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Differences between first and second ejaculations regarding seminal plasma calcium, magnesium and total antioxidant capacity in dual-purpose Fleckvieh bulls and their relationships with semen quality

Amir Khaki[1]*, 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

Artificial insemination is a well-established and widely used method for genetic improvement in cattle breeding industry. Recently, researchers have shown an increased interest in the cryoprotective effects of minerals and antioxidants on semen. Previous studies on calcium (Ca) and magnesium (Mg), two main macro-minerals, have mainly investigated their roles in mammalian spermatogenesis and fertility. In addition, the experimental data examining the semen content regarding these minerals and antioxidants from different animal species are rather controversial and there is no general agreement about their associations with semen quality. Therefore, this study was conducted to assess the seminal plasma concentrations of Ca, Mg and total antioxidant capacity (TAC) in first and second ejaculations of dual-purpose Fleckvieh bulls and to link them to the sperm characteristics of fresh and frozen-thawed semen. Sperm progressive motility after thawing was used to classify the data into three groups: < 40.00%, 40.00 to 50.00% and > 50.00%. The measurements of two minerals and TAC were carried out using spectrophotometry and enzyme-linked immunosorbent assays, respectively. The results showed that there were significant differences in several parameters of semen quality between first and second ejaculations. No significant differences were also found on Ca and Mg concentrations and Ca/Mg ratio. The TAC level was significantly higher in the first ejaculation than the second one. The findings of this study suggest that TAC is a potential marker for bull semen quality assessment in the frozen semen production industry.

Introduction

Semen banking (cryopreservation or semen freezing) is the process of collecting, storing and using semen for breeding, population control, therapeutic and scientific research purposes.[1] This method has been previously used for animal species such as bull, stallion, and ram. Of available parameters for semen quality assessment, some studies have suggested sperm motility and semen concentration as key factors for frozen-thawed samples.[2,3] Seminal plasma, the fluid medium in semen providing nutrients and protection for suspended sperm cells, has become the center of attention for studying the effect of its components on spermatozoa transportation from testis to female reproductive tract.[4,5] For example, the macro- and micro-minerals have been shown to affect sperm longevity and fertility suggesting their levels as indicators for sperm function and post-thawed viability.[6-8]

Calcium (Ca) is one of the main minerals in seminal plasma mainly associated with sperm capacitation.[9,10] In testis, Ca is involved in steroid hormone production.[11] A study on the cryoprotective potential of Ca testing different concentrations in fresh and frozen-thawed semen has indicated that its equilibrium is effective in cryodamage prevention.[9] Nevertheless, the relation between Ca levels and semen quality is controversial and previous findings have been inconsistent.

***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

Magnesium (Mg) is another important mineral in seminal plasma mainly secreted by seminal vesicles. It is known to affect sperm quality through maintaining osmotic balance essential for sperm function and fertility as well as cooperating with enzymes such as Ca^{2+}-dependent Mg^{2+}-ATPase.[12] The reproductive system could also be indirectly affected by Mg as a result of homeostatic balances between calcium, phosphorus and magnesium. This is particularly the case for Mg deficiency due to losing appetite.[13] The Mg level has been reported to be correlated with spermatozoa viability in ram [14] and it was found to be significantly higher in seminal plasma of a group of brown bear with high testosterone level.[15]

Oxidative stress is formed by reactive oxygen species over-production or antioxidant mechanisms exhaustion.[16] It contributes significantly to male infertility due to membrane damage of sperm affecting its motility and binding ability concomitant with mitochondrial DNA damage interfering ATP synthesis. Such high susceptibility is caused by poor endogenous antioxidants storage in low cytoplasm content of sperm. Therefore, seminal plasma protects gametes via its intracellular and antioxidant enzymes.[17,18] One of the reliable, inexpensive and easy to use marker for biological fluid oxidation status is the total antioxidant capacity (TAC). It was recommended to estimate total seminal fluid antioxidant level.[19]

In recent years, there has been growing interest in studying the relation between the concentrations of macro- and micro-minerals and oxidation-reduction. Moreover, some studies have investigated the association between these seminal plasma biochemical markers and semen quality in human, cattle, and buffalo.[19-21]

There are a few studies regarding the first and second ejaculations in bulls. However, it has been reported that first ejaculation in men is rich in sperm and contains epididymal and prostatic secretions such as acid phosphatase, citric acid, Mg and zinc, promoting the disulfide bridges formation, preventing premature chromatin decondensation and inhibiting endonuclease activity. In comparison, the second ejaculation exhibits a low sperm count and primarily contains secretions from the seminal vesicles.[22,23] Considering this, it can be inferred that sperm characteristics could be different in the first and second ejaculations which is the main hypothesis of this research.

To our knowledge, there is no report regarding the association between two frequent ejaculates in Fleckvieh bulls. Therefore, the aim of the present study was to measure the seminal plasma Ca, Mg and TAC in first and second ejaculations of dual-purpose Fleckvieh bulls and to link them to the sperm characteristics of fresh and frozen-thawed semen.

Materials and Methods

Animals. The semen collection was carried out over a three-month period from the middle of January to the middle of March of 2017. Eight healthy service bulls of the Fleckvieh breed aged between two to four years were used. The samples were taken during routine weekly semen collection at the Iran Simmental Cattle Breeding Center (height above sea level: 47 m, longitude: 52° 23′57.76″ E and latitude: 36° 30′ 18.55″ N) between 8 and 12 AM. Animals were fed three times a day with the following formula: Silage 18.00 kg, concentrate 9.00 kg, alfalfa 3.00 kg, straw, and water *ad libitum*, Ca 0.74%, P 0.53%, sodium (Na) 0.49%, Mg 0.29%, zinc (Zn) 375.00 ppm, manganese (Mn) 381.44 ppm, cobalt (Co) 1.01 ppm, selenium (Se) 2.75 ppm plus mineral and vitamin supplements.

Semen collection and processing. Sires' ejaculates were routinely collected by artificial vagina twice a week, both on the same day and a duplicate every time at 15-30 min intervals. The sexual preparation of bulls was performed by standing them in a collection area for 10 min after two false mounts. The artificial vagina was pre-warmed in the oven (Memmert, Schwabach, Germany) at 46.00 ˚C for 10 hr before usage. Each ejaculation was analyzed and frozen separately. Immediately after collection, semen volume was measured by a sterile graduated glass vial and then it was placed in a water bath at 34.00 ˚C. Sperm concentration of semen was determined using a SDM photometer (Minitube, Tiefenbach, Germany) calibrated for bull sperm cell counting.

To estimate 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. The sperms with abnormal motility such as non-progressive, spherical or twisted movement patterns were neglected. Viability of spermatozoa was estimated by observing 200 spermatozoa at a magnification of 400× using Eosin-Nigrosin staining technique (Minitube). To determinate sperm morphology, Spermac staining kit (Minitube, Onderstepoort, South Africa) was used. Abnormal heads (detached, decapitated and micro- and macrocephalic), mid-piece and tails as well as cytoplasmic droplets (proximal and distal) were considered as abnormal morphological characteristics (Figs. 1 and 2).

The seminal plasma was then separated by centrifugation of 2.00 mL of each ejaculate for 10 min at 3000 rpm followed by pipetting into 1.50 mL microtubes (Eppendorf, Hamburg, Germany). Subsequently, semen samples were re-centrifuged to eliminate the remaining cells. Right after careful microscopic examination for obtaining cell lysate-free seminal plasma, samples were frozen (– 80.00 ˚C).

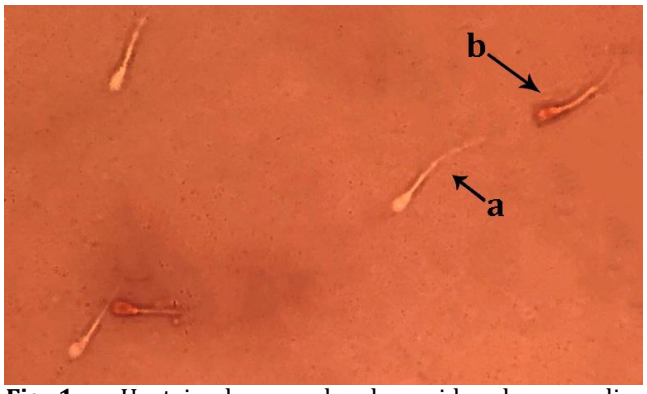

Fig. 1. a: Unstained sperm head considered as an alive spermatozoa; and b: Red-colored head regarded as a dead sperm, (Eosin-Nigrosin staining; 400×).

Semen freezing. Bulls' ejaculates were frozen according to the routine method of Iran Simmental Cattle Breeding Center, Amol, Iran, based on one step dilation method (semen packaging at room temperature). In short, immediately after semen collection and above-mentioned primary measurements, Steridyl CSS one-step extender (Minitube) was gently added (ratio of 1:1) to make pre-dilution solution and then it was kept in a water bath at 34.00 ˚C for 10 min. The following formula was used to calculate the number of semen doses and total extender volume:

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

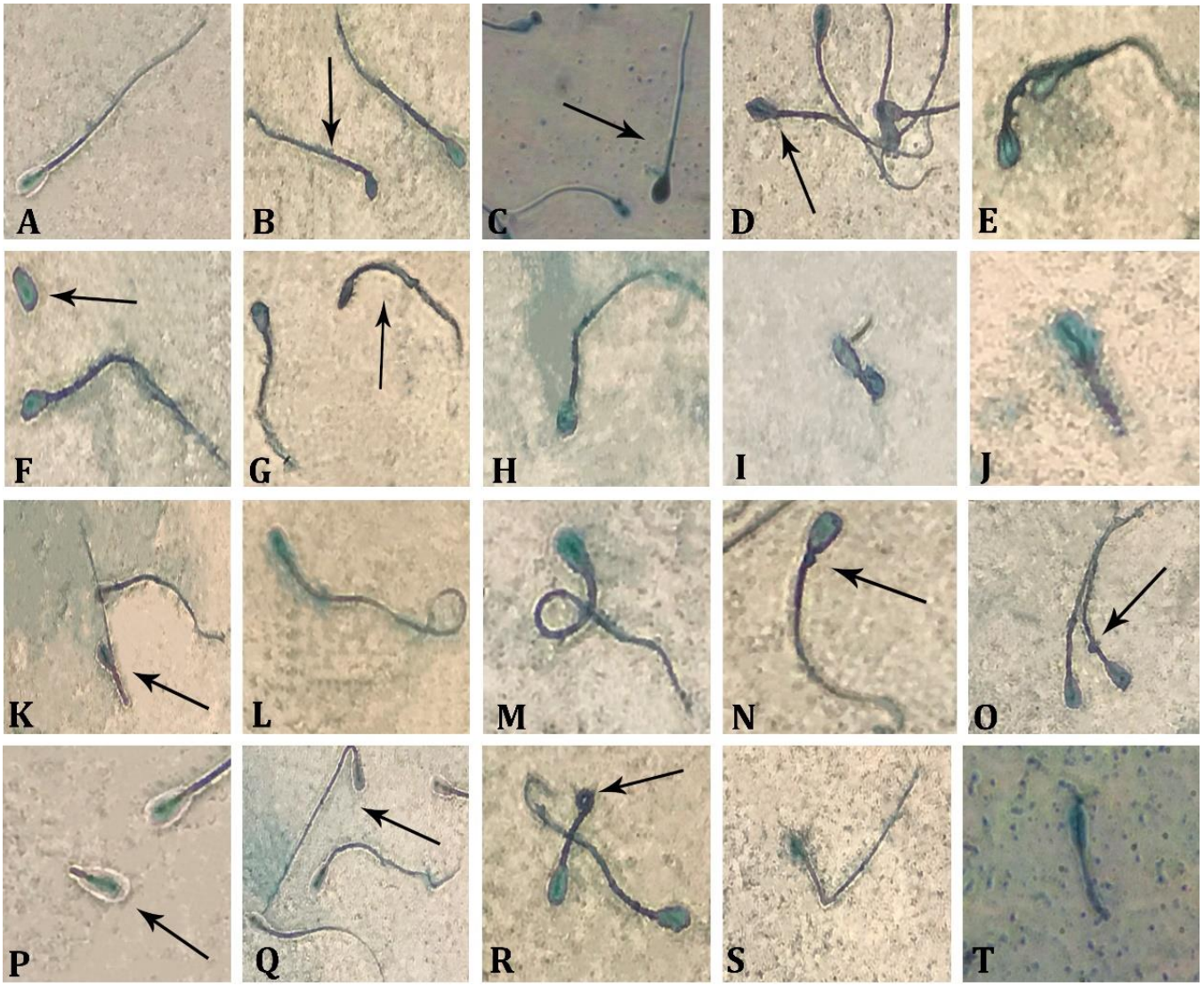

Fig. 2. Spermac® staining for detecting sperm abnormal morphology (400×). **A)** Normal spermatozoa; **B)** Microcephalic sperm; **C)** Macrocephalic sperm; **D)** Pyriform head; **E)** Double head; **F)** Detached heads; **G)** Elongated head; **H)** Broad head; **I)** Coiled-tail; **J)** Detached tail from mid-piece; **K)** Distal reflex; **L)** Terminally coiled tail; **M)** Shoe-hook tail; **N)** Proximal droplet; **O)** Distal droplet; **P)** Tail-stump defect; **Q)** Bowed mid-piece; **R)** Tail rotation until the mid-piece; **S)** Simple bent tail; and **T)** Dag defect (mid piece reflexes and coiled tail).

After 10 min, the pre-dilution solution was added to the final calculated extender. The mixture was then left at room temperature (20.00 - 24.00 ˚C) for 15 min. Finally, a MPP Uno automated filling and sealing apparatus (Minitube) was used to pack the diluted semen in 0.50 ml straws (Minitube). The freezing process was done by three-step procedure: Cooling in a refrigerator at 4 ˚C for 3 hr followed by freezing in a freezer at – 120 ˚C for 10 min and then placing in liquid nitrogen.

Computer-aided sperm analysis (CASA). For motility assessment of sperms after freezing, CASA system (Hooshmand Fanavar, Tehran, Iran) was used. The sperm progressive motility along with other motility-related parameters including 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]) were measured. Prior to analysis, the semen was frozen-thawed by putting the straws in a water bath at 37.00 ˚C for 40 sec.

Measurements of Ca, Mg and TAC. The concentrations of Ca and Mg in seminal plasma were detected by atomic absorption spectrophotometry (Varian Spectra AA-240FS, Palo Alto, USA). The TAC was measured with TAC commercial kit (ZellBio, Berlin, Germany) based on the manufacturer's instructions. Ascorbic acid action was regarded as a standard in the assay, which could detect TAC with 0.10 mM sensitivity (100 µmol L^{-1}). The absorbance of the sample was determined calorimetrically in wavelength of 490 nm.

Statistical analysis. Statistical analysis was performed using SPSS for Windows (version 24.0; SPSS Inc., Chicago, USA). Spearman's correlation coefficient (two-tailed) test was used to examine the inter-correlation between fertility parameters, different ejaculations and Ca/Mg ratio. Progressive motility of frozen-thawed (PMFT) semen was considered as the main marker for bull fertilization capacity as it was shown to be a good candidate for a range of variations.[2] Accordingly, all data classified into three groups: < 40.00%, 40.00 – 50.00% and > 50.00%. In the present study, Ca ratio to Mg (4.90 on average) was chosen as an independent factor and two groups were defined hence: > 4.90 and < 4.90.

Multivariate analysis of covariance (MANCOVA) was used for different ejaculates analyzing with all parameters except Lin, MAD, BCF and ALH as dependent variables, first and second ejaculates as a fixed factor and other factors as covariates. Mann-Whitney tests and Student *t*-test were performed for nan-parametric and parametric data comparison between two frequent ejaculations in which revealed results were similar to those reported here except in TAC status. Multivariate analysis of variance (MANOVA) was used to determine between-subject effects of first and second ejaculations compared to post thawing motility and different Ca/Mg ratios. Prior to executing statistical tests, data for volume, frozen-thawed semen viability (VFT), morphology before freezing, LIN and morphology after freezing were normalized using Johnson translation in Minitab (version 16.0, Minitab Ltd, Coventry, UK). A p value of ≤ 0.05 was considered significant. The total motility before freezing (TMBF) and progressive motility before freezing (PMBF) were excluded from MANOVA and MANCOVA evaluation because the visual based examination was performed in assessment procedure and data were not normalized by Johnson translation procedure.

Results

The data and abbreviations for all parameters in different ejaculations are summarized in Table1.One way MANCOVA test revealed significant differences between two frequent ejaculates (Pillai's T = 0.78, F = 5.52 and p = 0.00) and LIN (Pillai's T = 0. 93, F = 22.75 and p = 0.00), ALH (Pillai's T = 0.76, F = 4.86 and p = 0.00), MAD (Pillai's T = 0.69, F = 3.58 and p = 0.00) and BCF (Wilk's L = 0.88, F = 11.42 and p = 0.00) main effect on first and second ejaculations.

Table 1. Distribution of sperm quality characteristics in the first and second ejaculations of dual-purpose Fleckvieh bulls (n = 42). Data are presented as the mean ± standard error.

Parameters	First ejaculate	Second ejaculate
Ejaculate volume (mL)	8.16 ± 3.35	6.93 ± 2.15
Concentration (×10^6 mL^{-1})	1438.25 ± 270.32	957.75 ± 259.87
TMBF (%)	79.20 ± 5.05	79.52 ± 4.35
PMBF (%)	73.20 ± 6.93	74.52 ± 4.35
AMBF (%)	7.54 ± 3.94	8.00 ± 3.06
VBF (%)	85.16 ± 3.27	85.44 ± 5.34
PMFT (%)	48.59 ± 11.38	46.09 ± 11.71
VAPFT (µm sec^{-1})	44.78 ± 10.31	42.86 ± 10.85
VCLFT (µm sec^{-1})	62.97 ± 13.59	61.57 ± 16.44
VSLFT (µm sec^{-1})	39.19 ± 9.78	36.86 ± 9.86
LIN (%)	49.50 ± 8.40	48.11 ± 8.67
ALHFT (µm)	2.61 ± 0.52	2.67 ± 0.74
BCFFT (Hz)	1.03 ± 0.26	0.93 ± 0.29
MADFT	27.20 ± 6.07	26.57 ± 9.51
AMFT (%)	8.84 ± 4.54	8.22 ± 3.77
VFT (%)	64.55 ± 13.85	68.56 ± 11.72
Calcium (mg dL^{-1})	20.81 ± 5.20	18.43 ± 5.45
Magnesium (mg dL^{-1})	4.22 ± 0.77	4.17 ± 0.71
TAC (mmol L^{-1})	1.22 ± 0.18	1.10 ± 0.13
Ca/Mg ratio	5.15 ±1.70	4.51 ±1.39

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, LIN: 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, and TAC: Total antioxidant capacity.

Also, Student *t*–test and Mann Whitney were conducted to clarify exact differences between groups, which only TAC significant level (p = 0.52 versus 0.97) was different compared to MANCOVA evaluation. Based on MANCOVA analysis, significant difference was only observed in semen concentration between two repetitive ejaculations (df: 1, F: 19.17 and p: 0.00).

The semen quality differed in ejaculate to PMFT and ejaculate to Ca/Mg ratio (Table 2) indicating different factors influence sperm quality after thawing and different ejaculates might solely have a direct effect on sperm motility after cryopreservation process. Significant differences were observed in viability before freezing (VBF; df: 2, F: 2.07 and p: 0.14), volume (df: 2, F: 5.03 and p: 0.01) and TAC (df: 2, F: 7.23 and p: 0.002) in different PMFT groups.

The MANOVA comparison between two frequent ejaculations and Ca/Mg ratio revealed non-significant differences in Ca/Mg ratio analyzing. However, a meaningful difference was shown in ejaculate × Ca/Mg ratio analysis (Table 2) and between-subject effect parameters including concentration (df: 1, F: 7.35 and p: 0.01), abnormal morphology before freezing (AMBF; df: 1, F: 7.09 and p: 0.01), straight-line velocity of frozen-thawed semen (df: 1, F: 4.14 and p: 0.04), beat cross frequency of frozen-thawed semen (df: 1, F: 5.60 and p: 0.02), linearity of frozen-thawed semen (df: 1, F: 4.22 and p: 0.04) and TAC (df: 1, F: 8.64 and p: 0.00) which might indicate Ca/Mg ratio as a factor influencing first and second ejaculates quality.

The correlation coefficients evaluated between all data are presented in Table 3. A negative correlation was observed between volume and TMBF, PMBF, TAC and all CASA parameters excluding MAD which might indicate the negative effect of semen volume on sperm quality. Furthermore, a positive correlation was detected between volume and seminal plasma Mg content.

All sperm parameters after thawing excluding MAD had a significant and positive correlation with TMBF and PMBF as well as TAC. Also, a strong positive correlation was detected between CASA parameters after thawing.

Sperm concentration with AMBF and VBF as well as abnormal morphology of frozen-thawed (AMFT) semen and VBF with sperm characteristics did not have a meaningful correlation. Whereas, correlations with other semen parameters were observed. This might be related to the high semen quality of the bulls considered for semen production in our investigation.

The Ca/Mg ratio did not correlate with any sperm quality characteristics (p > 0.05), but Ca and Mg were correlated with volume and VFT, respectively.

Discussion

Development in methods of semen freezing procedure has caused sperm number reduction used for artificial insemination with acceptable conception rate and has increased the importance of each ejaculate characteristics. Moreover, semen quality parameters are different even at repetitive ejaculates from individual sire and investigation into diverse animal species has declared that there are differences in repetitive ejaculates.[20,24,25]

Sperm quality reduction was detected by increase collection frequency in boar and human.[25,26] In human, first ejaculation presents a lower volume, higher sperm concentration, higher motility rates, and lower sperm DNA fragmentation.[27] Although the semen concentrations of first to third jets of jackasses' sperm-rich fractions were decreased, no differences in vigor, semen volume, and sperm motility were observed.

On the other hand, the best sperm morphologies of this species were belonged to the first ejaculation[28] which is in contrast with the report about tomcats showing that first ejaculates contain significantly higher proportions of sperm morphological abnormalities as well as lower sperm count and motility.[29] But, it was believed that this differences may be due to the aging of spermatozoa in the epididymis of the tomcats.[29]

Previously, first and second ejaculates differences and chemical profile in different cattle breeds have been examined.[24,30,31] Seminal osmolarity, pH and amino acid constitute of Holstein bulls' repetitive ejaculates have been examined and reduction of these parameters in seminal plasma from first to the fourth ejaculate was found.[32]

Pickett and Komarek have detected the higher amount of lipid content and dry mater in the first ejaculation, but the higher content of dry matter and lipid was measured in second ejaculate spermatozoa.[33] Sperms in the first ejaculate have been shown to exhibit similar[34] or higher motility[30,35] compared to those in the second ejaculate.

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

Group	Pillai's trace	F	df	*p*	Eta2	Observed power*
PMFT ratio	0.93	2.19	20.00	0.01	0.46	0.96
Ejaculate	0.72	6.19	10.00	0.00	0.72	0.99
PMFT × ejaculate	0.88	1.99	20.00	0.02	0.44	0.94
Ca/Mg ratio	0.53	1.63	15.00	0.14	0.53	0.67
Ejaculate	0.81	6.26	15.00	0.00	0.81	1.00
Ejaculate × Ca/Mg ratio	0.61	2.21	15.00	0.04	0.61	0.83

* Computed using alpha = 0.05

PMFT: Progressive motility of frozen-thawed.

Table 3. Correlation coefficients of sperm quality characteristics in seminal plasma.

	Vol	Conc	TMBF	PMBF	AMBF	BF	PMFT	VAP	VCL	VSL	LIN	ALH	BCF	MAD	AMFT	VFT	Ca	Mg	TAC	Ca/Mg ratio
Vol	1	0.11	-0.44**	-0.51**	0.01	-0.17	-0.47**	-0.47**	-0.42**	-0.47**	-0.48**	-0.38*	-0.38*	-0.21	-0.04	-0.39*	-0.03	0.32*	-0.36*	-0.18
Conc		1	-0.08	-0.17	0.32*	-0.08	0.08	0.07	0.11	0.07	0.03	0.10	0.15	0.20	0.19	-0.14	0.04	0.00	0.25	0.06
TMBF			1	0.84**	-0.16	0.35*	0.84**	0.79**	0.69**	0.80**	0.80**	0.50**	0.77**	0.45**	-0.17	0.63**	-0.16	-0.21	0.25	-0.03
PMBF				1	-0.00	0.28	0.80**	0.74**	0.62	0.76**	0.79*	0.44**	0.67**	0.33*	-0.05	0.65**	-0.18	-0.09	0.16	-0.12
AMBF					1	-0.12	-0.03	-0.09	-0.07	-0.10	-0.09	-0.00	-0.12	-0.04	0.34*	-0.08	0.16	0.17	-0.32	0.05
BF						1	0.34*	0.23	0.17	0.23	0.36*	0.15	0.23	0.14	-0.08	0.31*	-0.25	-0.02	0.02	-0.16
PMFT							1	0.95**	0.86**	0.96	0.95**	0.66**	0.94**	0.62**	-0.02	0.60**	-0.13	-0.08	0.41**	-0.07
VAP								1	0.94**	0.99**	0.89**	0.78**	0.97**	0.73**	0.02	0.53**	-0.07	-0.04	0.46**	-0.05
VCL									1	0.90**	0.73**	0.93**	0.91**	0.90**	0.07	0.40**	-0.03	-0.07	0.48**	-0.00
VSL										1	0.92**	0.70**	0.96**	0.66**	0.01	0.56	-0.08	-0.03	0.45**	-0.06
LIN											1	0.49**	0.86**	0.42**	-0.08	0.66**	-0.23	-0.05	0.46**	-0.15
ALH												1	0.73**	0.94**	0.12	0.24	0.01	-0.09	0.40**	0.04
BCF													1	0.76**	-0.01	0.46**	-0.07	-0.02	0.48**	-0.05
MAD														1	0.07	0.14	0.00	-0.04	0.41**	0.01
AMFT															1	-0.26	0.19	0.19	-0.05	0.03
VFT																1	-0.32*	-0.02	0.25	-0.26
Ca																	1	-0.04	-0.11	0.82**
Mg																		1	-0.02	-0.58**
TAC																			1	-0.18
Ca/Mg ratio																				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, LIN: 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, Ca: Calcium, Mg: Magnesium,TAC: Total antioxidant capacity.

However, the second ejaculate showed higher fertility than the first one when used for artificial insemination without freezing.[35] It has been reported that no significant differences are observed in VSL, VCL or VAP between the first and second ejaculates in Japanese black bulls' fresh semen. However, the first ejaculates had higher ALH and lower BCF than the second ones. In visual inspections, no differences were detected in sperm motility between the first and second ejaculates in all bulls or between bulls.[24] In the present study, significant differences were detected between first and second ejaculations which is in consistent with Kanno and co-workers report.[24] Moreover, same result was reported in rams except in ejaculation volume alteration.[36] Furthermore, PMFT groups showed significant differences between two frequent ejaculations in our study which seems to be an original result and there is no report in this field.

Seminal plasma Mg content in the present study was detected as 4.22 ± 0.77 mg dL^{-1} and 4.17 ± 0.71 mg dL^{-1} in first and second ejaculations respectively, which there was no significant difference between them. A positive correlation was detected between semen volume and Mg content which same correlation was reported in Nili-Ravi buffalo bull semen.[37] Besides, buffalo bulls were reported to have correlation between Mg concentration and sperm characteristics which could be a possible reason for different previous studies.[21,37] Also, a correlation between stallion semen quality and Mg content wasn't observed in fresh and frozen-thawed semen quality assessment.[38,39] Tvrdá *et al*., have reported higher seminal plasma Mg in Fleckvieh bulls (7.65 ± 0.90 mg dL^{-1}) and significant positive correlation between visual basic detected motility and Mg content, not supporting our study. However, the difference between first and second ejaculations wasn't considered in their study.[7]

Seminal Ca concentration in different species such as ram (10.60 ± 1.02 mg per100 g), bull (44.10 ± 2.55 mg per 100 g), human (20.60 ± 1.65 mg per 100 g), dog (0.80 ± 0.260 mg per 100 g), rabbit (8.00 ± l.63 mg per 100 g), fowl (10.40+1.39 mg per 100 g), rooster (6.52 ± 0.40 mg dL^{-1}), diverse boar breeds (mean concentration of 0.994 mmol per L) and water buffalo (22.36 ± 0.52 mg dL^{-1}) was detected in previous studies.[21, 40-43]

In the current study, Ca concentrations of Fleckvieh bulls' seminal plasma were detected as 20.81 ± 5.20 mg dL^{-1} and 18.43 ± 5.45 mg dL^{-1} in the first and the second ejaculations, respectively. Interestingly, the controversial correlation was reported between Ca and semen quality in various animal species. It was reported that there is a positive correlation between fresh seminal plasma Ca concentration and sperm motility, concentration and volume in bull,[44,45] water buffalo, [21] stallion, [5,46] salmon fish.[47] Nevertheless, a negative correlation has also been reported between them.[8,48]

Also, a significant correlation was observed between poor sperm motility and low seminal plasma Ca level in the stallion.[49] Based on the current study, seminal plasma Ca concentration didn't show a significant difference between first and the second ejaculation. Although, tempt to be significant was observed in ejaculate × PMFT assessment. A negative correlation was detected between Ca concentration and VFT. In agreement with our results, positive correlation between impaired tail membrane

integrity and seminal plasma Ca concentration has been shown in boar semen assessment.[8]

Liang *et al*., have suggested the greater importance of the Ca/Mg ratio of seminal plasma compared to semen quality in repetitive ejaculates. They have illustrated that lower than 2.50 proportions of Ca/Mg ratio, Mg and total Ca concentration are associated with human sperm quality reduction.[50] Based on our investigation, a significant reduction wasn't detected in Ca and Mg contents in different analyses. However, the meaningful difference was observed in ejaculate × Ca/Mg ratio assessment demonstrating that Ca/Mg ratio might be one of the factors influencing sperm quality between first and second ejaculations.

In Holstein bulls, Ca and Mg contents were measured as 40.22 ± 1.35 and 6.32 ± 0.86 mg dL^{-1} respectively, and Ca was positively correlated with sperm concentration, but Mg was negatively correlated with concentration and seminal plasma volume.[44] Semen in different ram breeds was collected by electro-ejaculation and no significant correlation was reported between seminal plasma Ca and Mg and semen quality.[14] The Mg and Ca contents were also measured as 22.36 ± 0.52 mg dL^{-1} and 11.94 ± 0.36 mg dL^{-1} in water buffalo respectively, and significant differences were shown between high and other motility groups and Ca and Mg contents.[21] Moreover, a strong correlation was shown between sperm viability and Mg in Nili-Ravi buffalo seminal plasma.[37] Marwari stallions and Poitou jack's semen analyses have explored the correlation between visual-based sperm motility, pH, volume, concentration and hypo-osmotic swelling test percentage and seminal plasma Ca concentration. Talluri and co-workers have reported that there is no significant correlation between seminal plasma antioxidant status and Ca content.[5] Although, visual-based analysis of frozen-thawed semen has indicated no significant differences between Ca content and semen quality parameters and positive correlation between Ca and seminal volume in the stallion. Moreover, no correlation was observed between Mg and other sperm characteristics.[46]

Although Ca and Mg have critical roles in spermatogenesis and fertility, our findings demonstrated that measuring these elements in seminal plasma couldn't be markers for bull sperm quality assessment, supporting several former studies mentioned before.[5,14,46] However, Ca/Mg ratio might be a good indicator for semen quality assessment. Further studies are required to clarify the latter value and correlation with sperm parameters.

Recent studies have indicated a mutual relationship between the electrolyte and oxidative capacity of seminal plasma.[7] The strong relation between sperm quality characteristics and seminal plasma TAC was reported previously in buffalo,[23] so that, TAC had been chosen as a marker of ROS/TAC status in this study. Tvrdá *et al*., have examined the protective potential of seminal plasma against oxidative stress and the correlation between Ca and Mg and antioxidant capacity in which high correlation was reported between Ca and Mg and the antioxidant indicator and motility.[7] Holstein bulls eight consecutive ejaculates (four times in each day) were measured based on oxidative markers. The TAC as a key marker wasn't fluctuated in individual sires, but altered among different bulls.[20] According to our findings, TAC status in ejaculation and ejaculate × PMFT assessment tempt to be significant. Moreover, PMFT and ejaculate × Ca/Mg ratio analyses illustrated the significant effect of TAC status indicating that TAC could be suggested as the main marker for semen quality evaluation and post freezing quality prediction. Meaningful negative correlation was detected between seminal volume and abnormal sperm morphology and TAC. No correlation was detected between Ca, Mg and Ca/Mg ratio and TAC in seminal plasma. Eghbali *et al*., have reported that TAC could be a valuable marker for semen quality evaluation which our study confirmed its influence even at first and second ejaculations.[21]

In conclusion, our study represented complex interactions and associations between the two frequent ejaculates based on Ca and Mg contents, spermatozoa CASA assay and TAC status in the dual purpose Fleckvieh bulls' semen. Our results revealed that TAC could be suggested as the main indicator for semen quality examination in the frozen semen production industry. With the exception of seminal plasma Ca and Mg concentration correlation respectively with volume and VFT, no correlation was detected between other sperm quality parameters and these minerals. Due to the fact that Fleckvieh breed has recently attracted many breeders worldwide and on the other hand, there is no comprehensive information about biochemical properties of this breed semen, further studies are suggested for evaluation of micro-minerals and enzymes, especially in seminal plasma and spermatozoa and their correlation with semen quality and conception rate.

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Conflict of interest

The authors declare no conflict of interest was involved in this study.

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