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Original Article

The effect of different concentrations of taxifolin on the quality of frozen and thawed semen of Simmental cattle

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Abstract

Background: Artificial insemination (AI) is one of the most important reproductive technologies used to modify animals genetically. Using this method, the genetic composition of the herd can be improved and selected by choosing bulls with excellent genetic characteristics. Taxifolin (TXF), a plant flavonoid, has shown an antioxidative effect. **Aims:** The current study aimed to evaluate the impact of TXF on the quality of frozen-thawed semen in Fleckvieh (Simmental) dual-purpose bulls. **Methods:** Freezable semen specimens were obtained by an artificial vagina. Ejaculates were equally divided into six parts for six experimental groups, including without adding TXF to diluent (C), adding 25 (T25), 50 (T50), 100 (T100), 200 (T200), and 400 (T400) µM TXF. Sperms were frozen in a one-step dilution method. Semen factors, including motility, viability, sperm membrane integrity, DNA damage, and oxidant and antioxidant enzyme activities, were examined after thawing. **Results:** Our findings revealed that all semen quality parameters, antioxidant enzyme activities, and free radical levels were superior in TXF-treated groups compared to the control group, and the differences were noticeably higher in the T100 group than the other groups. **Conclusion:** Adding 100 µM TXF to diluent could improve the quality of bull frozen semen.

Key words: Semen quality, Simmental, Taxifolin

Introduction

Animal husbandry depends on artificial insemination (AI) as safe and reliable for natural mating in some species, especially cattle. Transmission of venereal diseases can significantly be reduced using AI in cattle. AI has advantages over natural mating, including increasing genetic improvement rates, reducing bull-to-female ratios, and reducing the risk of bull injury. Because domestic animals are raised to produce healthy milk and meat for humans, increasing the fertilization ability of bulls' semen can reduce human malnutrition due to the lack of animal proteins by increasing calf and milk production (Cojkic and Morrell, 2023).

While the quality of bull frozen semen is better than the other species, the motility and viability of thawed sperm are still significantly lower than that of fresh semen and diverge noticeably among different bull breeds. Therefore, semen freezing protocols have a long way to reach the optimum point. There are numerous diluents that have been enriched with chemicals and natural agents to reduce sperm damage during freezing procedures, having different success rates. So, research regarding improving the bull semen extender quality seems to be among the most essential branches of assisted reproductive technologies (Ugur *et al*., 2019). There are many reasons for the superiority of Simmental cattle, among which are proper performance even in heat stress conditions (Bartoň *et al*., 2007), longer yield period, ability to adapt to different environmental conditions, and more meat at the time of weaning (Gredler *et al*., 2009). The other reasons include resistance to diseases, especially digestive diseases, high sperm fertility (Perišić *et al*., 2009), more milk fat and protein (Götz *et al*., 2015), resistance to acidosis, extremely low growth of hoof and high resistance of hoof tissue, higher calving, better health of udders, a calm and contented breed, and no difficult birth (Filipčík *et al*., 2020).

Reactive oxygen species (ROS) essentially contribute to many pathogenic problems and diseases, including arteriosclerosis, diabetes, cancer, Parkinson's, AIDS, liver damage, and issues such as premature birth. In cows, ROS accumulation has been determined in diseases such as pneumonia, intestinal infection, mastitis, poisoning, and endometritis. Male infertility and

increased ROS levels are significantly negatively correlated with decreased sperm motility (Tvrdá *et al*., 2011). Damages related to DNA and peroxidation of the lipid membrane of sperm cells have demonstrated a significant relationship with high levels of free radicals. ROS can also reduce the binding ability of sperm to oocytes (Tvrdá *et al*., 2011).

Endogenous antioxidants include antioxidants in spermatozoa and seminal plasma. Plasma has three main enzymatic antioxidants, namely, catalase (CAT), glutathione peroxidase/glutathione reductase (GPx/GRD), and superoxide dismutase (SOD). Moreover, non-enzymatic antioxidants are ascorbate, vitamin E, urate, glutathione, pyruvate, vitamin A, albumin, hypotaurine, taurine, and ubiquitol. Oral antioxidants include vitamins C and E, flavonoids, carotenoids, and beta-carotene. Additionally, metal-binding proteins such as metallothionein, albumin, transferrin, ceruloplasmin, myoglobin, and ferritin act by inactivating the transport of metal ions, catalyzing free radical production (Sanocka and Kurpisz, 2004).

Taxifolin (TXF) is one of the flavonoids derived from plants and is mainly found in fruits and vegetables. TXF has shown promising pharmacological activities in managing inflammation, tumors, infections, and oxidative stress (OS), as well as cardiovascular and liver disorders (Abeydeera *et al*., 1998). TXF is a flavonoid obtained from the *Madliuca butracea* plant, which has anti-inflammatory activity on the oxidative and proliferative phases of inflammation and prevents the increase of serum aminotransferase activity during inflammation (Galeati *et al*., 2010). In a previous research, adding TXF to the semen extenders could improve the motility of sperms in fresh and frozen-thawed semen in some animals (Caamaño *et al*., 2023). Therefore, the present research study investigated the effect of adding TXF to the frozen-thawed semen of dual-purpose Simmental bulls (Fleckvieh) on sperm quality parameters. To the best of our knowledge, no research has focused on the impact of TXF on frozen-thawed semen quality in dual-purpose Simmental bulls.

Materials and Methods

Animals and experimental groups

In the current research, the clinical part and bull semen freezing was performed in Iran Simmental Cattle Breeding Center, Amard-Dam Company, Amol, Iran, located in Amol, Mazandaran province (36° 28′ 11″ N, 52° 21′ 3″ E, height above sea level: 47 m). The Ethics Committee of Urmia University approved the procedure of the present study (IR-UU-AEC-3/13).

The bulls had the same food ration and free access to drinking water. Using an artificial vagina (AV), the semen specimens were gathered twice a week. For this purpose, 12 dual-purpose Simmental (Fleckveh) bulls (in the age range of 3-7 years and with a weight of 900-1300 kg) were selected, and semen samples were collected three times from each bull during three months using a pre-warmed AV at 46°C in an oven. The samples were regarded as normal sperms and chosen for the experiment if they had abnormal sperm morphology <20% per ejaculation, progressive sperm motility >60%, and a concentration of >500 × 10^6/ml (Gil *et al*., 2003).

Immediately after semen collection and confirmation of sperm quality, each ejaculation was classified into equal parts of six for all experimental groups (n=6). The groups were those without the addition of TXF to semen extender (C), addition of 25 µM TXF (T25), addition of 50 µM TXF (T50), addition of 100 µM TXF (T100), addition of 200 µM TXF (T200), and addition of 400 µM TXF (T400).

Freezing of semen

Semen freezing was performed by the one-step dilution method (Tahmasbian *et al*., 2022). The procedure is summarized as follows:

A 1.500 ml Erlenmeyer flask was used to combine 750 ml of double-distilled water with a 250 g Triladyl extender (Minitube Company, Germany). The ready-to-use extender was prepared by adding 250 g of egg yolk to the resulting compound, a stock solution. Moreover, the extender was gently added to the semen with a ratio of 1:1 in order to prepare the pre-extender dilution, followed by placing the solution in a water bath at 32°C for 10 min. Eventually, the volume of the extender was determined as follows:

Number of doses: (semen volume × semen concentration × progressive motile sperm × morphologically normal sperm) / (sperm per dose [15 million])

Finally, the intended solution was obtained by adding the pre-extender to the determined volume of the diluent. Then, the flasks were put in a plastic container with a constant water level from the water bath at 32°C and placed at 20°C (room temperature) for 15 min. Subsequently, the diluted semen packed in 0.5 ml straws (Minitube Company, Slovakia) by the MPP Uno automated filling-sealing machine (Minitube Company, Tiefenbach, Germany) was adjusted via putting straws in a refrigerator at 4°C for 3 h. After being packed with French straws, the semen was frozen in an MT freezing (automatic) device (Minitube Company, Tiefenbach, Germany) at different temperatures (from +4 to -12°C at -4°C/min, -12 to -40°C at -40°C/min, and -40 to -140°C at -50°C/min). Eventually, the frozen straws were stocked in different goblets in the canisters of a container consisting of liquid nitrogen.

Motility of the sperm

CASA software (Hooshmand Fanavar Company, Tehran, Iran) was utilized to evaluate the mobility of the frozen-thawed semen. To thaw the frozen semen, the straws were placed in a water bath adjusted to 37°C for 40 s. Different parameters were investigated, including straight-line velocity (VSL), curvilinear velocity (VCL), progressive motility (PM), lateral head displacement (ALH), average path velocity (VAP), linearity (LIN [VSL/VCL]), straightness (STR [VSL/VAP]), and beat cross frequency (BCF). Finally, the analyses were

conducted by employing a light microscope equipped with a chamber (sperm meter, depth 10-micron, surface Graticule, 100x 0.1 SQMM) and a hot plate preserving the specimens at 37°C in order to prevent the decrease in the motility of sperms throughout the analysis.

Integrity of sperm membrane

A hypo-osmotic swelling (HOS) test was performed to predict the integrity of the sperm membrane. The HOS test can measure the sperm membrane's ability to preserve the balance between the spermatozoa and its environment. Fluid incursion causes the tail of the sperm to swell or coil because of the hypo-osmotic solution. The higher percentage of sperm with a coiled tail demonstrates that the membrane of that sperm is more functional and healthier. It should be noted that at least 200 spermatozoa were analyzed (with a magnification of ×400) under a phase contrast microscope for each specimen.

Viability of sperms

Eosin-nigrosin staining was performed to examine the percentage of sperm viability. One drop of the frozen-thawed semen specimen was combined with two drops of 4.00% Nigrosin and one drop of 2.00% Eosin using the sampler and then spread on the glass slide. Next, the sample was analyzed on a heated, dry plate, and immediately following (a maximum of 2 m), nearly 200 spermatozoa using a phase-contrast microscope (MBL 2000, Minitube Company, Germany). Dead sperms, because of the high permeability of their cell membranes, take on the color of Eosin and turn red, and the dead spermatozoa percentages are achieved accordingly.

DNA damage of sperms

DNA integrity was evaluated by the acridine orange staining method (Katayose *et al*., 2003). One ml of the prepared staining solution containing 0.019% acridine orange was placed on each of the slides for 5 min at room temperature, followed by washing the specimens with distilled water. Eventually, the specimens were examined by an epifluorescence microscope (Model GS7, Nikon Company, Japan), and 100-200 sperms from each slide were counted to calculate the number of sperms with damaged DNA. At the time of evaluation, normal sperms and sperms with damaged DNA are found in green and orange, respectively.

Evaluation of total antioxidant capacity

A colorimetric assay kit was employed to estimate the number of antioxidants in the semen (Naxifer™ - #TAC NS-15012, Total Antioxidant Capacity Assay Kit-TAC, Navand Salamat Company, Urmia, Iran). Notably, the assessment was based on a ferric-reducing antioxidant power assay. The amount of absorbance was read at 593 nm for 5 min and depicted by a standard curve. In addition, the capacity of the antioxidant was determined and presented as mmol/L.

Analysis of enzyme activity

Commercial kits were used to determine SOD and GPx activities (Nasdox™-Superoxide Dismutase Assay Kit-SOD, Navand Salamat Company, Urmia, Iran; Nagpix™-Glutathione Peroxidase Assay Kit-GPx, Navand Salamat Company, Urmia, Iran). The enzyme activity level of both parameters was reported as a unit per mg of plasma protein.

Determination of malondialdehyde level

A spectrophotometer by a commercial kit was utilized to estimate MDA levels (Nalondi™ Lipid Peroxidation Assay Kit [NID], Navand Salamat Company, Urmia, Iran). The basis for the evaluation was the reaction of MDA with thiobarbituric acid-reactive substances. The spectrophotometer was used against a distilled water blank in order to measure the absorbance at 550 nm. The results of MDA levels were expressed as nmol/ml.

Statistical analysis

SPSS software (version 26.0, SPSS Inc, Chicago, IL, USA) was employed to analyze the statistical data. Bonferroni correction was utilized to check multiple comparative trends, and differences were considered statistically significant at $P<0.05$.

Results

Integrity of plasma membrane

Examining the percentage of the plasma membrane continuity of the sperm by the HOS method revealed that the 100 µM TXF group could significantly ($P<0.05$) improve the plasma membrane continuity of the sperm after the freeze-thaw process in comparison to other treatment groups. Moreover, the findings of this research demonstrated that the groups of 25 and 200 µM have no significant differences. Based on the obtained data, all treatment groups could significantly increase plasma membrane integrity compared to the control group ($P<0.05$, Table 1, Fig. 1a).

Table 1: Mean (±SD) percentage of plasma membrane integrity, viability, and DNA damage of sperms in different treatment groups of TXF

Index	Control	25 µM TXF	50 µM TXF	100 µM TXF	200 µM TXF	400 µM TXF
Plasma membrane integrity of sperm (%)	51.29±1.06^{d}	56.62±1.51^{c}	62.90±1.35^{b}	66.39±1.18^{a}	57.67±1.34^{c}	59.35±1.72^{e}
Viability of sperm (%)	60.14±1.40^{d}	64.27±1.15^{c}	69.32±1.44^{b}	73.91±1.19^{a}	63.96±1.44^{c}	54.47±1.72^{e}
DNA damage of sperm (%)	13.54±0.42^{c}	9.30±0.61^{b}	6.73±0.58^{a}	5.24±0.46^{a}	9.79±0.25^{b}	16.94±0.67^{d}

SD: Standard deviation, and TXF: Taxifolin. Different letters (^{a}, ^{b}, ^{c}, ^{d}, and ^{e}) represent significant differences between the data ($P<0.05$) in rows

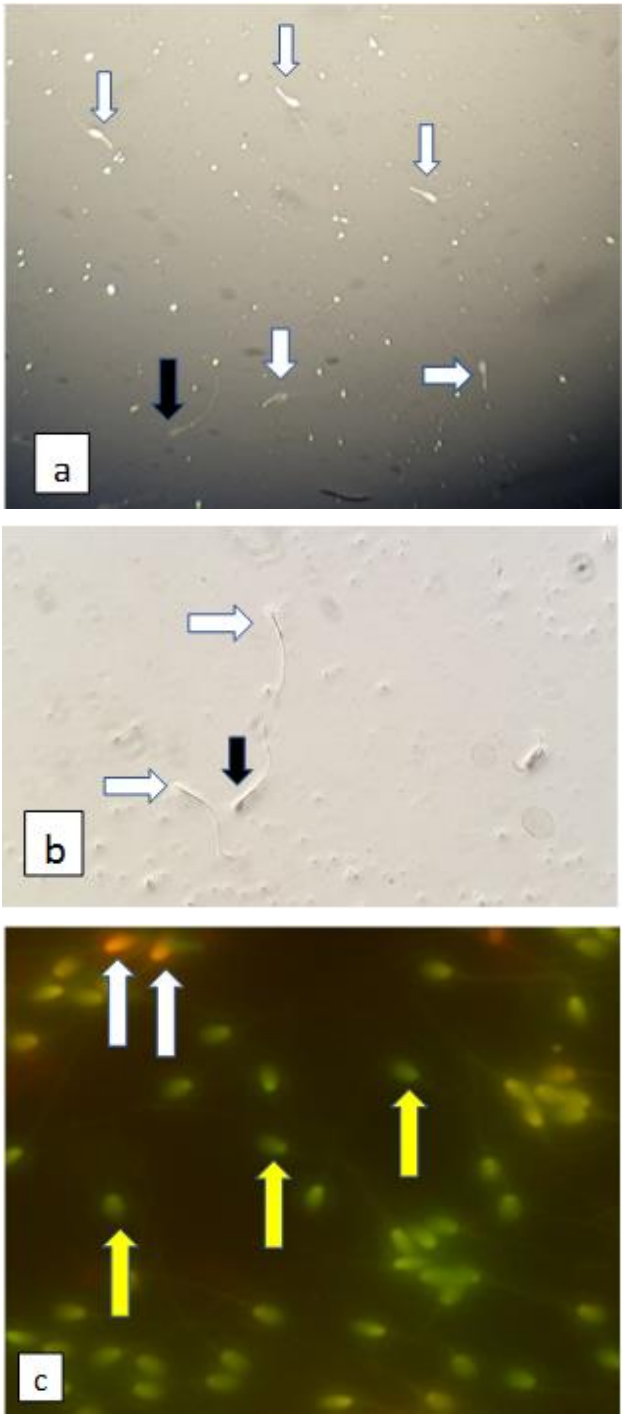

Fig. 1: Different semen factors. (**a**) Plasma membrane integrity of Simmental cow sperm after freeze-thaw process using the HOS method. White arrows indicate curved tail spermatozoa with a healthy plasma membrane, and black arrow represents smooth-tailed spermatozoa with an unhealthy plasma membrane, (**b**) Simmental cow sperm viability after the freeze-thaw process using the eosin-nigrosin method. White arrows show live, unstained sperms, and black arrow indicates dead sperms, and (**c**) Acridine orange staining to detect sperm with damaged DNA. Yellow and while arrows demonstrate sperms with healthy and damaged DNA, respectively. HOS: Hypo-osmotic swelling, (magnification ×400)

Viability

Investigating the percentage of sperm viability using eosin-nigrosin staining showed that adding 100 µM TXF to the semen diluent of Simmental cattle considerably increases ($P<0.05$) the percentage of viable sperm compared to other treatment and control groups. The 25, 50, and 200 µM TXF groups led to a noticeable increase ($P<0.05$), while the 400 µM group significantly reduced ($P<0.05$) live sperm percentage in comparison to the control group. Based on the examination of the viability of sperms, this index has no significant difference in the two groups containing 25 and 200 µM (Table 1, Fig. 1b).

DNA damage

The examination of sperm DNA damage demonstrated no meaningful difference ($P<0.05$) between the 50 and 100 µM TXF groups, but these treatment groups significantly ($P<0.05$) had less DNA damage than the other groups treated with TXF and the control group. Likewise, no significant difference ($P<0.05$) was found between the 25 and 200 µM groups. Based on statistical data analysis, the DNA damage of the sperms of the group containing 400 µM TXF increased noticeably ($P<0.05$) compared to the other treated and control groups (Table 1, Fig. 1c).

Motility and motility parameters

According to the results related to the overall sperm motility, the 100 µM TXF group had the highest ($P<0.05$) sperm motility percentage. There were no significant differences between the 25 and 200 µM groups with regard to this index. However, the overall sperm motility represented a considerable increase ($P<0.05$) in all treatment groups, except for the 400 µM TXF group compared to the control group ($P<0.05$, Table 2).

The rate of PM based on the CASA system indicated that the 100 µM TXF group had the highest value in comparison to the other treated and control groups ($P<0.05$). Likewise, this study found that in the group containing 25-100 µM TXF, the PM of sperm increased significantly with increasing TXF ($P<0.05$). In the 400 µM TXF group, PM demonstrated a significant decline ($P<0.05$), whereas it significantly increased in all other treatment groups ($P<0.05$) in comparison to the control group (Table 2).

The examination of the VSL, VCL, and LIN indexes revealed that the 100 µM TXF group had the highest ($P<0.05$) level when compared with the other treatment groups. In the present study, by increasing the amount of TXF from 25 to 100 µM, these indexes also increased significantly ($P<0.05$). In comparison, the addition of 400 µM TXF caused a significant decline in these indexes ($P<0.05$, Table 2).

The findings demonstrated that the 25 and 200 µM TXF groups and the control group did not have significant differences, but these groups had a significantly lower VAP index than the 100 and 50 µM TXF groups ($P<0.05$). Compared to all groups, the highest and lowest levels of this index were observed in the 100 and 400 µM groups ($P<0.05$, Table 2).

Based on the examination of the STR, ALH, and

Table 2: Mean (±SD) percentage of different motility indexes of sperms in different treatment groups of TXF

Index	Control	25 µM TXF	50 µM TXF	100 µM TXF	200 µM TXF	400 µM TXF
Spermatozoa motility (%)	56.22±1.51^{d}	61.65±1.47^{c}	65.39±1.68^{b}	70.18±1.02^{a}	61.89±1.43^{c}	52.20±1.39^{e}
Progressive motility (%)	19.34±1.26^{d}	25.88±1.69^{c}	29.84±1.93^{b}	34.56±1.44^{a}	24.73±1.92^{c}	15.17±1.36^{e}
VSL (µM/s)	18.32±1.64^{d}	19.18±1.62^{c}	27.74±1.59^{b}	29.90±1.52^{a}	19.41±1.17^{c}	22.36±1.62^{e}
VCL (µM/s)	36.55±1.07^{d}	41.63±1.57^{c}	44.56±1.44^{b}	48.37±1.50^{a}	40.94±1.21^{c}	31.70±1.28^{e}
VAP (µM/s)	31.75±1.90^{c}	31.40±1.13^{c}	35.35±1.52^{b}	39.77±1.32^{a}	31.45±1.30^{c}	25.67±1.60^{d}
LIN (%)	32.49±1.73^{d}	37.18±1.61^{c}	41.23±1.81^{b}	45.62±1.42^{a}	36.50±1.39^{c}	27.54±1.64^{e}
STR (%)	66.95±1.76^{a}	67.41±1.90^{a}	67.73±1.57^{a}	68.23±1.96^{a}	67.24±1.07^{a}	66.77±1.30^{a}
ALH (µM/s)	2.35±0.21^{a}	2.12±0.16^{a}	2.20±0.37^{a}	2.86±0.30^{a}	2.28±0.11^{a}	2.48±0.18^{a}
BCF (Hz)	2.45±0.18^{a}	3.15±0.25^{a}	3.07±0.21^{a}	3.37±0.16^{a}	2.40±0.24^{a}	2.07±0.15^{a}

SD: Standard deviation, TXF: Taxifolin, LIN: Linearity, VCL: Curvilinear velocity, ALH: Lateral head displacement, VAP: Average path velocity, VSL: Straight-line velocity, STR: Straightness, and BCF: Beat cross frequency. Different letters (^{a}, ^{b}, ^{c}, ^{d}, and ^{e}) demonstrate significant differences between the data (P<0.05) in rows

Table 3: Mean (±SD) TAC, SOD, GPx, and MDA in different treatment groups of taxifolin

Index	Control	25 µM taxifolin	50 µM taxifolin	100 µM taxifolin	200 µM taxifolin	400 µM taxifolin
TAC (mmol/L)	1.56±0.20^{d}	2.41±0.28^{c}	3.33±0.23^{b}	3.75±0.17^{a}	2.32±0.14^{c}	0.84±0.15^{e}
SOD (U/ml)	1.14±0.14^{a}	1.30±0.21^{a}	1.64±0.12^{a}	1.82±0.15^{a}	1.21±0.10^{a}	1.03±0.11^{a}
GPx (mU/ml)	75.41±2.27^{d}	84.67±2.83^{c}	90.80±2.48^{b}	96.59±2.79^{a}	82.69±2.80^{c}	69.75±2.64^{e}
MDA (mmol/L)	2.23±0.24^{d}	1.89±0.15^{c}	1.24±0.18^{b}	0.64±0.21^{a}	1.86±0.19^{c}	2.96±0.16^{e}

SD: Standard deviation, SOD: Superoxide dismutase, TAC: Total antioxidant capacity, MDA: Malondialdehyde, and GPx: Glutathione peroxidase. Different letters (^{a}, ^{b}, ^{c}, ^{d}, and ^{e}) indicate significant differences between the data (P<0.05) in rows

BCF indexes, no noticeable difference (P<0.05) was found in the treatment and control groups, and the addition of TXF had no effect on the treatment groups (Table 2).

Oxidative stress

The results represented that the 100 µM TXF group had the highest TAC and GPx indexes (P<0.05) among the studied groups. In this study, no considerable difference (P<0.05) was observed in these indexes between the 25 and 200 µM TXF groups. However, these groups have a significantly increased level in comparison with the control group (P<0.05). In addition, the group containing 400 µM TXF had a significantly decreased value in TAC and GPx indexes (P<0.05) than the control group (Table 3).

As regards SOD activity, the addition of TXF in different doses not only did not make any noticeable differences (P<0.05) between the treated groups when compared to the control group (Table 3).

Based on the findings, the MDA level was considerably (P<0.05) lower in the 100 µM TXF group than in the other treatment and control groups. In the current study, this index decreased significantly with increasing the amount of TXF added from 25 to 100 µM (P<0.05, Table 3).

Discussion

Overall, the findings confirmed the positive effect of adding TXF to the diluent on improving the quality of the frozen-thawed sperms of Simmental cows. Motility is one of the most important characteristics of spermatozoa fertility (Brito *et al*., 2004). This damage ultimately can lead to membrane integrity loss, deterioration of the membrane, a reduction in sperm motility, loss of fertility, leakage of intracellular enzymes, and damage to sperm DNA through OS and production of cytotoxic aldehydes (Brito *et al*., 2004). Antioxidant supplementation can avoid this process (Buffone *et al*., 2012).

Sperm motility is very important in facilitating passage via the cervix and connecting the fallopian tubes. It is essential for actual penetration into zona pellucida oocytes and cumulus cells (Rao *et al*., 2015). In this study, 25, 50, 100, and 200 µM of TXF resulted in a higher motility percentage in the treatment groups than in the control group. The dense fibers and the axoneme of intermediate segments in the sperm, which are known to be responsible for the movement of sperm, are protected by mitochondria that generate energy by oxidative phosphorylation. High levels of ROS can disrupt the motility of the sperm, and thus, ATP depletion causes axoneme damage (Rao *et al*., 2015).

The freezing process leads to cold shock damage, OS, intracellular ice crystal formation, and plasma membrane lipid and protein reorganization (Bailey *et al*., 2000). Freezing can cause damage to cell parts of the sperm, including the membrane (acrosome and plasma), chromatin, and mitochondria (Castro *et al*., 2016). This happens because, during the freezing and thawing procedure of the sperm, it undergoes extreme changes in temperature and osmolarity that cause the production of ROS (Nebel, 2007). Pressure on the plasma membrane during sperm freezing causes asymmetric modifications in the phospholipid bilayer membrane. It should be noted that the plasma membrane of the sperm of mammalians is enriched with unsaturated fatty acids that ROS simply damages in a reaction called “lipid peroxidation” (LPO) (Agarwal *et al*., 2003). This study found that 25, 50, 100, and 200 µM of TXF could maintain the integrity of the plasma membrane of the sperm after the freezing and thawing procedure. Intact plasma membrane integrity is necessary for sperm survival. The plasma membrane is

considered the entrance of materials from the outside into the cell or vice versa. The metabolism is disturbed by damage to the plasma membrane of the sperm, reducing sperm motility and capability to fertilize oocytes (Garner and Hafez, 2013).

In sperm-freezing, defense systems may require the integrated action of enzymatic and non-enzymatic antioxidants to overcome OS against ROS production. Previous research demonstrated that the freeze-thaw procedure can cause noticeable changes in the organization of the lipids of the sperm membrane (Gadea *et al*., 2008). This is probably because of the lack of endogenous antioxidant capacity and excessive ROS production in cells (Bilodeau *et al*., 2000). Experiments conducted on the human sperm also demonstrated that the freeze-thaw procedure can increase LPO activities (Alvarez and Storey, 1992). Sperm cells are exposed to oxidative attacks and fat oxidation because of double bonds in their carbon chain unsaturated fatty acids. Evidence indicates that the level of LPO is inversely related to sperm viability, motility, and function (Aitken *et al*., 1993). GPx, SOD, and CAT enzymes reduce LPO membranes by inhibiting superoxide and hydrogen peroxide ion (Sikka, 1996). During the freezing procedure of semen, LPO causes damage to the sperm because the plasma membrane of the sperm consists of phospholipids. Sperm is composed of unsaturated fatty acids, particularly susceptible to free radicals stimulating autocatalytic reactions and destructing double bonds (Catalá, 2013). Long-term LPO can cause damage to the lipid matrix structure, generating an unstable cell plasma membrane (Itri *et al*., 2014). ROS are physiologically involved in sperm function regulation. It affects the initiation of sperm motility after ejaculation through cyclic adenosine 3´,5´-monophosphate combination, and protein phosphorylation (Baumber *et al*., 2003). Superoxide anion is required at the physiological concentration to initiate hyperactivation, capacitation, and acrosomal reactions during fertilization (Thompson *et al*., 2014). Antioxidant concentration and storage periods significantly affect viability, motility, membrane integrity, and acrosome integrity (Itri *et al*., 2014). This implies that antioxidants can avoid the increase of free radicals and improve semen quality (Ahmadi *et al*., 2016). Biological antioxidants, including GSH, GPx, CAT, and SOD, exist in the semen, suppressing free radicals (Petruska *et al*., 2014). The capacity of the endogenous antioxidants of the sperm is probably impaired during the freezing and thawing procedure. Suppose there is a disturbance in the equilibrium between antioxidants and ROS. In that case, the OS may have negative impacts on DNA integrity (Baumber *et al*., 2003), oxidative metabolism inhibition (Makker *et al*., 2009), and sperm motility and viability reduction (Agarwal *et al*., 2014). In the current study, a decline was observed in the level of MDA, and an increase in the activity level of TAC and GPx in comparison with the control group in Simmental semen diluted with TXF doses, except for the 400 µM dose. Accordingly, the decline of MDA levels may positively reduce LPO by improving ROS scavenging systems. It is hypothesized that doses of 100, 50, 25, and 200 µM TXF may protectively affect the functional integrity of acrosome and mitochondria and improve the motility of the sperm after thawing without inhibiting LPO, respectively. It should be noted that the integrity of sperm DNA is of vital significance for the cells of the sperm. This study revealed that 100, 50, 25, and 200 µM of TXF can protect sperm DNA. Some researchers suggested that the DNA integrity of the sperm can more objectively represent the function of the sperm compared to sperm factors, including motility. DNA damage could be due to free radical damage in previous studies, but the applied antioxidants could protect against freezing, DNA, and morphological damage of the sperm after thawing.

Based on the present study's findings, it can be assumed that adding 100 and 50 µM of TXF to the Simmental cattle sperm diluent could lead to an increase in the fertility potential of the sperm.

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Conflict of interest

The authors declare no conflict of interest.

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