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Research Article

Nano-oleic Acid in Tris-Extender: Effects on Bull Sperm Cryopreservation, Fertility, and Post-thaw Antioxidant Status

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Abstract:

The aim of this study was to evaluate the impact of supplementing Tris-extender with different levels of nano-oleic acid (NOA) on sperm characteristics, and enzyme activity and antioxidant status of post-thawed sperm medium of bull semen. Semen was collected by artificial vagina from 5 Holstein bulls, then pooled, diluted by Tris-egg yolk extender supplemented with NOA at levels of (0, 1, 2, 4 and 6 µL/mL), equilibrated at 5°C for 4h, loaded in straws, and stored in liquid nitrogen, then thawed at 37°C for 30 s for evaluation. Supplementing Tris-extender with NOA (5 µL) increased (P<0.05) the percentages of progressive motility and membrane integrity after dilution, progressive motility, livability, and membrane integrity after equilibration, as well as increasing progressive motility, livability, and membrane integrity, and decreasing abnormality of sperm cells after thawing. Also, recovery rate of progressive motility, livability, and membrane integrity after equilibration and thawing was the highest for semen supplemented with NOA at level 1µL. Supplementing Tris-extender with NOA at a level of 5 or 2µL decreased (P<0.05) AST and ALT activities and increased (P<0.05) total antioxidant capacity level and glutathione peroxidase activity as compared to free-extender. Pregnancy rate of cows inseminated with 1µL NOA was higher (57.5%, P<0.05) than in free-extender (32.5%) or other levels of NOA (25-42.5%). In conclusion, addition of 1µL NOA in Tris-extender of bull semen can be used as a strategy for improving freezing ability and fertilizing capacity of cryopreserved bull spermatozoa.

1. Introduction

Artificial insemination (AI) is now widely utilized in most domestic species including mammals which requires a broad use of semen preservation techniques. In cattle, the use of cryopreserved spermatozoa of genetically superior animals is beneficial for the genetic improvement and transportation of spermatozoa over long distances. The thermal, osmotic, and toxic stress during freeze-thawing process leads to damage of sperm membrane damaged **(Giraud et al., 2000)**, resulting in a reduction of 50% in sperm viability **(Watson, 2000)**.

It is important to maintain the sperm freezing and fertilizing abilities of cryopreserved semen by developing an efficient system of cryopreservation (Gliozzi et al., 2003), because plasma membrane of mammalian sperm cells contain high level of polyunsaturated fatty acids (PUSFA), they are sensitive to reactive oxygen species (ROS) inducing lipid peroxidation during *in vitro* storage and causing male infertility (Surai and Sparks, 2001). Lipo-peroxidation irreversibly abolishes the fructolytic and respiratory activity, which may thereby result in a considerable decline in their respiratory rate and motility (Surai and Sparks, 2001). During cryopreservation (cooling and freezing processes), and thawing, sperm cells are severing different physical and biochemical challenges, and the survival of sperm cells is dependent mainly on the protective roles of different components of the extender used in semen preservation (Abdel-Khalek et al., 2024).

Fatty acid is the composite of a hydrocarbon chain, a methyl group, and a carboxylic acid group. The length of hydrocarbon chain ranges from 14 to 24 carbon atoms with different positions of carbon-carbon double bonds (Christie, 1973). Phospholipids contain two fatty acid chains (Vance and Vance, 1996) and the roles of phospholipids are primarily as constituents of biological membranes (Hames and Hooper, 2000) like sperm cells. Different studies have been conducted to evaluate the impact of PUSFA on quality of semen in different animal species after cooling, freezing, and thawing. In this context, bull sperm motility and viability were improved in semen chilled for 7 days by supplementation of citrate-based extender with alpha-Linolenic acid (ALA) at levels of 100 and 50 µM, respectively. While, motility and viability were maintained by palmitic acid at a level of 50 µM (Nakamura and Nara, 2004; Kiernan et al., 2013). Semen extender supplementation with linoleic acid improved motility and viability of frozen-thawed spermatozoa in bull semen (Takahashi et al., 2012). Addition of Docosahexaenoic acid (DHA) improved bull sperm quality after cryopreservation (Nasiri et al., 2012; Towhidi and Parks, 2012). Quality of frozen bull semen was improved in Tris or Bioxcell extender supplemented with ALA or DHA, but their combination impaired semen quality after freezing (Kaka et al., 2015a; Kaka et al., 2015b). On the other hand, sperm characteristics in frozen bull semen were not affected by supplementing citrate extender with omega-3 PUFAs (Abavisani et al., 2013; Kandelousi et al., 2013). In boar, Hossain et al. (2007) reported that addition of unsaturated free fatty acids in semen extender improved sperm motility and viability during *in vitro* incubation.

Oleic acid (OA) is one of fatty acids in sperm cells (Kelso et al., 1997; Lenzi et al., 2000). OA is found naturally in various animal and vegetable fats and oils. It is an odorless, colorless oil, although commercial samples may be yellowish due to the presence of impurities. In chemical terms, oleic acid is classified as a mono-unsaturated omega-9 fatty acid, abbreviated with a lipid number of 18:1 cis-9, and a main product of Δ9-desaturase (Nakamura and Nara, 2004). It was reported that sperm motility and viability were maintained by oleic acid at a level of 100 µM in bull semen extender (Kiernan et al., 2013). In earlier studies, it was reported that the fatty acids are easily incorporated into the cell membrane and act as substrates for ATP production via mitochondrial β-oxidation (Neill and Masters, 1971; Jaswal et al., 2011). In addition, incorporation of specific lipids has positive impacts on sperm cells, conferring greater protection during the cryopreservation process. In this respect, the addition of oleic-linoleic acid dimers (C18:1 n-9 and C18:2 n-9, respectively) improved freezing ability leading to increased motility of and viability of cryopreserved ram spermatozoa (Pérez-Pé et al., 2001; Marti et al., 2008).

Nanoparticles (NPs), defined by having at least one dimension within the range of 1–100 nm, have become increasingly common in a variety of medical and research fields (Barkhordari et al., 2013). Nano-particulates showed novel properties, such as a specific surface area, numerous active surface centers, high surface activity and catalytic efficiency, and strong adsorptive ability (Peters et al., 2004; Shi et al., 2010). Previous studies have evaluated the impact of different types of nano-particles (Shahin et al., 2020) and natural extracts of medicinal herbs (Khalil et al., 2018; Tvrdá et al., 2018) in semen extenders of different animal species, on freezing ability and fertility of sperm cells after cryopreservation. The information on the impact of nano-particles of fatty acids, especially oleic acid, on maintaining sperm characteristics in post-thawed bull semen is rare. Therefore, the aim of the current study was to evaluate the effect of NOA on freezing and fertilizing abilities of bull spermatozoa.

2. Materials and Methods

This study was conducted at the International Livestock Management Training Center (ILMTC), Sakha, belonging to the Animal Production Research Institute, Agricultural Research Center, Ministry of Agriculture, Egypt, in participation with Department of Animal Production, Faculty of Agriculture, Tanta University, during the period from August 2023 until January 2024.

Semen donors:

Five sexually mature healthy Holstein bulls (600 kg body weight and 4.5 years old) housed individually under semi-open sheds and allowed to drink all day were used as semen donors. All animals were with normal external genitelia. The testicular tone was glandular; all epididymal regions were present in right and left testes and both were equal in size and moved freely up and down within the scrotal pouches.

All animals were fed according to the live body weight as recommended by Animal Production Institute, every bull was fed on daily ration composed of 8 kg concentrate fed mixture (CFM), about 6 kg rice straw beside 40 kg green berseem (*Trifolium alexandrinum*) in winter, or 6 kg berseem hay in summer. The CFM was composed of 32% undecorticated cotton seed cake, 26% wheat bran, 22% yellow maize, 12% rice bran, 5% linseed meal, 0.5%limestone, 2%vines and 0.5% salt. The daily ration was given individually to all bulls, while drinking clean fresh water and mineral blocks were available for all bulls at all day times.

Semen collection:

The semen was collected from five bulls twice a week for 6 months (240 ejaculates from all bulls), using the conventional artificial vaginal method. The temperature of the inner lining rubber sleeve of the artificial vagina was adjusted to 41-43°C. Semen ejaculates were collected before feeding from 7.0 am to 8.0 am. A bull was used as teaser animal for sexual preparation.

Collected semen immediately was held in a water bath at 37°C before being transferred to the lab. Ejaculates having good mass motility (≥70%) were pooled for each collection day.

Experimental design:

Hydrothermal squeezes method was implemented for preparation of nano-particles of the oleic acid. About 2µL of commercial oleic acid was immersed in 60 mL distilled water containing 1-2 mL dimethyl sulfoxide (DMSO) in a 100 mL Teflon Beaker. The Teflon beaker was then autoclaved at 40 °C overnight for about 24 h. The extract was squeezed from the powder under the effect of temperature and pressure in the closed autoclave. Obtained extracts were then collected and centrifuged. Obtained extract stored in a cold dark place until use.

Tris-citric acid extender was used as a buffer, consisted of 1.675 g citric acid, 3.025 g tris–(hydroxymethyl-aminomethane), 0.75 g glucose and 7.0% glycerol. The control extender contained Tris, 15% fresh egg yolk (15 mL/100), while nano oleic acid was added to other four experimental extenders at levels of 1, 2, 4, and 6µL mL. Amounts of 0.25 g lincospectin and 0.005 g streptomycin were added to all extenders. All contents were thoroughly dissolved in bi-distilled water up to 100 mL.

Semen was diluted at a rate of 1:20 with each type of extender as the following:

T1: Tris-extender without addition (control extender).
T2: Tris-extender supplemented with 1µL NOA/mL.
T3: Tris-extender supplemented with 2µL NOA/mL.
T4: Tris-extender supplemented with 4µL NOA/mL.
T5: Tris-extender supplemented with 6µL NOA/mL.

After dilution, semen was equilibrated by cooling gradually in a refrigerator at 5°C for 4 hours. At the end of equilibration period, the extended semen was loaded

in 0.25 mL French straws using a semen filling machine, during filling in the straws extended semen was kept in ice water bath to keep its temperature at 5ºC. Straws were transferred into processing canister and located horizontally in static nitrogen vapor 4 cm above the surface of liquid nitrogen for 10 minutes, then the straws were placed vertically in a metal canister and immersed completely in liquid nitrogen container for storage at –196ºC. Frozen semen in the 1st experiment was thawed by dipping the frozen straws into a water bath at 37ºC for 30 seconds.

Sperm characteristics:

Progressive motility:

It was estimated by adding one drop of raw semen and cryopreserved semen (post dilution, post equilibrium and post thawed to a test tube containing 2 mL warm physiological saline (0.9% Nacl) and wormed it in 37ºC in water bath. The mixture was gently shaken and a drop of semen was taken from the test tube with a warm Pasteur pipette and placed on a worm slide. The drop was covered by a wormed cover slide and immediately examined under the 40x objective. The samples were graded according to the percentages of spermatozoa moving forward motion across the field of vision with normal vigorous swimming motion according to Amann and Hammerstedt (1980). Then the percentage of sperm motility was calculated.

Live sperm:

A smear from semen was made on a glass slide and was stained by eosin (1.67%) and nigrosine (10%) mixture stain (Hackett and Macpherson, 1965). Ten grams' nigrosine and 1.67 g eosin were dissolved in distilled water up to 100mL for the preparation of eosin-nigrosine stain at 37°C.One drop of the prepared stain was added to one drop of the fresh semen and were mixed on glass slide at 37°C and left for 2-3 minutes. Then, a thin smear was made by drawing a second slide across the stained semen. The slide was allowed to dry on the hot stage of research microscope and then examined under a high-power magnification (x 400). At examination, the dead spermatozoa (unstained ones) and live spermatozoa (stained ones) were counted in field of a total of 200 spermatozoa. Then the percentage of live spermatozoa was calculated.

Abnormal sperm:

Normal spermatozoa, when exposed to hypo-osmotic stress due to the influx of water, undergo swelling and subsequent increase in volume. The sperm plasma membrane surrounding the tail fibers appears to be more loosely attached than the membrane surrounding the head, so, the tail regions show the swelling effect more clearly (Foote and Kaproth, 1997; Ballester et al., 2007; Lodhi et al., 2008).

Plasma membrane integrity:

The plasma membrane integrity of spermatozoa was assessed using the hypotonic swelling test (HOST) as described by Jeyendran et al. (1984) after dilution, equilibration and freezing-thawing semen. HOST solution was prepared by dissolving 0.735 gm of sodium citrate dihydrate and 1.351gm fructose in 100 mL distilled water. The HOST was performed by mixing 50 µl of the semen sample to 500 µl of the pre-warmed (37°C) HOST solution and incubated at 37°C for 30min. After incubation, sperm swelling was assessed by placing 15 µl of well-mixed sample on a warm slide(37°C) which was covered with a cover glass before being observed under light microscope at 40x magnifications. At least 200 spermatozoa per slide were counted. The spermatozoa were classified as positive or negative based on the presence or absence of coiled tail.

Recovery rate:

Recovery rate (RR) of motile, live and plasma membrane integrity post equilibrium and post thawing were calculated according to the following formula:

RR (Post equilibration) = (Post-equilibrated sperm parameter (%)/Post-diluted sperm parameter (%).

RR (Post thawing) = (Post-thawed sperm parameter (%)/Post-diluted sperm parameter (%).

Enzyme activity and antioxidant biomarkers:

Antioxidant capacity parameters including levels of total antioxidant capacity (TAC) was determined in post-thawed semen according to (Ohkawa et al., 1979), also seminal plasma glutathione peroxide (GPx) was measured, while aspartate transaminase (AST) and alanine transaminase (ALT) activities were measured as described by Reitman (1957). All assays were achieved by using a spectrophotometer (Spectro UV-VIS Auto, UV-2602, Labomed, Los Angeles, CA, USA) and commercial kits (Biodiagnostic, Giza, Egypt) according to the manufacturer's instructions.

Fertility trial:

A total of 100 Holstein cows owned by small and medium scale breeding holders in different village in Kafrelsheikh Governorate were artificially inseminated with random frozen doses from five various extenders (20 cows each). Cows in heat were rectally examined to clarify the occurrence of estrus. Each cow was artificially inseminated with a single insemination dose (0.25 mLFrench straw, 20×10^6 motile spermatozoa) 8-14h after estrus behavior had begun. At the time of insemination, the frozen semen was thawed at 37C0 for 30 seconds. Using recto-vaginal technique and the universal insemination gun, the semen was deposited just next to the anterior end of the cervix. Cows were diagnosed for pregnancy on Day 50 post insemination, then pregnancy rate was calculated as following:

Pregnancy rate = (Number of conceived cows /numbers of inseminated cows) x100.

Statistical analysis:

The obtained data were statistically analyzed by a one-way ANOVA design using a software package (SAS, 2007). Significant differences among groups were tested by Duncan's multiple range test (Duncan, 1955) and set at $P<0.05$. The percentage values were subjected to arcsine transformation before performing the analysis of variance. Means were presented after being recalculated from the transformed values to percentages. Pregnancy rate was analyzed by Chi-square test at $P<0.05$.

3- Results

Physical sperm characteristics in post-diluted semen:

Data of the statistical analysis revealed that sperm characteristics including the percentage of progressive motility and membrane integrity of bull spermatozoa in post-diluted semen were affected significantly by supplementing Tris-extender with NOA. However, the effect of adding NOA had no effects on the percentage of sperm livability and abnormality was not significant. Results show that supplementing Tris-extender with NOA at a level of 1µL increased (P<0.05) progressive motility and membrane integrity, while supplementing Tris-extender with NOA at a level of 6µL increased (P<0.05) membrane integrity as compared to free-extender. On the other hand, supplementing Tris-extender with NOA at a level of 2µL decreased (P<0.05) membrane integrity in comparing to free-extender (Table 1).

Table 1. Effect of supplementing Tris-extender with NOA on sperm characteristics in post-diluted bull semen.

Extender	Sperm progressive motility (%)	Sperm livability (%)	Sperm abnormality (%)	Membrane integrity (%)
C (Control)	75.37±1.99^{b}	80.87±1.34	17.10±0.47	57.12±1.20^{b}
T1 (1 µL)	82.25±1.56^{a}	82.67±1.81	17.50±0.86	59.75±1.90^{a}
T2 (2 µL)	76.25±1.82^{ab}	79.75±2.49	18.12±0.47	55.50±1.25^{c}
T3 (4 µL)	78.75±1.25^{ab}	79.25±1.91	18.50±0.26	58.62±2.44^{ab}
T4 (6 µL)	76.62±1.75^{b}	78.12±1.91	18.50±0.26	59.00±2.44^{a}
P-value (significance)	0.0397	0.1433	0.4440	0.0024

a-c Means denoted within the same column with different superscripts are significantly different at P<0.05.

Physical sperm characteristics in post-equilibrated bull semen:

The analysis of variance showed that the effect of supplementing Tris-extender with NOA on the percentage of progressive motility, livability, and membrane integrity of bull spermatozoa in post-equilibrated semen was significant, while sperm abnormality was not affected by adding NOA. Supplementing Tris-extender with NOA only at a level of 1µL improved (P<0.05) progressive motility, livability, and membrane integrity, but sperm abnormality percentage tended to be lower as compared to free-extender and other levels of NOA in tris-extender (Table 2).

Table 2. Effect of supplementing Tris-extender with NOA on sperm characteristics in post-equilibrated bull semen.

Extender	Sperm progressive motility (%)	Sperm livability (%)	Sperm abnormality (%)	Membrane integrity (%)
C (Control)	67.37±1.75^{b}	74.50±1.56^{ab}	18.00±0.80	50.00±1.88^{b}
T1 (1 µL)	77.25±1.25^{a}	78.75±1.52^{a}	18.25±0.45	55.50±3.84^{a}
T2 (2 µL)	70.50±2.50^{ab}	73.25±2.58^{ab}	19.75±0.64	50.75±1.83^{b}
T3 (4 µL)	66.87±4.52^{b}	70.75±3.03^{b}	19.12±0.61	52.62±1.27^{ab}
T4 (6 µL)	70.25±2.05^{ab}	70.87±2.20^{b}	19.50±0.73	50.75±1.46^{b}
P-value (significance)	0.0466	0.0113	0.3346	0.0069

a-c Means denoted within the same column with different superscripts are significantly different at P<0.05.

Recovery rate of bull sperm characteristics post equilibration:

Recovery rate of all sperm characteristics after equilibration (Table 3) was affected significantly by supplementing Tris-extender with NOA. Results showed that recovery rates of progressive motility and livability were higher (P<0.05) for supplementing Tris-extender with NOA at levels of 1, 2, and 6µL as compared to free-extender and NOA at a level of 4µL. However, recovery rate of membrane integrity was improved (P<0.05) by supplementing Tris-extender with NOA at levels of 1, 2, and 4µL. as compared with free-extender and 6µL NOA in Tris-extender. It is of interest to note that supplementing Tris-extender with NOA at a level of 5 showed the highest recovery rate of progressive motility, livability, and membrane integrity compared with free-extender and other NOA levels in Tris-extender after equilibration.

Table 3. Effect of supplementing Tris-extender with NOA on recovery rate of bull sperm characteristics post equilibration.

Extender	Recovery rate		
	Progressive motility	Livability	Membrane integrity
C (Control)	89.39±1.78^{b}	92.12±1.45^{ab}	87.53±1.25^{b}
T1 (1 µL)	93.92±1.52^{a}	95.25±1.62^{a}	92.88±1.48^{a}
T2 (2 µL)	92.45±1.68^{a}	91.84±1.67^{ab}	91.44±1.67^{a}
T3 (4 µL)	84.91±2.35^{b}	89.27±1.23^{b}	89.76±1.43^{a}
T4 (6 µL)	91.68±1.46^{a}	90.71±1.38^{ab}	86.01±2.33^{b}
P-value (significance)	0.0117	0.0070	0.0228

a-c Means denoted within the same column with different superscripts are significantly different at P<0.05.

Physical sperm characteristics in post-thawed bull semen:

The effect of supplementing Tris-extender with NOA was significant on all sperm characteristics studied (Table 4). Supplementing Tris-extender with NOA only at a level of 1µL increased (P<0.05) progressive motility and livability and decreased (P<0.05) sperm abnormality percentage in post-thawed semen in comparison to free-extender and other NOA levels. However, increasing NOA level to 2µL improved (P<0.05) membrane integrity percentage as compared to free-extender and other NOA levels.

On the other hand, the highest level of NOA (6µL) showed a reduction in membrane integrity percentage compared with free-extender and other NOA levels. Generally, adding NOA to Tris-extender at a level of 1µL improved all sperm characteristic in bull semen after thawing.

Table 4. Effect of supplementing Tris-extender with NOA on sperm characteristics in post-thawed bull semen.

Extender	Sperm progressive motility (%)	Sperm livability (%)	Sperm abnormality (%)	Membrane Integrity (%)
C (Control)	43.75±1.25^{b}	45.25±1.77^{b}	40.25±1.43^{ab}	41.00±3.36^{c}
T1 (1 µL)	49.00±1.53^{a}	52.75±1.95^{a}	38.00±1.00^{b}	45.00±1.22^{a}
T2 (2 µL)	42.50±2.50^{b}	46.25±1.95^{b}	40.00±1.22^{ab}	42.25±0.62^{b}
T3 (4 µL)	41.75±1.25^{b}	43.50±1.70^{b}	42.25±1.37^{a}	41.75±3.09^{c}
T4 (6 µL)	36.25±3.14^{c}	39.75±1.65^{b}	42.75±1.88^{a}	35.50±3.27^{d}
P-value (significance)	0.0140	0.0476	0.0452	0.0236

a-c Means denoted within the same column with different superscripts are significantly different at P<0.05.

Recovery rate of bull sperm characteristics post thawing:

Recovery rates of all sperm characteristics after thawing were affected significantly by supplementing Tris-extender with NOA (Table 5). Recovery rates of all sperm characteristics were higher (P<0.05) by supplementing Tris-extender with NOA at levels of 5 compared with free-extender and other NOA levels. However, recovery rates of membrane integrity and progressive motility were increased (P<0.05) by 2 and 4µL of NOA, respectively, as compared to free-extender. On the other hand, recovery rate of all sperm characteristics was reduced (P<0.05) by supplementing Tris-extender with NOA at levels of 6µL as compared with free-extender and other NOA levels in Tris-extender.

Table 5. Effect of supplementing Tris-extender with NOA on recovery rate of bull sperm characteristics post thawing.

Extender	Recovery rate		
	Progressive motility	Livability	Membrane integrity
C (Control)	64.93±1.56^{a}	60.73±1.57^{c}	82.00±1.64^{b}
T1 (1 µL)	63.43±1.28^{a}	66.98±1.62^{a}	81.08±1.94^{a}
T2 (2 µL)	60.28±1.75^{b}	63.13±1.27^{b}	83.25±1.31^{a}
T3 (4 µL)	62.43±1.41^{ab}	61.48±1.37^{b}	72.62±1.79^{b}
T4 (6 µL)	51.60±1.69^{c}	56.08±1.84^{c}	69.95±1.47^{c}
P-value (significance)	0.0301	0.0290	0.0396

a-c Means denoted within the same column with different superscripts are significantly different at P<0.05

Total antioxidants capacity and enzyme activity of post-thawed bull sperm medium:

The effect of supplementing Tris-extender with NOA on activity of aspartate aminotransferase (AST) and alanine aminotransferase (ALT), total antioxidant capacity (TAC) level, and glutathione peroxidase (GPx) activity in post-thawed sperm medium was significant (Table 6). Supplementing Tris-extender with NOA at a level of 5 or 2µL decreased AST and ALT activities and increased TAC level and GPx activity as compared to free-extender (P<0.05). Supplementing NOA at a level of 4µL decreased ALT activity and increased TAC level (P<0.05), however, at a level of 6µL reduced (P<0.05) ALT activity only as compared to free-extender (Table 6).

Table 6. Total antioxidants capacity, enzyme activity (AST and ALT) and GPx in post-thawed sperm medium of bull semen extended with NOA.

Extender	AST (IU/l)	ALT (IU/l)	TAC (mM/l)	GPx (mU/mL)
C (Control)	36.40±0.60^{a}	35.88±1.06^{a}	0.16±0.020^{b}	3.31±1.20^{c}
T1 (1 µL)	32.30±0.95^{b}	31.38±0.62^{b}	0.26±0.004^{a}	5.91±0.16^{a}
T2 (2 µL)	33.20±0.91^{b}	31.86±1.18^{b}	0.21±0.002^{a}	4.08±0.05^{b}
T3 (4 µL)	36.95±1.53^{a}	31.52±0.72^{b}	0.20±0.007^{a}	3.97±0.12^{bc}
T4 (6 µL)	33.40±1.95^{ab}	30.08±0.45^{b}	0.18±0.013^{b}	3.39±0.25^{c}
P-value (significance)	0.0132	0.0001	0.0001	0.0059

a-b Means denoted within the same column with different superscripts are significantly different at p<0.05.

Fertility trail:

Pregnancy rate of cows inseminated with cryopreserved bull semen supplement with different NOA levels in Tris-extender is shown in Fig. 1. Semen with 1µL NOA showed higher (P<0.05) pregnancy rate (57.5%) than in free-extender (32.5%). Increasing level of NOA 2µL improved pregnancy rate (42.5%) as compared to free-extender but did not differ significantly from that in other NOA levels in Tris-extender. Further increase of NOA to 4µL showed higher pregnancy rate (42.5%) than free-extender, but the difference was not significant. However, the highest level of NOA in Tris-extender (6µL) was lower than that in free-extender (25%), but the difference was not significant (Fig. 1).

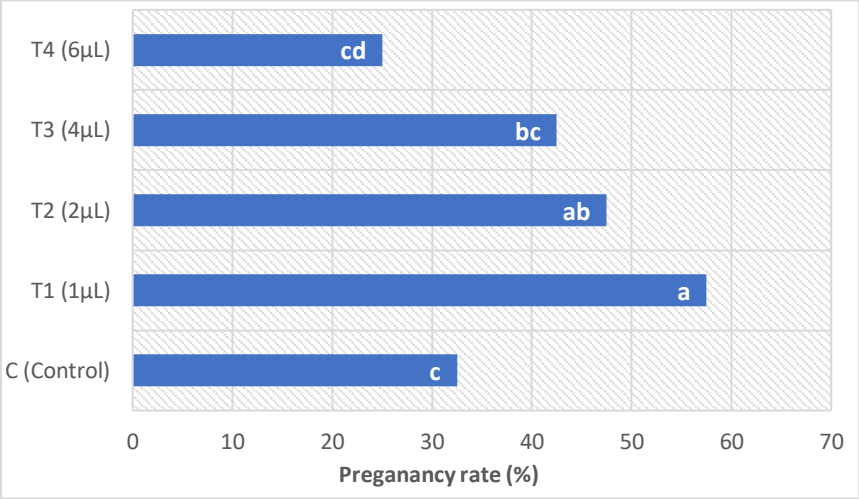

Figure 1. Pregnancy rate of cows artificially inseminated with bull semen with different levels of NOA in Tris-extender.

4. Discussion

The incorporation of biocompatible and environmentally sustainable additives has emerged as a critical strategy for enhancing animal productivity and improving the quality of animal products (El Basuini et al., 2023; Hussein et al., 2023; Shadrack et al., 2023). Poor semen quality is in relation to increasing the concentration of saturated fatty acids in sperm membranes, while increasing PUSFA concentrations showed positive correlation with integrity of sperm membranes. The in vitro or the dietary addition of suitable levels of fatty acid may alter the lipid profile of sperm membranes (Sampaio et al., 2015). In this context, some unsaturated fatty acids are able to increase sperm motility. OA and linoleic acids increased sperm viability as compared to the saturated fatty acids in boar (Fleming and Yanagimachi, 1984). The performances of oleic, linoleic and arachidonic acids were higher for motility, viability and acrosome reaction (Hossain et al., 2007).

The current study aimed to evaluate the effect of adding oleic acid nano-particles at different levels in Tris-extender on freezing and fertilizing abilities of bull spermatozoa. The obtained results reflected positive impacts of NOA at a level of 1µL in Tris-extender on sperm characteristics studied in terms of increasing progressive motility and membrane integrity after dilution, progressive motility, livability, and membrane integrity after equilibration, and improving all sperm characteristics after thawing. The pronounced improvement in sperm characteristics by NOA at a level of 1µL in Tris-extender was in association with the highest recovery rate of progressive motility, livability, and membrane integrity post equilibration and thawing.

In agreement with our results, the supplementation of OA (100 µM) or palmitic acid (75 µM) improved motility, membrane integrity, and acrosome integrity in liquid stored boar semen (Zhu et al., 2020). OA at a proper level and different storage times in semen extender has an ability to enhance

total and forward progressive motility, vitality, membrane integrity, and velocity of sperm cells as compared to free-extender at low temperature liquid storage. In this respect, Hashem et al. (2017) found that supplementing ram semen with OA (0.5 or 1 mM) increased forward motility, viability and curve linear velocity (VCl) of sperm cells at 24, 48, and 72 h. OA in semen extender increased the forward progressive motility percentage compared with the free-extender (Eslami et al., 2016). Furthermore, Pérez-Pé et al. (2001) studied the effect of different oleic-linoleic acid levels on protecting ovine sperm cells during preservation. They found that oleic-linoleic acid (37 µM) increased viability after incubation. Sampaio et al. (2015) reported that oleic-linoleic acid was poorer at a level of 74 µM than at a level of 37 µM; however, the protective effects were better compared to oleic-linoleic acid levels lower than 37 µM. Incorporation of OA and palmitic acid to the extender improved sperm quality in liquid stored semen (Turner et al., 2014). Generally, fatty acids play an important role in enhancing sperm movements and acrosome reaction. In comparison to the control, fatty acids increased motility, but the performance was higher for the unsaturated fatty acids than the saturated ones (Hossain et al., 2007).

The observed improvement in sperm characteristics by OA addition was attributes to that OA increased the mitochondrial activity by activation of β-oxidation, then enhancing the production of ATP **(Zhu et al., 2020)**. Fatty acids, like OA, have metabolic impacts and most of the long-chain, polyunsaturated fatty acids are derived from these fatty acids by chain elongation **(Mayes and Botham, 2003)**. Addition of oleic-linoleic acid enhances the osmotic resistance of sperm cells and the permeability of the membrane to water and cryoprotectants, which in turn contribute to the stabilization of the membrane and increase cryoresistance **(Glazar et al., 2009)**. The incorporation of PUSFA into the sperm membrane increases its flexibility **(Towhidi and Parks, 2012)**.

Oxidative stress occurs when the production of ROS exceeds the antioxidation capacity of semen and becomes harmful to the plasma membrane, resulting in induction of metabolites such as Malondialdihyde (MDA) (Aitken et al., 2012). It is suggesting the key role of the oxidative/nitrosative stress during semen preservation by reducing TAC level and elevating levels of MDA and Nitric oxide (NO) (Gundogan et al., 2010; Moradi et al., 2013; Hashem et al., 2017). OA treatment could affect the antioxidant defense system (Oudit et al., 2004). Hence, it seems to be indicative to evaluate the activities of the antioxidant enzymes following administration of oleate to the semen (Brown and Borutaite, 2007). In our study, the improvement in sperm characteristics by supplementing Tris-extender with NOA at a level of 1µL may be due to reducing the activity of AST and ALT, as markers of improving membrane integrity, and increasing the level of TAC and GPx activity, as markers of increasing antioxidant status in post-thawed sperm medium compared to free-extender and other levels of NOA. Similar results were reported Hashem et al. (2017), who found that OA in semen extender was able to promote level of total antioxidant capacity and activity of superoxide dismutase compared to free-extender. Also, OA ameliorates the level of MDA and nitric oxide of cooled ram sperm cells. Semen enrichment with OA can reduce the production of endogenous ROS during the cold storage period of semen. The total antioxidant activity values in the seminal plasma and the sperm cells were significantly higher in the oleate treated group (Oleate refers to a natural fatty acid found in various oils) compared with the control group. Semen enrichment with oleate reduces the lipid peroxidation of seminal plasma and sperm cells compared with the control group. OA might therefore provide protection in rooster spermatozoa via reducing sperm damage and improving antioxidant activity levels (Eslami et al., 2016). The increase in antioxidant markers following monounsaturated fatty acids treatment could be a result of their potency to elevate essential enzymatic antioxidants (Narang et al., 2004). It seems that, monounsaturated fatty acids could strengthen the antioxidant defense which is of the important factor in the viability of the cells (Oudit et al., 2004). Oleate reduces MDA level, increased activity of antioxidant levels, and improved the forward progressive motility of rooster spermatozoa (de Vries et al., 1997; Maedler et al., 2003; Yamasaki et al., 2008).

Artificial insemination in dairy cattle is provided with frozen-thawed semen (Andrabi, 2007) and pregnancy rate is directly related to sperm quality of the insemination (Pugliesi et al., 2014). The cryopreserved sperm survivability is affected by many factors such extender type (Moore and Thatcher, 2006)), method of package, equilibration period, freezing rate (Rodriguez et al., 1975; Robbins et al., 1976), thawing rate and handling of frozen straws before using (Moore and Thatcher, 2006). All factors were similar for all insemination, except for the level of NOA in semen extender. The cryopreservation process induces lipid peroxidation and can lead to an irreversible rearrangement of membrane lipids, which can compromise the sperm fertilizing capacity (Ricker et al., 2006). The essential metric for structural and functional aspects is integrity of sperm plasma membrane and its correlation with *in vivo* fertilizing ability of buffalo bull spermatozoa (Ahmed et al., 2016). The present results indicated beneficial impacts of OA addition on sperm membrane integrity after all cryopreservation processes. Sperm plasma membrane shields organelles from mechanical damage and serves as a filter for the interchange of intra- and extra-cellular substances. The plasma membrane's integrity is critical for spermatozoa because it impacts the metabolism associated with

motility and viability. Along with improving sperm characteristics by OA, Meizel and Turner (1983) showed that fatty acids, such as arachidonic and oleic acids, induced acrosomal reaction in hamster spermatozoa *in vitro* (Hossain et al., 2007). It was reported that that none return rate in bovine species ranges as widely as from 55 to 75% (Pickett et al., 1976). In the current study, supplementing Tris-extender of bull semen at a level of 1µL showed the highest pregnancy rate (57.5%) as compared to free-extender (32.5%) and other NOA levels (25-42.5%). Fertilizing ability is used for accurate evaluation of semen quality, and considered to be the best parameter to assess the quality of frozen semen (Vale et al., 1998). It is of interest to observe that increasing pregnancy rate was in relation to improving sperm characteristics, especially motility, membrane integrity, and livability of cryopreserved bull spermatozoa as well as the superiority of antioxidant status and enzyme activity of post-thawed sperm medium of semen extended with Tris-extender supplemented with NOA at a level of 5 µL in comparison with free-extender and other levels of NOA.

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