Original article Casp. J. Vet. Sci., 2024; 1(2): 135-144

Amol University of Special Modern Technologies

Caspian Journal of Veterinary Science

doi: 10.22034/cjvs.2024.210712

Journal homepage: https://Cjvs.ausmt.ac.ir/

Comparison of sperm quality, blood and seminal plasma element and enzyme levels in sub-fertile and healthy bull

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Article Info

Article history:

Received: 8 August 2024

Accepted: : 14 September 2024

Keywords:

Blood serum

Elements

Oxidative enzymes

Seminal plasma

Sperm quality

Abstract

The aim of this study was to compare the sperm quality, levels of zinc, copper, iron, calcium, and magnesium, and the levels of oxidative enzymes catalase, superoxide dismutase, and glutathione peroxidase in seminal plasma and blood serum of a sub-fertile bull with those of seven healthy fertile bulls. Semen was collected using an artificial vagina and frozen using a one-step dilution method. Blood samples were taken from the caudal vein. Sperm viability and morphology were examined using eosin-nigrosine staining, and sperm motility was assessed using CASA software. Element levels were measured by atomic absorption spectroscopy, and enzyme levels were determined by ELISA. The results showed that all sperm quality indices (except for ejaculate volume and percentage of cytoplasmic droplets) were significantly lower in the sub-fertile bull compared to the healthy fertile bulls. Comparison of the seminal plasma and blood serum of this bull with those of the healthy bulls revealed that only the magnesium level in the seminal plasma was significantly lower, with no significant differences in other parameters. These findings suggest that measuring the levels of the studied elements and enzymes in blood serum and seminal plasma may not be reliable indicators of reproductive health in bulls, and that post-thaw progressive sperm motility may remain the most important indicator of reproductive health in bulls.

Introduction

Proper fertility in cattle is crucial for any livestock operation (Dar *et al.,* 2018). Fertility issues in bulls can increase calving intervals, lead to unwanted culling in the herd, and reduce milk production (Inchaisri *et al.,* 2010). Reduced fertility can be attributed to various factors in males, females, management indices, or the environment. Given that the fertility of thousands of cows in farms depends on a single bull through artificial insemination, selecting a bull with appropriate fertility potential for high production is of great importance (Selvaraju *et al.,* 2018). Recent molecular studies identify the male as a primary factor in infertility, which has been investigated from various aspects (Bellin *et al.,* 1998). Efforts to increase female cattle fertility have been extensive, but many cases of infertility in cattle are due to poor sperm quality, highlighting the importance of evaluating male reproductive capacity in artificial insemination (DeJarnette *et al.,* 2004).

Semen consists of a cellular (sperm) and liquid (seminal plasma) portion, which play a vital role in

sperm transfer through the male reproductive tract. Some studies have reported significant relationships between seminal plasma and fertility rates in cattle (Killian *et al*., 1993; Kumar *et al.,* 2015). Seminal plasma contains minerals and non-minerals essential for sperm nutrition and protection until fertilization (Aggarwal *et al.,* 2009). The mineral content of seminal plasma is related to its blood levels, directly and indirectly (through oxidative enzymes in the semen) affecting sperm quality (Akalın *et al.,* 2015). It has been reported that the composition of minerals and proteins in semen differs between fertile and sub-fertile animals (McCauley *et al.,* 1999). Fertility in animals is controlled by various factors, including DNA damage, oxidative stress, apoptosis pathways, and chemokines, which are directly related to the mineral content of seminal plasma (Hidiroglou *et al.,* 1979; Dar *et al.,* 2018).

Studies have shown that zinc plays a crucial role in sperm structural stability and acrosin in the reproductive system (Kvist *et al.,* 1990; Riffo *et al.,* 1992). The prostate gland is the primary source of zinc in seminal plasma, providing greater stability in sperm chromatin in each ejaculate (Evenson *et al.,* 1993). It has been reported that zinc concentration in seminal plasma is much higher than in blood, indicating its essential role in testicular growth, sperm physiological function, and motility (Wong *et al.,* 2000; Colagar *et al.,* 2009). Reduced zinc levels in seminal plasma are associated with decreased fertility potential (Caldamone *et al.,* 1979) and increased DNA damage (Richthoff *et al.,* 2002). Zinc may also have a regulatory role in the capacitation process and acrosome reaction (Riffo *et al.,* 1992; Ali *et al.,* 2007). Insufficient zinc levels are associated with inadequate secondary sexual gland development and hypogonadism (Prasad, 1991).

Copper accelerates oxidative stress processes and reduces glucose consumption (Skandhan, 1992). The main organ secreting copper is the prostate, but unlike zinc, it is also secreted into seminal fluid from other glands (Valsa *et al.,* 1994). It has been suggested that asthenozoospermia is associated with low serum copper concentrations (Yuyan *et al.,* 2008,). A significant positive correlation between seminal copper concentration and sperm forward movement in cattle has been reported, with higher copper concentrations observed in normal ejaculates compared to abnormal ones (Mullis *et al.,* 2003). A significant positive correlation between plasma copper levels and sperm volume has been reported (Akinloye *et al.,* 2011). Higher than normal copper-to-zinc ratios have been reported in infertile men with asthenozoospermia, suggesting a link between zinc-to-copper ratios and infertility (Fuse *et al*., 1999; Wong *et al.,* 2001; Yuyan *et al.,* 2008).

Iron is crucial for many organs due to its high oxidation capacity (Craig *et al.,* 2009). It appears that iron induces lipid peroxidase catalysis, resulting in decreased sperm quality and motility (Braughler *et al.,* 1986). The seminal transferrin level, likely originating from sertoli cells, correlates with sperm production in humans and cattle and is an effective indicator of sertoli cell function (Sylvester *et al.,* 1994). Lactoferrin is secreted into seminal plasma by vesicular glands (Buckett *et al.,* 1997). Citrate acts as a physiological ligand for iron in seminal plasma, keeping it catalytically inactive (Menditto *et al.,* 1997). Mannose receptor binding on the sperm head requires calcium (Benoff *et al.,* 2000).

Calcium is a key factor in sperm capacitation and affects sperm motility. Sperm hyperactivation, which helps penetrate the cumulus matrix, is a process directly dependent on calcium (Meseguer *et al.,* 2004). Magnesium may play a role in sperm motility and is also recognized as an indicator of the secretory function of seminal vesicles (Abou-Shakra *et al.,* 1989).

It has been reported that the magnesium concentration in seminal plasma is higher than in blood (Omu *et al.,* 2001). Reduced magnesium may play a role in premature ejaculation. Blood magnesium, as well as seminal magnesium, is associated with this condition (Aloosh *et al.,* 2006).

Among important antioxidant enzymes is superoxide dismutase. The primary source of extracellular superoxide dismutase in seminal plasma is the prostate gland, although the epididymis is also suggested as a source (Peeker *et al.,* 1997). A linear relationship between superoxide dismutase activity in human seminal plasma and sperm concentration has been shown, with a significant linear correlation between sperm motility and enzyme activity also reported (Nissen *et al.,* 1983). The protective role of superoxide dismutase against the toxic effects of reactive oxygen species on ram sperm has been demonstrated (Kasimanickam *et al.,* 2006). Catalase has been identified in the seminal plasma of many mammals, with the main source being the accessory sex glands in humans (Zini *et al.,* 2002). As hydrogen peroxide is the main reactive oxygen species damaging to sperm, catalase plays a crucial role in sperm protection, with the prostate being the enzyme source (Ball *et al.,* 2000; Koskinen *et al.,* 2002). Glutathione

peroxidase, like superoxide dismutase, affects sperm function by preventing lipid peroxidation of the sperm membrane, ultimately improving sperm motility (Sanchez-Gutierrez *et al.,* 2008). From production to acquiring fertilization capacity, glutathione peroxidase plays a vital role in sperm function, with its absence leading to reduced sperm fertilization capacity (Hall *et al.,* 1998).

Based on the mentioned points, this study aimed to evaluate sperm quality indices and measure the levels of zinc, copper, calcium, magnesium, and iron in blood and seminal plasma, as well as the oxidative enzyme levels of catalase, superoxide dismutase, and glutathione peroxidase in a sub-fertile Simmental bull compared to healthy fertile bulls.

Materials and Methods

This study utilized seven healthy fertile dual-purpose Simmental (Fleckvieh) bulls from the Iran Simmental Cattle Breeding and Semen Production Center located in Amol, Mazandaran province, Iran, which had previously undergone reproductive capacity evaluation and were approved by the Iran National Breeding Center. Another Simmental bull, aged 36 months, with normal physical characteristics, libido, and scrotal circumference (41 cm), had also passed all necessary disease tests and quarantine periods, was provided for initial evaluation and entry into frozen semen production. It is noteworthy that all bulls were from a single purebred Simmental herd under identical management and feeding conditions. Initial evaluations indicated that the sperm quality of this bull was not suitable for frozen semen production, thus, it was used as the sub-fertile bull in this study. The feed composition, percentages, and amounts were listed in Table 1.

Table 1. Composition, percentage, and amounts of the diet for dual-purpose Simmental German reproductive breed bulls (Fleckvieh).

Combinations	Amount (Kg)	Crude Protein (%)	NDF (%)	ADF (%)	Fat (%)	Ash (%)	DM (%)
Concentrate [a]	9	14.68	16.8	13.1	3.2	7.4	89.2
Silo	18	8.5	54.5	32.7	1.8	5.7	25
Alfalfa	3	16.8	44.7	34.6	2.5	9.7	1.88
Straw	*Ad libitum*	3.9	70.3	45.5	1.1	9.8	94.8
Mineral	*Ad libitum*	-	-	-	-	-	-
Water	*Ad libitum*	-	-	-	-	-	-

supplement[b] [a] Calcium: 0.74%, Phosphorus: 0.53%, Sodium: 0.49%, Magnesium: 0.29%, Zinc: 375 ppm, Manganese: 44.381 ppm, Cobalt: 0.01 ppm, Selenium: 75.2 ppm, and Vitamin additives (Vitamin A: 7500 IU/kg, Vitamin D3: 1000 IU/kg, Vitamin E: 10 mg/kg). [b] Magnesium: 1.2%, Sodium: 7%, Iron: 355 mg/kg, Zinc: 1560 mg/kg, Copper: 390 mg/kg, Manganese: 1560 mg/kg, Selenium: 5.7 mg/kg, Cobalt: 3 mg/kg, Iodine: 5.15 mg/kg. Source: The data were calculated by the Animal Nutrition Laboratory, Faculty of Veterinary Medicine, University of Tehran.

Sperm sampling was performed in five stages over a three-month period (from January to March) using an artificial vagina (five ejaculations from each bull, totaling 40 ejaculations). The average, maximum, and minimum temperatures and humidity at the time of sperm collection were included in Table 2. Immediately after sperm collection, sperm quality indices such as ejaculate volume and semen concentration were examined. Sperm concentration was measured using a photometer device (Minitube, Tiefenbach, Germany) SDM.

Table 2. Temperature and humidity values at the sperm collection site during the research period.

Index	Humidity ± Standard deviation	Temperature ± Standard deviation
Maximum	60	17
Minimum	50	5
Average	57.42 ± 3.62	9.37 ± 3.37

To measure the levels of elements and investigate oxidative enzyme activities, 2 ml of semen from each ejaculation was separated and transferred to the laboratory of the Faculty of Veterinary Medicine, University of Applied Science and Technology, Amol, for centrifugation. Samples were centrifuged at 3000 rpm for 10 minutes. After centrifugation, the supernatant was taken with a sampler and in samples where the seminal plasma was not clear, one drop of plasma was inspected under a microscope for sperm presence and re-centrifuged if sperm were present. The obtained seminal plasma samples were then transferred to 1.5 ml microtubes and stored at -70°C until testing.

Blood samples from the bulls were taken via the caudal vein (10 ml each) at the beginning, middle, and end of the study period. The collected blood samples were centrifuged at 900g for 15 minutes, and the resulting serums were transferred to microtubes and stored at -70°C until biochemical analysis.

Sperm freezing was performed using the one-step dilution method. In this study, the semi-prepared commercial extender Triladyl 200g (Triladyl; Minitube, Tiefenbach, Germany), prepared by adding 750 ml of double-distilled deionized water and 250 g of egg yolk, was used for freezing. The dilution and freezing process is briefly described as follows: after evaluating the sperm, the extender was slowly added to the sperm in a 1:1 ratio to prepare the pre-extender solution. This solution was left at 34°C in a water bath for 10

minutes.

The formula for calculating the number of sperm doses produced is as follows; Final sperm dose count = ejaculate volume × sperm concentration × percentage of progressive motility × percentage of normal spermatozoa ÷ number of sperm per straw (15×10^6).

Based on the above formula and considering that the sperm were packaged in 0.5 ml straws, the required amount of final extender was obtained, and then the pre-extender was added to the final extender and kept at room temperature for 15 minutes. At this stage, the sperm were packaged using an automatic filling and sealing device (Minitube, Tiefenbach, Germany) MPP Uno at room temperature and placed on special trays for 0.5 ml straws, then transferred to a refrigerator at 4°C. To reach equilibrium, the straws were kept in the refrigerator for 4 hours. For freezing, the straws were placed at -120°C in a MT Freezer device (Minitube, Tiefenbach, Germany) for 10 minutes. Finally, the frozen sperm were placed in goblets floating in liquid nitrogen and transferred to sperm storage containers.

To examine the percentage of live sperm and sperm morphology, eosin-nigrosin staining (Minitube, Tiefenbach, Germany) was used. After the sperm were placed in the final extender, one drop of this mixture combined with one drop of 2% eosin and two of 4% nigrosin using a sampler, placed on a glass slide, and spread out. The spread samples were dried on a warm plate, and at least 200 sperm were examined under a phase-contrast microscope (Minitube, Tiefenbach, Germany) MBL 2000 at 400x magnification. Dead sperm, due to their high cell membrane permeability, absorbed the eosin stain and appeared red, distinctly different from live sperm, which did not absorb the stain, thus determining the percentage of dead sperm. At least 200 sperm per slide were examined for abnormal morphology in the head, midpiece, tail, and presence of cytoplasmic droplets.

Sperm motility before freezing was visually examined using a phase-contrast microscope and post-freezing using a CASA software (HFT CASA, Hooshmand Fanaver, Iran) equipped with a microscope containing a warm stage. To determine the percentage of progressive motility and post-freezing sperm motility indices, each straw was individually placed in a water bath set at 37°C for 30 seconds, then the sealed end of the straw was cut open vertically, and the contents were transferred into 1.5 ml Eppendorf tubes, kept in a floating rack in the water bath until analysis. 5 µl of this solution was placed on a specific sperm analysis slide with the CASA device (Sperm meter, Depth 10-micron, Surface Graticule, 100 x 0.1 sqmm), and at least 5 microscopic fields and 1000 spermatozoa were analyzed. The indices examined included the percentage of progressive sperm motility (PM), curvilinear velocity (VCL), straight-line velocity (VSL), average path velocity (VAP), mean angular displacement (MAD), amplitude of lateral head displacement (ALH), beat cross frequency (BCF), and linearity (LIN).

Calcium, magnesium, zinc, copper, and iron concentrations were measured using an atomic absorption device (Varian AA240FS, USA). Briefly, 1 ml of serum or seminal plasma was digested with 5 ml of concentrated nitric acid 69% (Merck, Germany). The wavelengths for measuring the elements were: zinc (213.9 nm), copper (324.7 nm), iron (248.3 nm), calcium (422.8 nm), and magnesium (285.2 nm). Calibration curves were prepared with standard solutions of different concentrations for each element, and the activity of catalase, superoxide dismutase, and glutathione peroxidase enzymes were measured using commercial kits (Zellbio, Germany) by ELISA according to the manufacturer's instructions.

In this study, SPSS 22 software was used for data analysis. Comparisons of group means were analyzed using the independent T-test. All results were presented as mean ± standard deviation.

Results

Our findings indicated that all sperm quality indices (except ejaculate volume and cytoplasmic droplet percentage) were significantly weaker in the sub-fertile bull compared to healthy fertile bulls. According to two of the mentioned indices, the percentages of abnormal spermatozoa and progressive motility post-freezing in this bull were 26.09 ± 3.81 and 16.91 ± 1.9, respectively, and based on the standards mentioned in reputable sources (Parkinson *et al*., 2009), they were of poor quality and thus could not be used as a reproductive and productive bull. A comparison of this bull's seminal plasma with that of healthy fertile bulls showed that magnesium levels were significantly lower than in healthy fertile bulls, while other elements and oxidative enzymes showed no significant differences (Table 4). Serum blood analysis showed no significant differences in any of the elements and oxidative enzymes between the sub-fertile bull and healthy fertile bulls (Table 5).

Table 3. Comparison of sperm quality indices between the sub-fertile bull and healthy fertile bulls. Data are presented as mean ± standard error. Different superscript letters (a and b) in a row indicate significant differences in the respective index.

Index	Sub-fertile bull	Healthy fertile bulls
Ejaculate volume(ml)	6.45 ± 0.48 [a]	8.3 ± 0.61 [a]
Semen concentration(×10⁶)	965 ± 164.68 [a]	1305 ± 62.35[b]
Total Sperm motility before freezing (%)	65.67 ± 1.52[a]	78.16 ± 0.95[b]
Progressive Sperm motility before freezing (%)	60.67 ± 1.52	71.87 ± 1.31[b]
Live spermatozoa before freezing (%)	63.5 ± 3.58	84.39 ± 0.79[b]
Total abnormal morphology before freezing	18.75 ± 1.57	8.01 ± 0.66[b]
Abnormal Head morphology before freezing	6.58 ± 0.69	2.36 ± 0.43[b]
Abnormal Midpiece morphology before freezing (%)	2.3 ± 0.36	1.43 ± 0.16[b]
Abnormal tail morphology before freezing (%)	8.94 ± 1.4	3.64 ± 0.36[b]
Cytoplasmic Droplet before freezing (%)	0.93 ± 0.38	0.58 ± 0.07[a]
Total sperm motility after-freezing (%)	37.44 ± 2.45	70.68 ± 2.12[b]
Progressive Sperm motility after freezing (%)	16.91 ± 1.9[a]	45.8 ± 2.25[b]
VAP (µm/S) after freezing	16.23 ± 1.3[a]	42.99 ± 2.05[b]
VCL (µm/S) after freezing	25.73 ± 1. 93[a]	62.23 ± 2.8 [b]
VSL (µm/S)after freezing	12.65 ± 1.27[a]	37.09 ± 1.9[b]
LIN (%)after freezing	23.21 ± 1.79[a]	47.11 ± 1.66[b]
ALH (µm)after freezing	1.33± 0.11[a]	2.66 ± 0.11[b]
BCF (Hz)after freezing	0.27 ± 0.11[a]	0.97 ± 0.05[b]
MAD after freezing	11.73 ± 1.23[a]	26.37 ± 2.72[b]
Live spermatozoa after freezing (%)	40.78 ± 3.89[a]	63.33 ± 2.72[b]
Total abnormal morphology after freezing(%)	26.09. ± 3.81[a]	8.44 ± 0.73[b]
Abnormal Head morphology after freezing (%)	8.38 ± 1.04[a]	2.03 ± 62.35[b]
Abnormal Midpiece morphology after freezing (%)	2.81 ± 0.53[a]	1. 7 ± 0.19[b]
Abnormal Tail morphology after freezing (%)	13.831 ± 1.93[a]	4.25 ± 0.4[b]
Cytoplasmic droplet before freezing (%)	1.07 ± 0.42[a]	0.46 ± 0.09[a]

Table 4. Comparison of element and oxidative enzyme levels in semen plasma between a sub-fertile bull and healthy fertile bulls.

Index	Sub-fertile bull	Healthy fertile bulls
Zinc (mg/dl)	7.09 ± 122.33[a]	11.54 ± 131.17[a]
Copper (mg/dl)	0.22 ± 2.28[a]	0.14 ± 2.17[a]
Iron (mg/dl)	3.83 ± 27.77[a]	2.25 ± 26.60[a]
Calcium (mg/dl)	2.53 ± 19.45[a]	1.35 ± 20.02[a]
Magnesium (mg/dl)	0.23 ± 3.52[a]	0.17 ± 4.27[b]
Catalase (U/ml)	2.00 ± 16.02[a]	3.36 ± 23.37[a]
Superoxide Dismutase (U/ml)	0.10 ± 8.50[a]	0.44 ± 9.29[a]
Glutathione Peroxidase (U/ml)	0.06 ± 1.10[a]	0.31 ± 1.40[a]

Table 5. Comparison of element and oxidative enzyme levels in blood serum between a sub-fertile bull and healthy fertile bulls.

Index	Sub-fertile bull	Healthy fertile bulls
Zinc (mg/dl)	24.51 ± 92.73[a]	13.45 ± 137.41[a]
Copper (mg/dl)	23.39 ± 98.47[a]	7.98 ± 74.80[a]
Iron (mg/dl)	0.10 ± 2.32[a]	0.15 ± 2.18[a]
Calcium (mg/dl)	0.90 ± 8.68[a]	0.40 ± 9.44[a]
Magnesium (mg/dl)	0.08 ± 2.53[a]	0.13 ± 2.62[a]
Catalase (U/ml)	7.79 ± 12.29[a]	2.63 ± 19.02[a]
Superoxide Dismutase (U/ml)	2.22 ± 10.64[a]	0.35 ± 8.81[a]
Glutathione Peroxidase (U/ml)	1.85 ± 4.09[a]	0.60 ± 2.77[a]

Discussion

The aim of this study was to compare the sperm quality indices, levels of certain elements, and oxidative enzymes in the blood serum and semen plasma of a sub-fertile bull with those of healthy fertile bulls. Examination of the sperm quality indices revealed that, except for semen volume and the percentage of cytoplasmic droplets, all indices showed significant differences between the sub-fertile bull and the healthy fertile bulls. This indicated poor sperm quality in the sub-fertile bull, thus justifying its classification as sub-fertile in this study. Typically, bulls with low fertility are removed from the herd and are not used as breeding bulls in genetic improvement centers or for sperm production. Consequently, there is limited information available on the sperm quality indices, biochemical characteristics of the blood serum, and semen plasma of these animals. Therefore, we aimed to investigate the levels of certain important elements and oxidative enzymes, which have been identified to play significant roles in male fertility, in the sub-fertile bull and compare them with those in healthy fertile bulls from the Iranian Simmental cattle breeding and frozen semen production center. It has been established that post-thaw progressive motility of sperm is the most practical indicator for estimating reproductive capacity in bulls. (Zini *et al*., 2000),) The low average of this index in the studied bull, based on standards provided in the literature, can confirm the subfertility of the bull in question(Shamsi *et al*., 2010). A study reported that there is no significant statistical difference in blood and semen plasma zinc levels between fertile and infertile individuals. However, a weak correlation was observed between blood plasma zinc concentrations and sperm count, motility, and abnormal forms (Wong *et al*., 2001). It has been reported that zinc levels in semen plasma are higher than in blood (Xu *et al*., 1994), which is consistent with our findings for both the subfertility.

Additionally, (Egret-Cruz *et al*., 2002) reported that zinc levels in the blood and semen of infertile men are lower than those in healthy fertile men. Although in this study the zinc levels in the blood and semen plasma of the sub-fertile bull were lower than those in healthy fertile bulls, this difference was not statistically significant. (Akinloye *et al*., 2011) reported that serum zinc concentration in oligozoospermic patients is higher compared to healthy fertile individuals. Our study showed that although serum zinc levels were higher in healthy bulls, the concentration did not differ significantly between the sub-fertile bull and the healthy fertile bulls. There is a weak correlation between blood plasma copper levels and human sperm motility (Wong *et al*., 2001). Aydemir *et al*. (2006) reported that the copper levels in the semen plasma of infertile men are significantly higher than in fertile individuals, suggesting that copper may mediate oxidative damage and play a significant role in spermatogenesis and male infertility. Huang *et al*. (2000) also found higher copper levels in the semen plasma of men with low sperm motility. However, Wong *et al*. (2001) indicated no difference in copper levels between the blood and semen plasma of infertile and fertile men. Additionally, Masani *et al*. (2003) showed that copper has a toxic effect on the epithelium of the seminiferous tubules in rams, manifesting as decreased motile sperm percentage and increased abnormal sperm. Although in our study, copper levels in both the blood serum and semen plasma of the sub-fertile bull were higher than those in healthy fertile bulls, this difference was not statistically significant. In buffalo, semen plasma copper levels do not correlate with any semen quality parameters (Eghbali *et al*., 2010). It has been reported that blood serum copper levels are higher than those in semen plasma (Wong *et al*., 2001; Akinloye *et al*., 2011), which is consistent with our findings. Moreover, Wong *et al*. (2001) stated that the calcium levels in the blood serum and semen plasma of fertile and infertile men do not have a statistically significant difference. Similarly, although our study found that the calcium levels in the blood and semen plasma of healthy fertile bulls were higher than those of the sub-fertile bull; this difference was not statistically significant.

It has been reported that the magnesium concentration in semen plasma is higher than in blood (Omu *et al*., 2001), which is consistent with our findings. However, an important observation from our study is that the magnesium levels in the semen plasma of fertile bulls were significantly higher than those of the sub-fertile bull. Similarly, Eghbali *et al*. (2010) reported a significant positive correlation between semen plasma magnesium levels and sperm motility and viability in buffalo.

Aydemir *et al*. (2006) stated that iron levels in the semen plasma of infertile individuals are significantly higher, suggesting that iron may mediate oxidative damage and play a critical role in spermatogenesis and infertility. Additionally, another study suggested that high blood serum iron levels could be associated with low sperm motility (Khaki *et al*., 2017). Although our study found higher iron levels in both the serum and semen plasma of the sub-fertile bull compared to healthy fertile bulls, this difference was not statistically

significant. Alavi Shoushtari *et al*. (2009) reported a significant positive correlation between catalase levels and both sperm motility and viability, suggesting that catalase in semen plasma provides protective effects against oxidative damage to sperm. Khosrowbigi and Zarragami (2007) showed that individuals with abnormal semen had significantly lower catalase levels than healthy fertile individuals, and they reported a direct correlation between catalase activity and sperm concentration, motility, and morphology. Although our study found lower catalase levels in the semen plasma of the sub-fertile bull compared to healthy fertile bulls, this difference was not statistically significant. In contrast, Shamsi *et al*. (2010) reported a significant difference in blood serum antioxidant levels between infertile men and healthy fertile individuals, which contradicts our findings.

It has been reported that the activity of the enzyme superoxide dismutase is higher in men with oligozoospermia compared to those with normal sperm production (Sanocka *et al*., 1996). Additionally, the activity of this enzyme in the semen plasma of infertile men is significantly higher than in fertile men (Zini *et al*., 2000). Siciliano *et al*. (2001) demonstrated that the antioxidant capacity of this enzyme in individuals with decreased sperm motility does not differ from that in naturally fertile individuals; however, in those with decreased sperm motility and count, it is lower than in naturally fertile samples. Another study found no significant difference in the activity of this enzyme in semen plasma between healthy fertile men, men with reduced sperm count in semen, and men with reduced sperm motility, and no correlation was observed between this enzyme and sperm concentration and motility (Hsieh *et al*., 2002). Another study indicated that superoxide dismutase activity in the semen of men with oligozoospermia is higher than in men with normal sperm production (Tkaczuk-Wlach *et al*., 2002). In our study, the activity of the superoxide dismutase enzyme in both blood and semen plasma of the sub-fertile bull showed no significant statistical difference compared to the healthy fertile bulls.

In research, it has been stated that the activity of glutathione peroxidase in seminal fluid does not have a significant correlation with semen quality indices, and there is no difference in the activity of glutathione peroxidase between individuals with normal sperm production and those with reduced sperm count and motility (Hsieh *et al*., 2006).Tramer *et al*. (2004) also demonstrated no difference in glutathione peroxidase activity between naturally fertile men and those with reduced sperm motility. These findings align with the results of our study. However, it has been indicated that glutathione peroxidase can protect mammalian spermatozoa from decreased motility caused by spontaneous lipid peroxidation (Alvarez *et al*., 1989). On the other hand, it has been reported that glutathione peroxidase activity has a positive relationship with semen samples from individuals with reduced sperm motility (Dandekar *et al*., 2002). In contrast, another study showed that glutathione peroxidase activity is lower in infertile individuals compared to fertile individuals (Alkan*et al*., 1997). Additionally, another research pointed out that glutathione peroxidase activity is higher in healthy individuals than in infertile ones (Giannattasio *et al*., 2002). However, in our study, no significant statistical difference in glutathione peroxidase activity was observed between the sub-fertile bull and the healthy fertile bulls. Moreover, a study reported no significant statistical difference in glutathione peroxidase activity in the serum of healthy fertile bulls, which were categorized into excellent, good, and average groups based on sperm quality (Khaki *et al*., 2018).

Although the results of this study indicated that measuring the levels of the elements and enzymes examined in blood serum and seminal plasma might not be reliable indicators of reproductive health in bulls, and that the percentage of progressive sperm motility after freezing is likely the most important indicator of reproductive health in bulls, it is important to note that the differences in biochemical characteristics between a sub-fertile bull and healthy fertile bulls, which were not statistically significant, might have yielded different results if the study had included a larger number of sub-fertile bulls. However, considering that sub-fertile bulls are typically culled from herds in the livestock industry, finding an appropriate sample size for this category of bulls is very challenging. Therefore, the findings of this study can still be considered valuable.

Acknowledgment

This research has been financially supported by Amol University of Special Modern Technologies, Amol, Iran.

Conflict of Interest

The authors do not have any potential conflict of interest to declare.

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