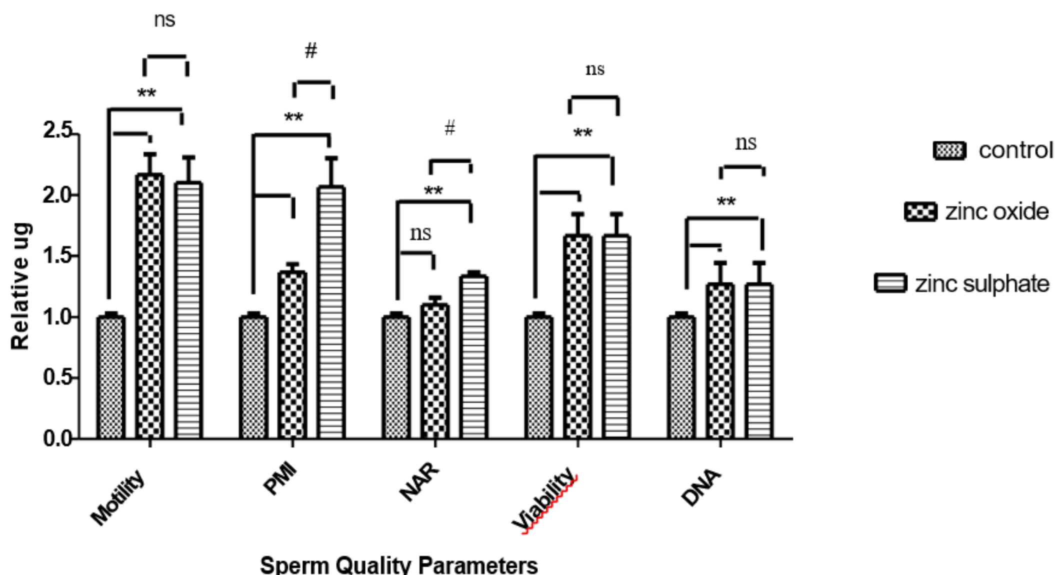

Figure 3. **Relative comparison of zinc oxide (50µg) and zinc sulphate (100µg)** shown are the bar graphs of effect of zinc oxide (50µg) and zinc sulphate (100µg) on post thaw sperm quality parameters (motility, plasma membrane integrity, normal apical ridge, viability and chromatin integrity) in sub fertile buffalo bulls. Bars showing *, **, # differ significantly (P<0.05) whereas "ns" shows non-significant (P>0.05) difference.

In-Vivo fertility (%)

Data on the in vivo fertility rate of low fertile buffalo bull sperm cryopreserved in extender containing zinc oxide (50 µg/mL) and zinc sulphate (100 µg/mL) are given in Table 1. The fertility rate of low fertile buffalo bull sperm in terms of pregnancy rate was significantly higher in the extender having zinc sulphate (100 µg/mL), i.e., 54%, as compared to zinc oxide (50 µg/mL), i.e., 40%. The results demonstrate that the use of this extender led to a higher pregnancy rate for the low fertile buffalo bull sperm. The control group used here is normal one. These result surprisingly shows that if we treat the semen of low fertile bull and supplement with zinc sulphate @ 100ug / zinc oxide @ 50ug it could be reached at level of control of normal bulls.

Table 1. In-vivo fertility trial of low fertile bull semen supplemented with Zinc Oxide (50 µg/mL) and Zinc Sulphate (100 µg/mL) and control (normal bull semen).

Extender type	No. of Insemination Recorded	Pregnancies achieved	chi-square	p-value
Control	50	22 (44%)		
Zinc Oxide (50 µg/mL)	50	26 (52%)	0.3802	0.826872
Zinc Sulpahte (100 µg/mL)	50	27 (54%)		

Discussion

The livestock industry prefers bulls with high fertility because they have a significant economic impact on production (Abdollahi-Arpanahi et al., 2017). As semen from low fertile bulls causes lower pregnancy rates, which increases the expense of housing these bulls and non-pregnant cows (Parisi et al., 2014), these bulls are culled and wasted. The current study was designed to improve the quality of low fertile buffalo bull through two steps. In the first step, glass wool filtration was performed with fresh semen from low fertile bulls before cryopreservation to remove dead, moribund and abnormal sperm, aiming to minimize oxidative stress by reducing ROS production

due to dead cells. In the second step, the filtered semen from low fertile bull was diluted in an extender supplemented with different concentrations of zinc compounds (sulfates and oxides). The post-thaw quality and in vivo fertility of zinc compounds (sulfates and oxides) supplemented semen from low fertile buffalo bull were assessed. The post-thaw quality of low fertile bull sperm was increased by supplementing the extender with zinc sulfate at a concentration of 100µg/mL and zinc oxide at a concentration of 50µg/mL.

Even from highly fertile bulls, ejaculates include 10 to 40% dead sperm (boar: Sancho et al., 2004; bull: Murphy et al., 2014; stallion: Janett et al., 2003; buck: Barkawi et al., 2006; ram: Azawi and Ismaeel, 2012). This dead percentage may increase under certain conditions, such as ageing or adverse environmental conditions (Todini et al., 2007). In present study semen was collected from low fertile buffalo bull, these ejaculates have higher number of dead sperm which is treated with glass wool filtration. As the sperm move through the glass wool fibers, glass wool filtration separates dead sperm that stick to the glass wool fibers (Henkel et al., 2001). These dead or dying sperm are the sources of ROS, and glass wool filtration only selects the sperm that are morphologically normal, viable, and having intact acrosome (Anzar et al., 1997; Januskauskas et al., 2005). In the current study, semen filtrates after glass wool treatment was assessed, and only ejaculates containing live sperm with forward motility and intact acrosome were selected for further processing in study.

Semen collected from low fertile bull has low quality and its motility and fertility is further reduced after freeze thaw steps. In the present study, perm progressive motility of low fertile bull is increased by supplementing the extender with zinc oxide and zinc sulphate at lower concentration (50µg and 100 µg respectively) as compared to control. Our findings showed that adding zinc compound to semen extender increases semen quality after freezing in a dose-dependent manner. According to studies, zinc acts as an antioxidant, particularly as a co-factor of copper/zinc superoxide dismutase (Cu/Zn SOD), which is crucial for the sperm's defense against reactive oxygen species and peroxidative damage (Sikka , 2001). The Lipid composition of buffalo sperm plasma membrane markedly differs from that of other cells, as it contain higher amount of polyunsaturated fatty acids (PUFAs). These PUFAs are more vulnerable to lipid peroxidation deterioration during the processing and freezing of sperm, leading to their deterioration. Reactive oxygen species cause the lipids peroxidation of the sperm plasma membrane, leading to destruction of the lipid matrix. This oxidative stress impairs the sperm motility, membrane functionality, and fertility by damaging the sperm DNA (Aitken, Baker 2004). The improved antioxidant capacity of zinc compound in the current study may have contributed to better sperm motility by reducing the oxidative damage to sperm caused by lipid peroxidation (Hidiroglou and Knipfel, 1984). Zinc sulphate supplementation at 250µg concentration significantly reduced the motility that might be due to imbalance in oxidant and antioxidant ratio, also high level of zinc in semen hinders sperm oxygen consumption, causing motility to drop to harmful levels (Dorostkar et al ., 2014).

When extender was supplemented with zinc compound, the membrane integrity of sperm from low fertile bull was higher, possibly due to zinc's membrane-stabilizing action resulting from its interaction with some functional groups in the intrinsic part of the sperm membrane, such as OH and CH (Dorostkar et al ., 2014), this may be reason for the sperm plasma membrane intactness with zinc compound supplementation in present study. However, adding a higher concentration of zinc sulphate might deplete all ROS and thereby have harmful effects on functional parameters such as sperm plasma membrane integrity (Martin-Hidalgo et al., 2011). The improved percentage of intact acrosomes due to the addition of zinc sulphate may be attributed to its antioxidant effects, which can inhibit lipid peroxidation by regulating phospholipase activity and stabilizing the acrosome membrane through zinc's membrane-stabilizing action, thereby influencing fluidity changes (Eggert-Kruse et al., 2002). Zinc has been found to stabilize acrosomal enzymes such as phosphatase, acrosin, and phospholipase, which could explain the higher percentage of intact acrosome. Zinc has also been found to maintain and suppress lysosome destabilization in the acrosome, which is a highly specialized form of lysosome (Kumar et al., 2006). These facts of zinc may be reason for

higher sperm acrosome integrity and sperm viability of semen collected from of low fertile bull when supplemented in extender in present study.
The positive effects of zinc compounds on the quality of the sperm have been previously reported in stallions (Kchalifa et al., 2006), goats (Liu et al., 2014), buffalo bulls (Alavi-Shoushtari et al., 2009), and humans (Kvist and Bjorndahl, 1985). The Membrane-stabilizing action of zinc, which prevent the leakage of enzymes, proteins, and other vital sperm components and improve the functional life span of sperm, may be the cause of the improved sperm quality (Kumar et al ., 2006). Similarly reported by Betteger and O'Dell (1981) that zinc stabilizes RNA, DNA, lysosomes, and ribosomes which may improve the normal functioning and survival rate of sperm. In the current study, semen from low-fertility bulls is treated with zinc compound supplementation for the first time, and the improved post-thaw quality may be a result of the aforementioned positive effects of zinc.
In the current study, fertility rates were found to be higher with zinc oxide and zinc sulphate at concentration 50 µg/mL and 100 µg/mL respectively when supplemented in the extender for semen collected from low fertile bull. Zinc ions play a crucial role in sperm capacitation as they regulate important processes necessary for fertilization competency. Previous studies have highlighted the significance of calcium ion influx for capacitation, zinc ions efflux is the gatekeeper to this calcium ion influx, in same way some lower quality of zinc ion may also be requirement for effective acrosomal exocytosis (Kerns et al., 2018). In the current study, the in vivo fertility of buffalo using semen from low fertile bull, supplemented with zinc compounds is attributed to the reduction of reactive oxygen species by zinc, which can have detrimental effects on sperm function through the oxidation of lipids, proteins, and DNA (Flaherty and Fournier, 2017). Sperm have the capacity for zinc ion loading, which is required for restoring their pre-capacitation zinc signature, thereby reducing premature and pathological sperm capacitation (Kerns et al., 2018) and improving fertility.

Conclusions

Supplementation of zinc oxide at concentration 50 µg/mL and zinc sulphate at concentration 100 µg/mL in the extender improved the sperm post thaw quality parameters of sperm and yielded higher in vivo fertility rate with semen collected from low fertile Nili-Ravi buffalo.

Acknowledgments

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Authorship contribution statement

AUH and SQ planned the study. SQ, AA, TR, RB and AUH were involved in data analysis and manuscript writing. All the authors read the final manuscript draft.

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