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Differences between some biochemical components in seminal plasma of first and second ejaculations in dual-purpose Simmental (*Fleckvieh*) bulls and their relationships with semen quality parameters

Amir Khaki[1,2]*, Atefeh Araghi[1], Mehdi Lotfi[2], Alireza Nourian[2]

[1] Department of Clinical Sciences, Faculty of Veterinary Medicine, Amol University of Special Modern Technologies, Amol, Iran; [2] Iran Simmental Cattle Breeding Center, Amard-Dam Company, Amol, Iran.

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Abstract

This study aimed to evaluate differences in seminal plasma zinc (Zn), copper (Cu), iron (Fe), catalase (CAT), superoxide dismutase (SOD), and glutathione peroxidase (GPx) levels in the first and second ejaculations and their relationships with semen quality parameters in *Fleckvieh* bulls. Repetitive ejaculates were separately collected, analyzed, and frozen from the sires. Progressive motility of frozen-thawed semen (PMFT) was considered the main factor for more data classification into three following groups: <40.00%, 40.00 - 50.00%, and >50.00%. Seminal plasma trace elements and enzymes were determined using atomic absorption spectrometry and ELISA, respectively. The results revealed significant differences between the first and second ejaculations. Semen concentration, SOD, GPx, and Fe were different in ejaculations. Although PMFT groups in different ejaculations did not show significant differences, there was significant alteration between different PMFT groups and first and second ejaculations. All frozen-thawed semen CASA parameters (except lateral head displacement) were associated with fresh motility parameters and before and after thawing sperm viability. Also, a correlation between seminal Zn concentration with fresh semen gross and progressive motility, average path velocity, and beat cross frequency, Cu with SOD and Fe and semen concentration was observed. CAT was associated with fresh and frozen-thawed sperm motility parameters except for lateral head displacement and angular displacement. Although our findings showed differences between the first and second ejaculations in some parameters, PMFT, which is the most important indicator for estimating bull fertility, was not different between them.

Introduction

The physiological function of the seminal plasma constituents is a matter of debate. There is much inter-species variation in the composition of seminal plasma. Hence, it has been challenging to ascribe absolute functions to many of its constituents. Although there is an agreement that the wide variety in the composition of the seminal plasma between species indicates no critical role for it, however, it seems that seminal plasma contributes to important functions of spermatozoa in bovine species.[1] Also, it has been suggested that seminal plasma could protect sperm membrane from damage during the cryo-preservation with binding to phosphatidylcholine, which induces one of the main mechanisms of cold shock.[2]

The correlation between trace minerals concentration and antioxidants status in seminal plasma was determined to be related to their cooperation with the production of metalloproteins, enzymatic and non-enzymatic anti-oxidants.[3] Although the physiological status of reactive oxygen species (ROS) in seminal plasma is necessary for sperm capacitation and penetration and sperm - oocyte interaction in bovine, the excessive ROS level induced deleterious effect on sperm characteristics.[3]

Seminal fluid contains the highest concentration of Zn compared to other organelles and body fluids in mammalians.[4] It has been reported that Zn levels arrange the antioxidant capacity of seminal fluid, which could influence sperm morphology.[5] Also, its positive effect was reported on semen concentration and motility[6] and head

***Correspondence:**

Amir Khaki. DVM, DVSc
Department of Clinical Sciences, Faculty of Veterinary Medicine, Amol University of Special Modern Technologies, Amol, Iran.
E-mail: a.khaki@ausmt.ac.ir

to the mid-piece attachment of spermatozoa.[7] Another essential trace mineral is copper (Cu). The Cu deficiency leads to a reduction in libido and semen quality and testicular damage and male animals' sterility.[8] On the other hand, Cu accumulation in the testis induces detrimental effects on spermatogenesis and male sex hormone depletion.[9] Positive correlation is reported between Cu seminal plasma concentration with sperm motility and viability[10-12] However, in contrast, excessive level of Cu concentration has a detrimental effect on sperm parameters.[13] Positive correlation between bull sperm motility and Fe concentration reported in previous studies indicated the importance of this micro-mineral in bull fertility.[10,14] Detrimental effect of Fe deficiency on spermatogenesis might be due to its indirect effect because of anemia, especially in combination with Cu deficiency in which sub-fertility and infertility are considered the main subsequences.[15]

Recent researches suggested Catalase (CAT), Superoxide dismutase (SOD), and Glutathione peroxidase (GPx) were the essential markers for semen quality assessment in bull and buffalo, and human.[3,16,17] CAT preservative potential against oxidation-reduction in semen seems to be more effective than SOD.[18] It has been suggested that high seminal SOD and GPx concentrations might have cryoprotective potential on buffalo frozen-thawed semen.[19]

Investigation on repetitive ejaculations alterations could be of interest for researchers, mainly based on their biological components and semen quality parameters. First and second ejaculates have been compared to elucidate their semen characteristics differences and correlations with frozen-thawed semen quality.[20]

The present study was conducted to determine differences of seminal plasma Zn, Cu, and Fe concentrations as well as CAT, SOD, and GPx activity in the first and second ejaculations of dual-purpose Simmental (*Fleckvieh*) bulls and evaluation of their relationship with semen quality in fresh and frozen-thawed stages.

Materials and Methods

Animals. This study was conducted on eight healthy service breeding *Fleckvieh* bulls (age range, 2-6 years), from December 2016 to April 2017. The samples were collected during routine weekly semen collection (total number of 121 semen sample) at the Iran Simmental Cattle Breeding Center (height above sea level: 47m, 36° 28′ 11″ N, 52° 21′ 3″ E) between 8:00 and 12:00 AM. Mean humidity and temperature of the study period were 57.42 ± 3.62 (Maximum 60 and Minimum 50) and 9.37 ± 3.73 (Maximum 17 and Minimum 5), respectively. The animals were fed three times per day based on following *Fleckvieh* bulls' daily diet formula: Silage 18 kg (8.50% Crude protein, 54.50% neutral detergent fiber (NDF), 32.70% acid detergent fiber (ADF), 1.80% FAT, 5.70% Ash, and 25.00% dry matter), concentrate 9.00 kg (14.68% Crude protein, 16.80% NDF, 13.10% ADF, calcium 0.74%, phosphorus 0.53%, sodium 0.49%, magnesium 0.29%, zinc 375 ppm, mn 381.44 ppm, cobalt 1.01 ppm, selenium 2.75 ppm plus mineral and vitamin supplements.

Semen collection, processing, and freezing. First and second ejaculates of semen were routinely collected by artificial vagina twice a week with 15-30 min intervals. The sexual preparation of bulls was performed by 10 min standing in the collection area after three false mounts. The artificial vagina was pre-warmed in an oven (Memmert, Schwabach, Germany) at 46.00 ˚C before usage. After collection, semen volume, and concentration was measured using a sterile graduated glass vial and SDM photometer (Minitube, Tiefenbach, Germany) calibrated for bull sperm cell counting, respectively. Two mL of each ejaculation was separated and centrifuged 10 min at 3000 rpm. Subsequently, recovered seminal plasma was re-centrifuged to diminish the remaining cells and kept at −80.00 ˚C for further investigation. For estimating fresh sperm motility, two small drops of diluted semen were put on a glass slide and analyzed using a binocular phase contrast microscope (Minitube) equipped with a warm stage at a magnification of 200×. Only the progressive motile spermatozoa were considered. One step dilation method (room temperature semen packaging) was performed for sperm freezing.

For semen freezing, one step dilution method (room temperature semen packaging) was performed as briefly described below:

- Preparation of Pre-extender dilution: Gently addition of the extender (Steridyl CSS; Minitube, Tiefenbach, Germany) to the semen (with a ratio of 1:1) and place in a water bath at 34.00 ˚C for 10 min.
- Calculation of final extender volume as following formula: Number of doses = (semen volume × semen concentration × progressive motile sperm × morphologically normal sperm) / (sperm per dose [15 ×10^6]).
- Preparation of final solution with adding the pre-extender to final calculated extender volume and then left it at room temperature (20.00 - 24.00 ˚C) for 15 min.
- Packaging the 0.50 mL straws (Minitube, Čeľadice, Slovakia) with MPP Uno automated filling and sealing machine (Minitube).
- Equilibration stage: Carrying out the packed straws at 4.00 ˚C for 3 hr in the refrigerator.
- Freezing stage: Putting the equilibrated straws at –120 ˚C for 10 min in MT freezer freezing device (Minitube).
- Storing the frozen semen in liquid nitrogen containers.

Sperm viability and morphology evaluation. Eosin-Nigrosin (Minitube) and Spermac (Minitube, Wellington, South Africa) staining were used for viability and morphological abnormality assessment by examining 200 sperm per sample under 400x magnification, respectively.

Computer-assisted sperm analysis (CASA). Frozen thawed sperm motility was analyzed by CASA system (Hooshmand Fanavar, Tehran, Iran) after thawing at 37.00 ˚C for 35 - 45 sec which following parameters were evaluated: Progressive motility (PM), curvilinear velocity (VCL), straight-line velocity (VSL), average path velocity (VAP), lateral head displacement (ALH), beat cross frequency (BCF), degrees of deviation (MAD) and linearity (LIN [VSL/VCL]). All analyses were performed by light microscopy equipped with a hot plate that maintained sample at 37.00 ˚C and chamber (sperm meter, depth 10 µm, surface graticule, 100 × 0.1 mm^2) to avoid sperm quality reduction during the assessment.

Measurements of micro minerals and antioxidants status. Zn, Cu, and Fe status in seminal plasma were measured by flame atomic absorption spectrometry (AA240FS; Varian Inc., Palo Alto, USA). The CAT, SOD, and GPx in seminal plasma were determined as instructed in their commercial kit (ZellBio GmbH, Ulm, Germany), and all preparations and detection steps were performed according to the manufacturer's direction.

Statistical analysis. All data were expressed as mean ± SEM. Correlation interaction was analyzed by Spearman's correlation coefficient (two-tailed) test. For better illustration of the interaction between motility and other semen parameters, progressive motility frozen-thawed (PMFT) was chosen as the main motility factor based on a recent study which indicated progressive motility as the main predictive factor for semen evaluation[21] and sample classified to following three groups: <40.00%, 40.00 - 50.00% and >50.00%. The differences between first and second ejaculations and between-subject effects of PMFT various groups compared to different ejaculates were determined by multivariate analysis of variance (MANOVA) test. Also, for determining the accuracy of analysis and predicting different errors in analysis between first and second ejaculations, all data were compared using student t-test and Mann-Whitney test for parametric and non-parametric data, respectively, which showed the same results.

Results

All values and abbreviations of two repetitive ejaculations were represented in Table 1.

Semen quality in first and second ejaculations. The analysis by MANOVA showed significant differences between first and second ejaculations in all parameters (Table 2). Moreover, the between-subject effect determined significant alterations in concentration, Fe, SOD, and GPx between first and second ejaculations. Furthermore, variations in VFT, MADFT, and Cu between first and second ejaculations (Table 3) were different.

Differences of three PMFT groups and different ejaculations. As mentioned before, PMFT was chosen for data classification. Our findings confirmed that there were significant differences in the three PMFT groups. However, there was no significant difference when two repetitive ejaculates were compared in three PMFT groups (Table 2). The between-subject effect in PMFT x Ejaculate analysis showed a significant difference in volume (Vol), total motility before freezing (TMBF), progressive motility before freezing (PMBF), viability before freezing (VBF), viability of frozen-thawed semen (VFT), CAT and SOD. Moreover, tend to be significant was observed in Zn (Table 3).

Zinc, iron, and copper concentration in seminal plasma. Based on our results, trace element concentrations in first and second ejaculations were detected in the following descending order: Cu < Fe < Zn. Only Fe concentrations were different between the two ejaculations. Alterations of Cu and Zn were not meaningful between two repetitive ejaculations and different PMFT groups (Table 3).

Table 1. Sperm quality characteristics and seminal plasma concentrations of trace elements and antioxidant enzymes in first and second ejaculations of dual-purpose Simmental (*Fleckvieh*) bulls (n = 121). Data are presented as the mean ± standard error.

Parameters	First ejaculate	Second ejaculate
Vol (mL)	8.14 ± 3.00	7.55 ± 2.58
Conc. (×10^6 mL^{-1})	1453.05 ± 278.05	1056.85 ± 297.93
TMBF (%)	78.30 ± 4.01	78.15 ± 3.86
PMBF (%)	73.00 ± 4.80	73.18 ± 3.87
AMBF (%)	8.06 ± 2.33	8.63 ± 2.20
VBF (%)	85.76 ± 2.87	85.17 ± 4.38
PMFT (%)	45.81 ± 8.43	45.36 ± 8.20
VAPFT (µm sec^{-1})	41.91 ± 8.63	41.87 ± 9.36
VCLFT (µm sec^{-1})	59.80 ± 13.10	59.17 ± 14.05
VSLFT (µm sec^{-1})	37.50 ± 8.45	36.70 ± 8.65
LINFT (%)	47.15 ± 8.02	46.70 ± 8.36
ALHFT (µm)	2.50 ± 0.42	2.49 ± 0.56
BCFFT (Hz)	0.92 ± 0.18	0.88± 0.24
MADFT	27.07 ± 4.88	24.94 ± 7.29
AMFT (%)	10.11 ± 2.91	11.13 ± 2.71
VFT (%)	63.54 ± 13.21	66.94 ± 10.30
Zn (mg dL^{-1})	125.46 ± 30.38	123.66 ± 28.90
Cu (mg dL^{-1})	2.07 ± 0.47	2.24 ± 0.56
Fe (mg dL^{-1})	22.42 ± 7.02	28.53 ± 7.57
CAT (mmol L^{-1})	17.34 ± 8.62	16.62 ± 8.15
SOD (mmol L^{-1})	11.16 ± 1.55	11.80 ± 1.22
GPx (mmol L^{-1})	1.20 ± 0.60	0.91 ± 0.50

Vol: Ejaculate volume, Conc.: Concentration, TMBF: Total motility before freezing, PMBF: Progressive motility before freezing, AMBF: Abnormal morphology before freezing, VBF: Viability before freezing, PMFT: Progressive motility frozen-thawed, VAPFT: Average path velocity frozen-thawed, VCLFT: Curvilinear velocity frozen-thawed, VSLFT: Straight-line velocity frozen-thawed, LINFT: Linearity, ALHFT: Lateral head displacement frozen-thawed, BCFFT: Beat cross frequency frozen-thawed, MADFT: Degrees of deviation frozen-thawed, AMFT: Abnormal morphology frozen-thawed, VFT: Viability frozen-thawed, Zn: Zinc, Cu: Copper, Fe: Iron, CAT: Catalase, SOD: Superoxide dismutase, and GPx: Glutathione peroxidase.

Table 2. Significant multivariate effects (at $p < 0.05$ level).

Group	Pillai's trace*	F	df	*p*-value	Eta2	Observed power†
Ejaculate	0.59	6.59	22.00	0.00	0.59	1.00
PMFT ratio	0.75	4.47	28.00	0.00	0.37	1.00
Ejaculate	0.41	5.23	14.00	0.00	0.41	1.00
PMFT × Ejaculate	0.28	1.22	28.00	0.21	0.14	0.92

* Pillai's trace is a test statistic produced by a MANOVA; df: degree of freedom; †Computed using alpha = 0.05.

Antioxidant enzymes status in seminal plasma. The meaningful difference in SOD and GPx was detected in first and second ejaculations and PMFT groups (Table 3). On the other hand, CAT and SOD were different in three PMFT groups, which could determine the differences between repetitive ejaculations based on enzymatic antioxidant status.

Correlations between data. The results of the correlation between all parameters in the present study are mentioned in Table 4. Data analysis showed that all CASA parameters exhibited a positive correlation together and also with TMBF, PMBF, VBF, and VFT. A positive and significant correlation was detected between CAT and CASA parameters except ALH, TMBF, and PMBF. A negative correlation was detected between Vol with some of CASA parameters (VAP, VSL, and LIN) and VFT. Although a negative correlation was observed between SOD and concentration, a correlation between this enzyme with VFT and Cu status was positive. Also, a significant negative correlation between Zn with PMBF, TMBF and between Fe with concentration was detected.

Table 3. Within-Subjects effects in the different ejaculate and PMFT ratio.

Variables		df	F	*p*-value
Ejaculate				
	Conc ($\times 10^6$ mL^{-1})	1	57.21	0.00
	VFT (%)	1	2.49	0.11
	MADFT	1	3.59	0.06
	Cu (mg dL^{-1})	1	3.19	0.07
	Fe (mg dL^{-1})	1	21.23	0.00
	SOD (mmol L^{-1})	1	6.25	0.01
	GPx (mmol L^{-1})	1	7.72	0.00
PMFT				
	Vol (mL)	2	3.06	0.05
	TMBF (%)	2	23.41	0.00
	PMBF (%)	2	25.67	0.00
	VBF (%)	2	6.73	0.00
	VFT (%)	2	53.42	0.00
	Zn (mg dL^{-1})	2	1.96	0.14
	CAT (mmol L^{-1})	2	3.20	0.04
	SOD (mmol L^{-1})	2	3.23	0.04

Vol: Ejaculate volume, Conc: Concentration, TMBF: Total motility before freezing, PMBF: Progressive motility before freezing, AMBF: Abnormal morphology before freezing, VBF: Viability before freezing, PMFT: Progressive motility frozen-thawed, VAPFT: Average path velocity frozen-thawed, VCLFT: Curvilinear velocity frozen-thawed, VSLFT: Straight-line velocity frozen-thawed, LINFT: Linearity, ALHFT: Lateral head displacement frozen-thawed, BCFFT: Beat cross-frequency frozen-thawed, MADFT: Degrees of deviation frozen-thawed, AMFT: abnormal morphology frozen-thawed, VFT: viability frozen-thawed, Zn: Zinc, Cu: Copper, Fe: Iron, CAT: Catalase, SOD: Superoxide dismutase, and GPx: Glutathione peroxidase.

Discussion

This study represented considerable data on semen quality parameters of *Fleckvieh* bulls both in fresh and frozen-thawed conditions, associations between sperm characteristics with seminal plasma trace element concentrations, and antioxidant enzymes status in the first and second ejaculations.

Among all micro-minerals, it seems that Zn has a special place in male reproductive physiology. However, association between seminal plasma zinc concentrations and semen quality is still controversial. Some studies reported that there is no correlation between seminal zinc values with sperm motility in human[22] and just had a borderline correlation with sperm density.[7] Significant correlations between semen volume with sperm concentration and seminal zinc were reported in stallion.[23] However, Barrier-Battut *et al*. reported that the variability between 17 different stallions ejaculates concerning post thaw sperm motility could not be related to levels of Ca, Mg, Co as well as Zn.[24] On the other hand, Usuga *et al*. found negative correlation between zinc concentration with sperm viability and plasma membrane integrity in stallions.[25] In contrast, several studies reported that there were positive correlations between seminal zinc concentration with both total[16,26] and progressive[16,21,27] motility of sperm in bulls (cattle and buffalo) and human[28] which are in agreement with present study which showed meaningful association between seminal plasma zinc with sperm motility in fresh semen and BCF as well as VAP in frozen thawed one. Nevertheless, Sørensen *et al*. reported that high zinc concentration in seminal plasma has negative relationship with progressive but not total motility of healthy human spermatozoa.[29] We found no significant differences between seminal plasma Zn concentration in the first and second ejaculation, however, tendency to be different was detected in three PMFT groups that could be in agreement with a study that reported that seminal plasma Zn concentrations in forth ejaculation of Holstein bulls were quite lower than first one.[30]

Evaluation of seminal plasma Cu levels in the first and second ejaculations in *Fleckvieh* bulls showed no significant difference in our study, which is not reported before.

Table 4. Correlation coefficients of sperm quality characteristics in seminal plasma.

	Vol	Conc	TMBF	PMBF	AMBF	VBF	PMFT	VAP	VCL	VSL	LIN	ALH	BCF	MAD	AMFT	VFT	Zn	Cu	Fe	CAT	SOD	GPx
Vol	1	0.04	-0.16	**-0.18***	-0.01	-0.04	**-0.20***	**-0.19***	-0.14	**-0.20***	**-0.23***	-0.10	-0.14	-0.02	-0.02	**-0.23***	0.02	0.09	-0.13	-0.14	-0.05	0.14
Conc		1	-0.15	-0.16	-0.01	-0.12	-0.03	-0.05	-0.01	-0.03	0.00	-0.00	0.07	0.13	0.00	-0.12	0.02	-0.03	**-0.24****	-0.09	**-0.19***	0.10
TMBF			1	**0.98****	-0.04	**0.27****	**0.61****	**0.55****	**0.55****	**0.56****	**0.57****	**0.45****	**0.52****	**0.36****	0.03	**0.47****	**0.24****	-0.01	-0.09	**0.35****	0.04	0.07
PMBF				1	-0.03	**0.27****	**0.63****	**0.57****	**0.56****	**0.58****	**0.59****	**0.45****	**0.53****	**0.35****	0.06	**0.50****	**0.22***	0.00	-0.06	**0.35****	0.06	0.05
AMBF					1	-0.02	-0.03	-0.06	-0.04	-0.05	-0.05	-0.09	-0.12	-0.17	**0.72****	0.03	0.00	0.16	0.11	-0.05	0.12	-0.06
VBF						1	**0.35****	**0.27****	**0.28****	**0.30****	**0.27****	**0.22***	**0.30****	**0.23****	0.06	**0.38****	0.03	-0.12	0.07	0.17	0.15	0.10
PMFT							1	**0.94****	**0.90****	**0.95****	**0.91****	**0.77****	**0.88****	**0.63****	0.04	**0.80****	0.15	-0.04	-0.07	**0.27****	0.06	0.04
VAP								1	**0.94****	**0.96****	**0.85****	**0.85****	**0.88****	**0.71****	0.01	**0.77****	**0.18***	-0.01	-0.08	**0.19***	0.11	-0.00
VCL									1	**0.93****	**0.81****	**0.89****	**0.85****	**0.79****	0.05	**0.71****	0.17	-0.04	-0.08	**0.19***	0.07	0.03
VSL										1	**0.89****	**0.80****	**0.87****	**0.68****	0.01	**0.79****	0.16	-0.03	-0.08	**0.26****	0.10	0.01
LIN											1	**0.60****	**0.76****	**0.48****	0.01	**0.78****	0.11	-0.03	-0.10	**0.25****	0.06	0.15
ALH												1	**0.79****	**0.86****	0.00	**0.58****	0.16	-0.06	-0.07	0.10	0.01	-0.05
BCF													1	**0.72****	-0.04	**0.70****	**0.21***	-0.11	-0.14	**0.23***	-0.03	0.05
MAD														1	-0.12	**0.45****	0.12	-0.00	-0.09	0.03	0.07	0.01
AMFT															1	-0.01	0.00	0.14	0.13	0.02	0.01	-0.05
VFT																1	0.17	-0.02	0.06	0.12	**0.20***	0.03
Zn																	1	0.07	-0.16	0.11	0.01	-0.04
Cu																		1	0.07	-0.04	**0.19***	-0.11
Fe																			1	0.12	0.13	0.00
CAT																				1	-0.05	0.07
SOD																					1	-0.11
GPx																						1

Vol: Ejaculate volume, Conc.: Concentration, TMBF: Total motility before freezing, PMBF: Progressive motility before freezing, AMBF: Abnormal morphology before freezing, VBF: Viability before freezing, PMFT: Progressive motility frozen-thawed, VAPFT: Average path velocity frozen-thawed, VCLFT: Curvilinear velocity frozen-thawed, VSLFT: Straight-line velocity frozen-thawed, LINFT: Linearity, ALHFT: Lateral head displacement frozen-thawed, BCFFT: Beat cross-frequency frozen-thawed, MADFT: Degrees of deviation frozen-thawed, AMFT: Abnormal morphology frozen-thawed, VFT: Viability frozen-thawed, Zn: Zinc, Cu: Copper, Fe: Iron, CAT: Catalase, SOD: Superoxide dismutase, and GPx: Glutathione peroxidase. *$p \leq 0.05$ and **$p \leq 0.01$ based on Spearman correlation analysis.

After frequent men semen collection, in which the samples were taken in ten consecutive days, no correlations were found between Cu and all sperm parameters.[31] However, a positive correlation was detected between Cu level and monthly semen volume, concentration, progressive motility assessment in bulls.[32] Based on the current examination, seminal plasma Cu concentration did not show statistically significant differences in different PMFT and ejaculate groups; however, tend to be different was observed in two repetitive ejaculations (p = 0.77). No correlation was detected between Cu and all semen parameters consistent with the other reports in stallions,[23] rams[33,] and men[34]. However, Cu concentrations in human semen samples showed to be higher in normozoospermic men than asthenospermic patients.[35] On the other hand, significant correlations between sperm rapid progressive motility, normal sperm morphology, and sperm concentrations with seminal plasma Cu concentrations were observed in men.[36] Moreover, rooster semen concentration and sperm progressive motility was reported to be associated with seminal Cu levels.[37] Besides, Cu reduction in seminal plasma was suggested to reduce sperm motility in men.[35]

Positive correlations between fresh semen seminal plasma Fe concentrations with sperm motility and viability were detected in the horse, buffalo, and bull.[16,23,38] However, our results did not demonstrate a significant correlation between seminal plasma Fe with sperm viability and motility in fresh and frozen-thawed semen. On the other hand, a negative correlation between seminal Fe and normal morphology was found in bull, ram, fox, and human spermatozoa[33,39,40] as well as seminal Fe and sperm motility in men (fresh semen) and stallions (both fresh and frozen-thawed)[25,40] which did not support our results. However, no significant alterations and associations were observed between normal fertile and sub-fertile men on seminal plasma Fe and Zn levels.[41] In Consistent with the present study, a negative correlation was detected between Fe and semen concentration in ram.[33] To the best of our knowledge, no study determined Fe status in two or more repetitive ejaculations in bulls. The present study revealed significantly higher seminal plasma Fe concentrations in the first compared to second ejaculations.

Our results demonstrated that the CAT level was not changed between first and second ejaculations; however, it differed in three PMFT groups. Apart from fresh sperm total and progressive motility, the PMFT, VAP, VCL, VSL, LIN, and BCF showed a positive correlation with CAT status, which was consistent with pre and post-thawing CAT evaluations in buffalo bulls[17] and human fertile men semen assessment.[42] Also, association with sperm viability detected with CAT was not observed based on our data.[17]

The correlation between seminal SOD levels and semen quality is still at issue, and various studies reported positive, negative, and none significant associations.[43,44] In the current study, not only the seminal plasma SOD concentrations were significantly higher in the second ejaculation than the first one, but also different in various PMFT groups. On the other hand, seminal Cu concentration

was associated with VFT, which was in agreement with Eghbali *et al.* study in buffalo bulls.[45] In comparison between oligozoospermia and normozoospermia in human, higher seminal plasma SOD concentration and activity were observed in sub-fertile samples, consistent with our findings and the other results which illustrated semen with lower sperm concentration and motility had higher SOD concentrations.[46] It has been suggested that Cu level change directly influences SOD status in accurate SOD function[15], which was supported in the present current study. However, the correlation between SOD activity with human seminal plasma Zn and Fe concentrations and sperm concentration was detected in normozoospermic men[40], which was not supported by our survey. According to the results observed in our investigation, GPx concentration was different between first and second ejaculations, not associated with any semen parameters. This result was in agreement with Neagu *et al*. on dog fresh and frozen-thawed semen evaluation. Nevertheless, they showed that post-thaw semen velocity and linear motility were correlated with seminal GPx and SOD levels, respectively.[47] Furthermore, no correlation was detected between bull and human sperm motility and GPx status, which was in agreement with our data.[48,49] Baumber and Ball suggested that despite high seminal SOD and GPx concentrations, low antioxidative activity was detected in stallion seminal plasma by these two enzymes, which was in agreement with our results.[50]

In conclusion, the first and second ejaculations are different according to fresh and frozen-thawed semen quality parameters, although no significant difference was detected comparing PMFT regarding ejaculate groups analysis. Based on different PMFT group analyses, the current study results suggested that frozen-thawed sperm progressive motility could be a good indicator for categorizing the statistical groups to understand better the data analysis of the semen quality parameters differences. Two the best of our knowledge, this is the first study that evaluated two repetitive ejaculation trace elements and antioxidative enzymes status differences and their associations with semen quality parameters in dual-purpose Simmental (*Fleckvieh*) bulls. Our findings showed some differences between the first and second ejaculations in some of the sperm quality parameters, and PMFT, the most important indicator for estimating fertility of a bull, was not different between first and second ejaculations. It could be economically remarkable for commercial centers which produce bull frozen semen to collect both the first and second ejaculations from a sire on the day of semen collection to increase the center efficiency.

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Conflicts of interest

The authors have any conflicts of interest to declare.

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