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Comparison of sperm characteristics and antioxidant and oxidant levels in bull semen frozen with four widely used extenders

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Abstract

Sperm survives for a very short time in fresh semen, and slow cooling to 5.00 ˚C kills a large number of sperms. This study was aimed to compare the semen quality parameters and anti-oxidant levels in four extenders (manual, Triladyl, Steridyl and AndroMed). Semen samples were obtained from a total number of 12 dual-purpose Simmental bulls kept in the Simmental Cattle Breeding Center for a period of 3 months using an artificial vagina. Sperm viability, motility, abnormal morphology, plasma membrane integrity, DNA damage, chromatin quality, total antioxidant capacity (TAC) and lipid peroxidation were evaluated. The highest progressive motility, viability, plasma membrane integrity, and TAC and the lowest levels of malondi-aldehyde in the frozen-thawed semen belonged to the semen group frozen with Triladyl. Parameters of motility were higher in the frozen-thawed semen with Triladyl than in other groups, indicating a significant difference from the manual extender. Among the extenders studied, Triladyl was the most suitable for semen freezing in Simmental bulls.

Introduction

Slow cooling of undiluted semen to 5.00 ˚C kills a large number of sperms, necessitating the protection during cooling to extend its lifespan.[1] Freezing affects semen metabolism, leading to changes in metabolites that counteract oxidative stress and regulate sperm capacity and motility.[2]

Semen has a much higher buffering capacity than body fluids, exerted by bicarbonate/carbon dioxide, high protein, and low molecular weight compounds such as citrate, pyruvate and phosphate.[3] The freeze/thaw process leads to the production of reactive oxygen species (ROS) that impairs motility, membrane strength and sperm quality resulting in the early acrosomal reaction, loss of cell contents, decreased motility and sperm fertility.[4]

Under physiological conditions, the concentrations of ROS are subtly regulated by anti-oxidants, and the result of oxidative damage is formation of malondialdehyde (MDA) and other toxic by-products.[5] Anti-oxidants prevent ROS formation and lipid peroxidation. Superoxide dismutase, glutathione peroxidase and catalase are known as anti-oxidants for sperm function. Free radicals are unstable and highly reactive compounds leading to cell damage and death.[6,7]

Egg yolk has been used as one of the main components of cow ejaculate extenders.[8] Low-density lipoproteins in egg yolk micelles and casein in milk stabilize the membrane and maintain sperm function during storage.[9] The plant source of lecithin is used in various cold protectors based on soy lecithin Biocephos plus, Bioxcell and AndroMed.[10] The removal of animal bio-products from the freezing protocol has been suggested due to incompatibilities in different individual qualities of the particles in egg yolk.[11] The ROS are among the most important free radicals.[12] Mass motility and degree of progressive sperm motility are common in most clinical andrologists in semen analysis.[13]

The reason for breeding the Simmental cow is to produce the maximum milk and meat at the minimum cost while using the least forage and concentrate.[14]

This project was aimed to produce the highest quality frozen bovine sperms and find the difference between diluents for freezing bovine sperm and their effects on sperm quality parameters and antioxidant levels of thawed frozen semen in the freezing process.

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Materials and Methods

The Animals Ethics Committee of Urmia University approved the animals selected for this study (No. IR-UU-AEC-1861.AD.3). After obtaining the consent and permissions from Iran Simmental cow breeding and sperm production center, semen samples were taken from 12 Simmental dual-purpose bulls (Falkfieh) kept in the Simmental Cow Breeding and Production Center, in at least four to five stages over a period of 2 - 3 months using the synthetic vagina in 31 ejaculations. Alternative to the components of animal origin in semen expanders is soy lecithin, a natural blend of phosphatidylcholine and several fatty acids such as stearic, oleic and palmitic.[15]

Animals. The semen collection was carried out over a 4-month period from late December 2019 to mid-April 2020. Overall, 12 healthy breeding bulls of the Simmental breed aged 4 - 7 years old were used for this research. The samples were taken during routine weekly semen collection time at the Simmental Cattle Breeding Center (height above sea level: 47.00 m, longitude: 52° 23′57.76″ E, and latitude: 36° 30′ 18.55″ N) between 8:00 - 12:00 A.M. Animals were fed three times a day with the composition mentioned in Table 1.

Semen collection and experimental groups. Semen samples from each bull were collected by a pre-warmed artificial vagina at 46.00 ˚C in an oven (three repetitions for each cow). Measurement of the semen concentration was carried out by the SDM photometer (Minitube, Tiefenbach, Germany) calibrated for bull sperm cell counting.

Extender preparation. The manual, 29.00 g of sodium citrate (Sigma-Aldrich, St. Louis, USA) was dissolved in 1,000 mL of double-distilled water followed by adding 70.00 mL glycerol (Sigma-Aldrich), 250 mL egg yolk, 1.00 g streptomycin (NASR pharmaceutical, Tehran, Iran) and 0.70 g penicillin (NASR pharmaceutical) to 680 mL of the prepared sodium citrate solution. Triladyl (Sigma-Aldrich) included Tris, citric acid, glucose, buffer, glycerol, pure water and antibiotics (tylosin, gentamicin, spectinomycin, and lincomycin) in 250 g bottles. Double-distilled water (750 mL) was combined to a 250g Triladyl diluent in a 1,500 mL Erlenmeyer flask. The resulting compound was a stock solution to which 250 g of egg yolk was added. Steridyl (Minitube), contains the same compound as Triladyl as well as egg yolks, in 500mL bottles. The final diluent, 750 mL of pure double-distilled water was simply added to a complete package of Steridyl diluent in a 1,500 mL Erlenmeyer flask. AndroMed (Minitube) consists phospholipids, Tris, citric acid, sugar, anti-oxidants, buffers, glycerol, antibiotics and the purest water in 200 and 1,000mL bottles. The current research used 200 mL bottles of this diluent. Each pack of 200 mL of this diluent contained 11.40 mg tylosin, 57.20 mg gentamycin, 68.60 mg spectinomycin and 34.40 mg lincomycin. To prepare the final diluent, 800 mL of double-distilled water was simply added to a complete package of 200 mL AndroMed diluent in a 1,000 mL Erlenmeyer flask.

Semen freezing. The diluted semen was packed in 0.50 mL straws (Minitube) with the MPP Uno automated filling and sealing machine (Minitube). Equilibration of the packed semen was done by placing straws in a refrigerator set at 4.00 ˚C for 3 hr.

Sperm motility analysis. To thaw the frozen semen, the straws were placed in a water bath adjusted at 37.00 ˚C for 40 sec. All analyses were performed by a light microscope (Nikon, Tokyo, Japan) equipped with a hot plate and the samples were maintained in a chamber (sperm meter, depth 10.00 µm, surface graticule, 100 × 0.10 mm^2) at 37.00 ˚C to avoid the decline in the sperm motility during analysis.

Sperm viability and morphology assay. Eosin-Nigrosin staining was used (Fig. 1A). Eosin-Nigrosin staining was used. After the sperms were placed in the final diluent, 1.00 drop of sperm was mixed with 1.00 drop of 2.00% Eosin and 2.00 drops of 4.00% Nigrosin with the sampler and spread. Spread on a hot, dry plate and immediately after (maximum 2 min) at least 200 spermatozoa were examined under the microscope (Nikon). Due to the high permeability of their cell membrane, dead sperms take on the color of Eosin and become red, thus, the percentage of dead spermatozoa is obtained.[16,17]

Table 1. Compositions and amounts of Fleckvieh bulls' daily meal.

Ingredient	Amount (kg)	Crude protein (%)	NDF (%)	ADF (%)	Fat (%)	Ash (%)	Dry matter (%)
Concentrate mix*	9.00	14.68	16.80	13.10	3.20	7.40	89.20
Silage	18.00	8.50	54.50	32.70	1.80	5.70	25.00
Hay	3.00	16.80	44.70	34.60	2.50	9.70	1.88
Straw	*Ad libitum*	3.90	70.30	45.50	1.10	9.80	94.80
Mineral supplement†	*Ad libitum*	-	-	-	-	-	-
Water	*Ad libitum*	-	-	-	-	-	-

* Calcium 0.74%, Phosphorus 0.53%, Sodium 0.49%, Magnesium 0.29%, Zinc 375 ppm, Manganese 381.44 ppm, Cobalt 1.01 ppm, Selenium 2.75, and vitamin additives (vitamin A 7,500 U kg^{-1}, vitamin D3 1,000 U kg^{-1}, and vitamin E 10.00 mg kg^{-1}).

† Magnesium 2.10%, Sodium 7.00%, Iron 355 mg kg^{-1}, Zinc 1,560 mg kg^{-1}, Copper 390 mg kg^{-1}, Manganese 1,560 mg kg^{-1}, Selenium 7.50 mg kg^{-1}, Co 3.00 mg kg^{-1}, and Iodine 15.50 mg kg^{-1}.

NDF: neutral detergent fiber, ADF: acid detergent fiber, and Ash: total mineral content of a diet.

Source: Data calculated by Animal Nutrition Laboratory, College of Agriculture and Natural Resources, University of Tehran, Karaj, Iran.

Assessment of membrane integrity. The hypo-osmotic swelling test (HOST) was carried out following the technique used by Revell and Mrode. Percentages of sperm reacted in a HOST and the sperm with swollen or coiled tails were determined for all bulls (Fig. 1B).[18,19]

Aniline blue staining. Aniline blue staining was used to assess the maturity of the sperm nucleus. This analysis was based on the fact that during spermiogenesis, protamine was replaced by histone in the nucleus chromatin, which was a very important replacement in sperm density and stability. In this staining method, immature sperms turn blue due to high histone, while adult sperms undergo less staining (Fig. 1C).[16] The sperm chromatin quality was examined by Aniline blue, as an indicator of significant chromatin/DNA damage. The air-dried fixed smears were stained for 5 min in 5.00% aqueous Aniline blue solution (5.00 g Aniline blue (Sigma-Aldrich), 4.00% acetic acid (Sigma-Aldrich) in double-distilled water, pH = 3.50). The percentages of spermatozoa stained with Aniline blue and Aniline blue positive were determined by counting 400 spermatozoa per slide under a bright field microscope (Nikon, Tokyo, Japan).[20,21]

Acridine orange staining. Acridine orange staining was performed to investigate DNA damage and identify denatured, double-stranded DNA segments in the chromatin of the sperm at a low pH rate. In green sperms DNA was normal and in yellow to red sperms DNA were damaged (Fig. 1D).[4,16]

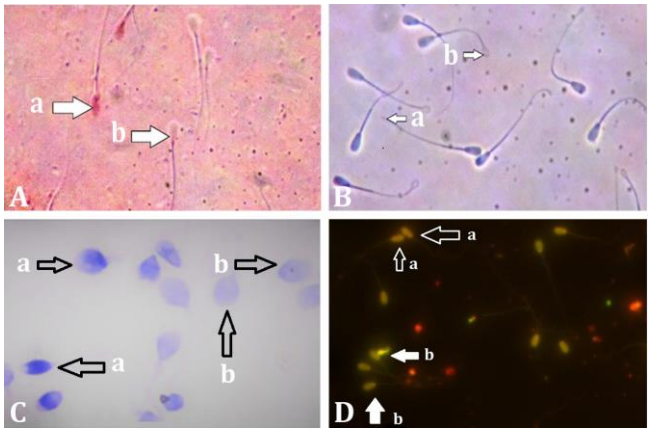

Fig. 1. A) Evaluation of live and abnormal sperms by Eosin-Nigrosin staining. a: Dead sperm with red head due to plasma membrane permeability, b: Impermeable live sperm with blue head (200×). **B**) Evaluation of the sperm membrane health in hypoosmotic solution, a: Sperm with a healthy plasma membrane and twisted tail, b: Unhealthy sperm without reaction (400×). **C)** Evaluation of excess histone by the Aniline blue method. a: Immature sperms turn blue due to high histone, b: mature sperms undergo less staining (400×). **D)** Evaluation of DNA damage by Acridine orange staining, a: DNA damaged (yellow-red), b: Normal sperm (green), (200×).

Total antioxidant capacity (TAC) assessment. The TAC was measured using a kit (Naxifer™; Navand Salamat Co., Urmia, Iran) in seminal plasma based on the manufacturer's protocols. Briefly, the diluted seminal plasma (1:10) was mixed with stock solutions step by step. Then, the TAC content in the final solution was detected using a spectrophotometer (Thermo Fisher Scientific, Waltham, USA) at 593 nm.[22]

Evaluation of lipid peroxidation. The products of lipid oxidation included oxidized cholesterol MDA, lipid hydroperoxides, and other aldehydes and ketones.[23] This method was performed according to the protocol of Draper and Hadley.[24,25] Seminal plasma MDA status was detected by the thiobarbituric acid (TBA; Sigma-Aldrich). Subsequently, TBA reagent was added to the samples based on the kit instruction and the final solution was read by a spectrophotometer (Thermo Fisher Scientific, Waltham, USA) at 586 nm.[26]

Statistical analyses. The obtained data were analyzed by SPSS Software (Version 26.0; IBM Corp., Armonk, USA). One-way ANOVA test was used for data with normal distribution and the Kruskal-Wallis was used for data with abnormal distribution to compare the four experimental groups. A *p*-value of less than 0.05 was considered statistically significant.

Results

Sperm characteristics in fresh and frozen-thawed semen diluted in different extenders are shown in Table 2. In fresh diluted semen, curvilinear velocity (VCL), straight-line velocity (VSL) and average path velocity (VAP) were significantly higher in Steridyl and AndroMed than in the manual ($p < 0.05$; Table 2). Moreover, VSL and VAP were significantly higher in semen diluted with AndroMed than Triladyl. The mean angular displacement (MAD) of the Triladyl, Steridyl, and AndroMed groups was significantly ($p < 0.05$) higher than the manual group. The linearity (LIN) of fresh semen diluted in the AndroMed was significantly higher than the manual ($p < 0.05$; Table 2).

The highest progressive motility (PM) and viability of frozen-thawed semen could be observed in the Triladyl, which was higher than the manual group (Table 2). Motility parameters such as VCL, VSL, VAP, amplitude of lateral head displacement (ALH) and beat-cross frequency (BCF) with Trilady were higher than in other groups indicating a difference with the manual group. Plasma membrane integrity in the Triladyl was higher than in the manual group ($p < 0.05$; Table 2). The percentage of DNA damage with Triladyl and AndroMed was lower than in the manual group ($p < 0.05$; Table 2). The percentage of chromatin integrity in the Triladyl was higher than in the manual group ($p < 0.05$; Table 2). The TAC of the Triladyl and AndroMed groups was higher than the manual group ($p < 0.05$), whereas, the MDA of the Triladyl, Steridyl and AndroMed groups was lower than the manual group ($p < 0.05$; Table 2).

Table 2. Comparison of sperm quality characteristics for the fresh semen extender in different extenders. Data are presented as the mean ± standard deviation. All the semen quality parameters and blood serum antioxidant enzymes were compared in all groups and classified based on fresh sperm progressive motility and post thawing.

Parameters	Manual	Triladyl	Steridyl	AndroMed
Fresh semen extender				
PM (%)	69.78 ± 12.40^{a}	76.61 ± 10.46^{a}	78.21 ± 8.52^{a}	75.21 ± 9.28^{a}
VCL (µm sec^{-1})	36.68 ± 14.38^{a}	42.22 ± 10.87^{ab}	48.15 ± 12.98^{b}	52.12 ± 15.24^{b}
VSL (µm sec^{-1})	19.33 ± 8.20^{a}	20.55 ± 6.67^{ab}	27.41 ± 9.72^{bc}	31.04 ± 10.88^{b}
VAP (µm sec^{-1})	24.44 ± 9.48^{a}	26.40 ± 6.99^{ab}	33.08 ± 9.74^{bc}	36.29 ± 11.43^{b}
MAD	26.58 ± 14.72^{a}	34.43 ± 14.96^{b}	33.49 ± 15.29^{b}	34.07 ± 18.67^{b}
ALH (µm)	1.96 ± 0.71^{a}	2.10 ± 0.45^{a}	2.18 ± 0.41^{a}	2.27 ± 0.54^{a}
BCF (Hz)	2.18 ± 1.95^{a}	2.11 ± 1.82^{a}	2.09 ± 1.71^{a}	3.22 ± 7.09^{a}
LIN (%)	42.45 ± 8.98^{a}	43.17 ± 8.57^{ab}	48.39 ± 9.94^{ab}	48.69 ± 8.61^{b}
Viab (%)	82.02 ± 8.03^{a}	83.33 ± 8.21^{a}	82.47 ± 9.55^{a}	81.27 ± 6.37^{a}
Morph (%)	7.87 ± 3.81^{a}	7.50 ± 5.73^{a}	7.97 ± 5.23^{a}	7.19 ± 4.89^{a}
Head (%)	2.67 ± 2.29^{a}	2.93 ± 4.22^{a}	2.63 ± 3.22^{a}	2.59 ± 3.23^{a}
Mid.P (%)	1.50 ± 0.93^{a}	1.18 ± 0.88^{a}	1.16 ± 0.78^{a}	1.10 ± 1.01^{a}
Tail (%)	2.51 ± 1.82^{a}	2.47 ± 1.84^{a}	2.80 ± 2.80^{a}	2.54 ± 1.82^{a}
Cy.D (%)	1.17 ± 1.10^{a}	0.91 ± 1.14^{a}	1.37 ± 2.25^{a}	0.95 ± 0.93^{a}
HOST (%)	57.93 ± 10.89^{a}	61.59 ± 11.52^{a}	62.43 ± 11.24^{a}	64.03 ± 12.23^{a}
Frozen-thawed semen extender				
PM (%)	44.91 ± 16.13^{a}	55.69 ± 14.39^{b}	49.08 ± 12.88^{ab}	52.41 ± 15.67^{ab}
VCL (µm sec^{-1})	24.81 ± 11.73^{a}	32.60 ± 14.43^{ab}	27.49 ± 10.83^{b}	40.25 ± 16.92^{b}
VSL (µm sec^{-1})	10.65 ± 5.12^{a}	13.13 ± 5.49^{ab}	13.74 ± 5.33^{ab}	18.78 ± 8.93^{b}
VAP (µm sec^{-1})	14.72 ± 6.94^{a}	19.01 ± 7.97^{ab}	17.75 ± 6.91^{ab}	24.54 ± 11.03^{b}
MAD	22.93 ± 11.13^{a}	32.05 ± 14.47^{ab}	22.51 ± 9.28^{a}	34.88 ± 13.24^{b}
ALH (µm)	1.64 ± 0.68^{a}	2.09 ± 0.78^{ab}	1.64 ± 0.60^{a}	2.28 ± 0.85^{b}
BCF (Hz)	2.32 ± 1.29^{a}	3.29 ± 1.31^{b}	2.33 ± 1.05^{ab}	3.33 ± 1.43^{b}
LIN (%)	24.33 ± 8.80^{a}	29.81 ± 7.21^{a}	29.20 ± 7.56^{a}	30.98 ± 9.82^{a}
Viab (%)	46.18 ± 9.31^{a}	57.59 ± 12.86^{b}	54.47 ± 12.41^{b}	49.64 ± 12.26^{ab}
Morph (%)	11.15 ± 5.67^{a}	10.16 ± 6.37^{a}	11.17 ± 7.22^{a}	10.69 ± 6.14^{a}
Head (%)	3.65 ± 3.32^{a}	3.27 ± 3.10^{a}	3.44 ± 4.26^{a}	3.14 ± 2.70^{a}
Mid.P (%)	1.63 ± 1.56^{a}	1.24 ± 0.85^{a}	1.61 ± 1.07^{a}	1.48 ± 1.14^{a}
Tail (%)	5.07 ± 3.93^{a}	5.21 ± 5.56^{a}	5.55 ± 5.10^{a}	5.32 ± 4.72^{a}
Cy.D (%)	0.78 ± 0.91^{a}	0.43 ± 0.73^{a}	0.56 ± 0.49^{a}	0.74 ± 0.87^{a}
HOST (%)	34.07 ± 8.39^{a}	41.95 ± 9.15^{b}	37.73 ± 11.12^{ab}	39.07 ± 10.14^{ab}
DNA damage (%)	33.35 ± 3.85^{a}	25.19 ± 3.41^{b}	29.65 ± 6.44^{a}	27.13 ± 4.76^{b}
Chromatin Integrity (%)	2.21 ± 3.53^{a}	1.94 ± 3.19^{b}	2.18 ± 9.15^{ab}	2.07 ± 9.15^{ab}
TAC (mmol L^{-1})	1.08 ± 0.25^{a}	2.49 ± 0.17^{b}	1.33 ± 0.20^{a}	2.17 ± 0.13^{b}
MDA (nmol mL^{-1})	6.39 ± 0.22^{a}	2.77 ± 0.35^{b}	5.19 ± 0.41^{c}	3.04 ± 0.39^{b}

PM: progressive motility, VCL: curvilinear velocity, VSL: straight-line velocity, VAP: average path velocity, ALH: amplitude of lateral head displacement, MAD: mean angular displacement, BCF: beat cross-frequency, LIN: linearity, Viab: viability, Morph: abnormal morphology, Head: head abnormality, Mid.P: mid-piece abnormality, Tail: tail abnormality, Cy.D: cytoplasmic droplet, and HOST: hypo-osmotic swelling test, TAC: total antioxidant capacity, MDA: malondialdehyde, VCL: curvilinear velocity, and STR: straightness.
^{abc} Different letters in the same line indicate a significant difference between the groups ($p < 0.05$).

Discussion

Abdel-Aziz Swelum *et al.* conducted a study in the University of Saudi Arabia to examine the effectiveness of Triladyl, Steridyl, AndroMed, SHOTOR and Optixcell for camel semen maintenance. In the pre-cryopreservation evaluation, PM was higher with SHOTOR. SHOTOR and Triladyl had better DNA viability and integrity. Triladyl resulted in plasma membrane integrity than other extenders. In the post-thaw evaluation, the PM parameter was significantly higher in Triladyl. The VAP, VSL, VCL parameters were higher in Triladyl. The highest amount of parameter LIN was observed in Triladyl and AndroMed.

The highest parameter of DNA and plasma membrane integration was obtained with SHOTOR. In the present study, PM, straightness (STR), LIN, ALH, BCF, VAP, VSL, and VCL showed better post-thaw evaluation, and the best performance was recorded in Triladyl. The sperm stored in Triladyl had more efficient flagellar structures. The best sperm quality after thawing, the lowest sperm abnormality, the best DNA integrity, plasma membrane integrity and increased motility were observed in SHOTOR and Triladyl.

In our study, fresh sperm showed the most PM and viability of frozen semen in Triladyl and no difference was observed between the experimental groups in terms of sperm morphology parameters. The parameters of PM,

VAP, VSL, VCL, and HOST Triladyl were different from the other groups concerning frozen semen, while there was no statistically significant difference between the experimental groups concerning the LIN parameter. The results of our research on Simmental Bull were consistent with the results of Abdel-Aziz Swelum *et al.*[27]

Suhardi *et al*., conducted a study on three factors of motility, viability and abnormality of Bali bull sperm with AndroMed and Triladyl egg yolk-tris stored at 4.00 ˚C.[28] AndroMed and egg yolks were compared. Sperm motility, viability and abnormalities were observed and compared, confirming the superiority of egg yolk-tris to AndroMed in three parameters sperm motility, viability, and a lower percentage of abnormalities when stored for four days at 4.00 ˚C.

In our study, fresh sperm showed the most PM and viability of frozen semen in Triladyl, while, no significant difference was observed between the experimental groups in terms of sperm morphology parameters.[28]

In a research study in 2015, Chaudhari *et al*., investigated the relative efficacy of egg yolk and soya milk-based on semen cryopreservation of buffalos (*Bubalus bubalis*).[15] They evaluated the comparative effects of ordinary egg yolk (TFYG) and commercial soybean-based (Bioxcell and Optixcell) on semen cryopreservation of *Bubalus bubalis* in terms of sperm motility, viability, morphology and acrosome integrity. Parameters such as greater sperm motility and viability and less abnormal morphology in semen were found in TFYG than in the Bioxcell in sperm post-cryopreservation examination. Compared to our study, the highest PM and viability of frozen semen were observed in Triladyl. Plasma membrane integrity in the Triladyl was higher than in the manual group and only the morphology was not different from the control group. The results obtained by Chaudhari *et al*. were consistent with our results.[15] Chaudhari *et al*. suggested that soy-based Bioxcell had fewer protective effects on maintaining the integrity of the sperm plasma membrane and indirectly reflected on sperm motility which was consistent with our findings.[15]

In a study of African buffalo elephants by Herold *et al.* the effect of equilibrium time of semen diffusers on sperm quality was investigated after transplantation of bonds such as motility, longevity and complete acrosome parameters commonly used for semen.[29] The use of AndroMed resulted in symptoms of much higher percentages of lost acrosomes than Triladyl and ROFB groups.[21] The results of our research in the three parameters of motility, survival and acrosome test were consistent with Herold's *et al.* research on sperm count after cryopreservation.

In a study by Fukui *et al.* at the Animal Reproduction Laboratory, the fertility of intrauterine ewes with thawed frozen semen using a soy-based semen (AndroMed) was compared to intrauterine ewes inoculated with thawed frozen semen using Tris-based extenders containing egg yolk or bovine serum albumin (BSA). The present results indicated that a semen enhancer containing egg yolk can be replaced with the non-animal AndroMed.[30]

Our study on frozen sperm, the most PM and viability of frozen semen were observed in the Triladyl, which was higher than the manual group. Parameters VCL, VSL, VAP, ALH, and BCF with the Triladyl were higher than the other groups. Plasma membrane integrity in the Triladyl was higher than in the manual group.

Another study, conducted by Al-Bulushi *et al.*, Oman Animal Research and Center, evaluated the quality of semen in Triladyl, green buffer and Optixcell with fresh semen.[30] Two extenders based on egg yolk (Biladyl or green buffer and Triladyl) and five extenders based on synthetic and non-animal compounds (Optixcell, EquiPlus, INRA96, Bioxcell, AndroMed) were diluted and stored at 4.00 ˚C for 48 hr.

The survival parameters, acrosome integrity and total sperm motility were then examined for maximum sperm motility and healthy acrosome after 48 hr of storage with Triladyl and green buffer. After 48 hr of storage with Triladyl, the results were consistent with our study concerning the parameters of viability and motility of fresh sperm and the acrosome test performed on frozen sperm.[31]

In a 2012 study at the German Farm Animal Biological Institute, John Beran and his colleagues ranked extenders in terms of sperm motility including reductions in sperm motility in fresh and post-thaw ejaculates. During the entire thermodynamic test and in the proportion of live and dead sperm after thawing, the difference in sperm motility during entire thermodynamic experiment was significant. Triladyl significantly increased the percentage of motility and live sperm after melting.[8] Ejaculation was collected once a week from four Holstein bulls. Each of 20 ejaculate samples from each bull was extended with 4 different developing agents AndroMed, Bioxcell without egg yolk, Triladyl and Optidyl with ionized egg yolk and used fresh. The results confirmed a significant difference in the volume, density and activity of sperm as well as in the share of live and dead sperm after collection. In reducing sperm motility in fresh ejaculate, after thawing, as well as in the proportion of live and dead sperm after thawing, the extenders that are ranked in terms of sperm motility are Optidyl, Triladyl, AndroMed and Bioxcell, indicating the higher quality of artificial insemination doses was produced using egg yolk extenders. The difference in sperm motility was significant. Egg yolk expanders after thawing significantly increased the percentage of viable sperm. The results of John Beran's research were consistent with our research.[8]

According to our study in post-cryopreservation evaluation, PM and survival had better results with Triladyl. VAP, ALH and BCF parameters were higher with Triladyl indicating differences with the manual group.

The potential predictive parameter for semen fertilization capacity in bovines is the percentage of PM.[7] Triladyl had better performance regarding this important parameter than the other groups.

Concerning the total anti-oxidant evaluation and lipid peroxidation, no research has been conducted related to midwifery and reproductive diseases. The results of this study could be considered as the first evaluation of the parameter of total anti-oxidant capacity. Triladyl did not show a statistical difference with the manual group, while, MDA of Triladyl, Steridyl, and AndroMed groups was significantly lower than that of the manual group.

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Conflict of interest

The authors declare no conflict of interest.

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