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Calcium, Magnesium and Total Antioxidant Capacity (TAC) in Seminal Plasma of Water Buffalo *(Bubalus Bubalis)* Bulls and their Relationships with Semen Characteristics

Mahdi Eghbali[1]
Sayed Mortaza Alavi-Shoushtari[1*]
Siamak Asri-Rezaei[1]
Mohammad-Hassan Khadem Ansari [2]

[1]Department of Clinical Science, Faculty of Veterinary Medicine, Urmia University, Urmia, Iran
[2]Department of Clinical Biochemistry, School of Medicine, Urmia University of Medical Sciences, Urmia, Iran

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Abstract

In order to determine calcium (Ca), magnesium (Mg) content and total antioxidant capacity (TAC) of seminal plasma in buffalo and to study their associations with the semen characteristics, 54 semen samples were collected from 10 buffalo bulls; semen quality was evaluated, seminal plasma was then harvested by centrifugation and its Ca and Mg content were estimated and its TAC determined. The Ca and Mg content of the seminal plasma (Mean ± SEM) were recorded as 22.36 ± 0.52 mg dl^{-1} and 11.94 ± 0.36 mg dl^{-1} respectively, while, its mean TAC value was 1.50 ± 0.02 mmol L^{-1}. The mean Ca value was highly associated with sperm progressive motility, gross motility, viability (P = 0.000 for all), negatively with semen volume (P = 0.01), and with Mg and TAC values (P = 0.000 for both). The mean Mg values was highly associated with sperm progressive motility, gross motility and viability and seminal plasma Ca and TAC (P = 0.000 for all) and negatively associated with semen volume (P = 0.014). The mean TAC values was highly associated with sperm progressive motility, gross motility and viability and seminal plasma Ca and Mg (P = 0.000 for all). For further clarification of these associations, the data was categorized in three groups of excellent (Ex, >90% motile, n = 33), good (Go, 80-89% motile, n = 15) and moderate (Mo, <79% motile, n = 6) according to their percentage of sperm motility. The mean progressive motility in Ex group was 92.24 ± 0.51%, in Go group it was 81.66 ± 0.62 %, and in Mo group it was 71.66 ± 1.05 %. The mean Ca, Mg and TAC values were respectively recorded as 25.12 ± 0.29 mg dl^{-1}, 13.78 ± 0.20 mg dl^{-1}, and 1.57 ± 0.009 mmol L^{-1} in Ex, 18.74 ± 0.63 mg dl^{-1}, 9.14 ± 0.33mg dl^{-1}, and 1.42 ± 0.044 mmol L^{-1} in Go, and 17.34 ± 0.18 mg dl^{-1}, 8.06 ± 0.25 mg dl^{-1}, and 1.23± 0.05 mmol L^{-1} in Mo groups. The associations in groups are discussed. These results show that seminal plasma Ca and Mg content and TAC are associated with semen characteristics, and synergistically have an effect on motility and viability of the spermatozoa after ejaculation, which are important factors in semen fertility.

Keywords: Buffalo; Seminal plasma, Macro-elements, TAC

[*]**Corresponding author:**
Sayed Morteza Alavi-Shoushtari, DVM, DVSc
Department of Clinical Science, Faculty of Veterinary Medicine, Urmia University, Urmia, Iran.
E-mail address: m.alavi@mail.urmia.ac.ir

Introduction

Physiologically, calcium (Ca) is classified as either intracellular or extracellular. The skeleton is a major reservoir for providing Ca for both the extra- and intracellular pools. Intracellular Ca has a key role in many important physiological functions including hormone secretion, glycogen metabolism and cell division. Extracellular Ca provides Ca ion for the maintenance of intracellular Ca, bone mineralization, blood coagulation and plasma membrane potential. Calcium stabilizes the plasma membrane and influences its permeability and excitability.[1] A higher proportion of the non-skeletal Ca is present within cells than in extracellular fluids, but most of the intracellular Ca is bound to proteins in cell membrane, mitochondria and nucleus. The concentration of Ca ion in intracellular fluid is reduced considerably by this binding.[2] Calcium is a part of the second messenger system in many cell functions. In diacylglycerol inositol phosphate system induced by gonadotropins for instance, inositol phosphates are primarily involved in controlling calcium channels in the cell membrane and redirecting intracellular calcium, and allowing calcium to enter the cell.[3] Calcium is needed for stimulation of steriodogenesis in Leydig cells of the testis. The disruption of the mitochondria membrane by Ca^{2+} allows the protein kinase to simulate side-chain cleavage of cholesterol which is the first step in steroidogenesis.[4]

Magnesium (Mg) is the second most prevalent intracellular cation and is involved in the metabolic activity of the cell. Within the cell, most of the Mg is bound to proteins and negatively charged molecules, 80% of cytosolic Mg is bound to ATP, and MgATP is the substrate for numerous enzymes. The nucleus, mitochondria and endoplasmic reticulum contain significant amounts of Mg. Approximately 0.5% to 5.0% of the total cellular Mg is in a free form. Transport of Mg across the cell membrane is regulated by a specific Mg transport system.[1]

Intracellular Mg is involved in the activity of hormone receptor complex in the cell membrane. After the hormone binds to receptor, the affinity of G complex for Mg^{2+} increases, catalyzing the exchange of GTP for GDP on the Gα protein. The Gα-GTP complex is now the 'active' form, and the active Gα-GTP may actually dissociate from the Gβγ complex and enter the cytoplasm.[3]

Reactive oxygen species (ROS) play a role in male infertility, where excessive amounts impair spermatozoal motility. Epididymal antioxidant enzymes protect spermatozoa from oxidative damage in the epididymal lumen. Antioxidant secretions (superoxide dismutase, glutathione peroxidase, glutathione reductase and catalase) from the seminal vesicle into the seminal fluid protect spermatozoa after ejaculation.[5]

Hydrogen peroxide (H_2O_2) is a reactive oxygen species that at low concentration is toxic to sperm. Hydrogen peroxide (H_2O_2) inhibits not only sperm viability but also the acrosome reaction, sperm-egg binding, and oocyte penetration. Antioxidant enzymes activate the decomposition of H_2O_2 into water and oxygen, thus removing an initiator of free radical chain reactions leading to lipid peroxidation.[6,7]

Little information is available about Ca and Mg values as well as total antioxidant capacity of seminal plasma in buffaloes. This study was carried out to: (1) estimate the Ca, Mg contents and total antioxidant capacity of the seminal plasma in buffalo bulls, (2) test whether any association exists between these parameters and semen characteristics.

Materials and Methods

Animals. Fifty four semen samples were collected with a bovine artificial vagina from 10 sexually mature buffalo bulls (4 - 5 years old) kept in Iran's Northwest Buffalo Breeding Center, Urmia (37° 33 ´

N, 45° 4′ E) at weekly intervals (5 to 6 samples from each bull) during the summer 2007.

Semen evaluation. Immediately after collection, the ejaculate was placed in a 37 ˚C water bath and the volume was recorded. Semen motility was evaluated immediately after collection. Gross motility was scored from 0 to 5 on a wet mount of neat semen at ×100 magnification. The percentage of progressively motile spermatozoa was estimated by microscopic examination at × 400 magnification on a pre-warmed slide (37 ˚C), and a subjective assessment of the progressive statement was recorded according to the procedure of Ax *et al.*(2000).[8] Sperm concentration was measured using standard hemocytometer (Hausser Scientific, Horsham, PA. USA) methods, the percentage of viable spermatozoa was estimated by viewing 200 spermatozoa under ×1000 magnification using eosin-nigrosin staining method of Barth.[9] The semen samples were cooled to room temperature and transported to the laboratory within 2 hours.

Preparation of Seminal Plasma. Fresh semen was centrifuged (Clements 2000, England) at 5,000 rpm for 10 min., the supernatants were transferred into 1.5 ml tubes, re-centrifuged to eliminate the remaining cells and kept frozen (− 20 ˚C) until further analyses.

Determination of minerals and TAC contents. Seminal plasma was diluted (1:10) by double de-ionized water and the calcium and magnesium content was measured by atomic absorption spectrophotometry (Shimadzu Asc-6100, Japan). The TAC content of the seminal plasma was determined by using a kit (Antioxidant Capacity Assay Kit, Randox Chemical Co. Ann Arbor, MI, USA).

Data analysis. The obtained data was analyzed using SPSS software (Version 16.5 for Windows; SPSS Inc., Chicago, IL, USA) computer program. Results are quoted as arithmetic mean ± standard error of mean (S.E.M) and significance was attributed at $P < 0.05$. Pearson's correlation coefficient (two tailed) test was used to examine the association between all the parameters of the semen. The data were categorized in three groups of excellent (Ex), good (Go) and moderate (Mo) according to their motility rate. The comparison of the semen parameters, minerals and TAC contents of the seminal plasma in groups of samples was carried out by one-way ANOVA, variance homogeneity of samples was examined by Levene's test, Duncan's test was used for the multiple comparison and LSD values were calculated in all groups.

Results

The results of the semen evaluation as well as calcium, magnesium contents and TAC of seminal plasma of 54 samples are summarized in Table 1. The mean Ca and Mg values were recorded as 22.36 ± 0.52 mg dl^{-1} and 11.94 ± 0.36 mg dl^{-1} respectively, while for the TAC values it was 1.50 ± 0.02 mmol L^{-1}. The mean calcium and magnesium values were highly positively associated with sperm gross motility, progressive motility, viability, seminal plasma TAC ($P = 0.000$ for all); seminal plasma Ca content was highly positively associated with seminal plasma Mg content ($P = 0.000$) and was highly negatively associated with semen volume ($P = 0.01$). Seminal plasma Mg content was negatively associated with semen abnormal morphology ($P = 0.023$) and semen volume ($P = 0.014$).

In order to have a better insight and make the range of variations narrower, the data was categorized in three groups of excellent (Ex, > 90% motile, n = 33), good (Go, 80-89% motile, n = 15), and moderate (Mo, <79% motile, n = 6) quality according to their progressive motility rates.

Table1. Characteristics of the buffalo semen (Mean ± SEM) n = 54

Ejaculate volume (ml)	3.07 ± 0.17
Sperm concentration ($\times 10^6$ cells/ml)	1374.14 ± 61.22
Progressive motility (%)	87.02 ± 1.06
Gross motility (Score)	3.59 ± 0.16
Abnormal morphology (%)	6.53 ± 0.32
Viability (%)	89.68 ± 0.94
Seminal Plasma Calcium (mg dl^{-1})	22.36 ± 0.52
Seminal Plasma Magnesium (mg dl^{-1})	11.94 ± 0.36
Seminal Plasma TAC (mmol L^{-1})	1.50 ± 0.019

The mean values for progressive motility were recorded as 92.24 ± 0.51% in Ex, 81.66 ± 0.62 % in Go, and 71.66 ± 1.05 % in Mo groups, which were significantly different ($P < 0.05$ for all). The comparison of the data between three groups is presented in Table 2. The mean Ca value in Ex group (25.12 ± 0.29 mg dl^{-1}) was highly positively associated with sperm progressive motility (r = 0.501, P = 0.003), semen concentration (r = 0.450, P = 0.009) and positively correlated with sperm viability (r = 0.430, p = 0.012) and seminal plasma TAC values (r = 0.397, P = 0.022). The mean Ca value in Go group (18.51 ± 0.73 mg dl^{-1}) was highly positively correlated with seminal plasma Mg (r = 0.879, P = 0.000), but in Mo group (17.11 ± 0.53 mg dl^{-1}) it had a highly significant association with Mg values (r = 0.961, P = 0.002), and had an association with gross motility (r = 0.893, P = 0.017). The Mg values in Ex group (13.78 ± 0.21 mg dl^{-1}) was highly correlated with sperm motility (r = 0.571, P = 0.001), viability (r = 0.612, P = 0.000), and Ca (r = 0.690, P = 0.000), and was associated with sperm concentration (r = 0.398, P = 0.022), but in Go group (9.02 ± 0. 32 mg dl^{-1}), it was highly associated with Ca content (r = 0.879, P = 0.000) only, and in Mo group, Mg content (8.55 ± 0.25 mg dl^{-1}) was highly associated with Ca (r = 0.961, P = 0.002) and was associated with gross motility (r = 0.836, P = 0.038) and with TAC activity (r = 0.839, P = 0.037). Seminal plasma TAC activity in Ex group (1.5 ± 0.009 mmol L^{-1}) was highly associated with sperm motility (r = 0.829, P = 0.000) and viability (r = 0.656, P = 0.000) and associated with seminal plasma Ca values (r = 0.397, P = 0.022). In Go group (1.38 ± 0.041 mmol L^{-1}) it was highly associated with sperm viability (r = 0.802, P = 0.000), and in Mo group (1.29 ± 0.024 mmol L^{-1}) it was associated with seminal plasma Mg values (r = 0.839, P = 0.037).

Discussion

The total Ca content of the buffalo seminal plasma obtained in this study was recorded as 22.36 ± 0.52 mg dl^{-1} (Mean ± SEM) which was highly correlated with sperm gross motility, progressive motility and viability (P = 0.000 for all), volume (P = 0.01) and also with seminal plasma Mg values and TAC (P = 0.000). The mean Ca value was lower than the figure (32.42 ± 3.10 mg dl^{-1}) reported by Sansone (2000) for seminal plasma in buffalo bulls.[10] The mean calcium value in the bovine seminal plasma has been reported as 28 mg dl^{-1} [11] and 24.6 ± 7.1 mg dl^{-1}.[12] Abdel-Rahman *et al*. (2000) recorded a figure of 19.2 ± 1.3 mg dl^{-1} for Ca content of seminal plasma in the ram.[13]

Pesch *et al*. (2006) reported seminal plasma Ca content in the stallion as 2.9 mmol L^{-1} (~11.6 mg dl^{-1}) while Barrier-Battut *et al*. (2002) reported it as 5.75 ± 4.2 mg dl^{-1} in 'Selle Francais' stallions.[14,15]

Table 2. Comparison of the results of the different groups of samples (Mean ± SEM)

	Groups			Total (54)
	Excellent(n=33)	***Good(n=15)***	***Moderate(n=6)***	
Gross motility (Score)	4.03 ± 0.14 [a]	3.23 ± 0.33 [b]	2.4 ± 0.35 [c]	3.59 ± 0.16
Progressive motility (%)	92.24 ± 0.51[a]	81.66 ± 0.62 [b]	71.66 ± 1.05 [c]	89.68 ± 1.04
Viability (%)	94.00 ± 0.48 [a]	85.26 ± 0.95 [b]	77.00 ± 2.94 [c]	89.68 ± 0.94
Abnormal morphology (%)	6.06 ± 0.36 [a]	6.81 ± 0.62[a]	8.45 ± 1.17 [b]	6.53 ± 0.32
Concentration ($\times 10^6$ cells/ml)	1376.84 ± 65.10 [a]	1584.86 ± 125.66[a]	859.5± 150.78 [b]	1374.14 ± 61.22
Volume (ml)	2.76 ± 0.15 [a]	3.86 ± 0.47 [b]	2.83 ± 0.30 [a b]	3.07 ± 0.17
Seminal plasma Calcium (mg dl^{-1})	25.12 ± 0.29 [a]	18.74 ± 0.63 [b]	17.34 ± 0.18 [b]	22.36 ±0.52
Seminal plasma Magnesium (mg dl^{-1})	13.78 ± 0.20 [a]	9.14 ± 0.33 [b]	8.06 ± 0.25 [b]	11.94 ± 0.36
Seminal Plasma TAC(mmol L^{-1})	1. 57 ± 0.009 [a]	1.42 ± 0.044[b]	1.23 ± 0.052[c]	1.50 ± 0.019

Different superscripted letters (a, b and c) in rows denote a significant difference ($P < 0.05$).

In this study, the Mg content of the seminal plasma was recorded as 11.94 ± 0.36 which was highly positively associated with sperm gross and progressive motility and viability and TAC ($P = 0.000$ for all), as well as with Ca, and negatively with semen volume ($P = 0.014$) and sperm abnormal morphology ($P = 0.023$). Our figure for Mg content of seminal plasma samples was higher than 6.64 ± .039 mg dl^{-1} reported by Sansone *et al*. (2000) [10] for buffalo bulls; 9.8 ± 1.9 mg dl^{-1} in the bovine [12] and 8.6 ± 0.6 mg dl^{-1} in the ram[13]. Pesch *et al*. (2006) reported seminal plasma Mg values as being 3.1 mmol L^{-1} (~7.53 mg dl^{-1}) and Barrier-Battut *et al*. (2002) reported it as 3.63 ± 1.9 mg dl^{-1} in the stallion.[14,15] Seminal plasma TAC was recorded as 1.5 ± 0.019 mmol L^{-1} which was highly associated with sperm gross and progressive motility and viability as well as with Ca and Mg values ($P = 0.000$ for all).

Association between Ca and Mg content of seminal plasma and sperm motility was best depicted in the Ex group while in the Go and Mo groups Ca and Mg values, which were lower than that in the Ex group, had no associations with sperm progressive motility and viability, and in the Mo group showed an associations only with gross motility.

Tanaka *et al*. (1984) working with bovine seminal plasma, reported that Mg^{2+} is essential to mediate ADP-ribosylation and to inhibit endonuclease activity.[16] ADP-ribosylation may be involved in DNA repair in eukaryotic cells. This post-translational event may protect chromosomal and extrachromosomal DNA

from rapid random degradation which may occur following damage to this nucleic acid. Blocking of endonucleotic activity may be a vital process to maintain damaged cells viable during the period of DNA repair. By activation of endogenous Ca^{2+}, Mg^{2+} dependent endonuclease, an extensive degradation of DNA has been observed following treatment of mouse liver slices with DNA-damaging agents.[16] A similar function for Ca^{2+} and Mg^{2+} can be supposed in the sperm nucleus.

Beltran-Parrazal *et al*. (2006) reported that mitochondrial movements occur by means of molecular motors, including kinesin, dynein, and myosin, and several linkers and anchors that enable transit along microtubules and actin filaments.[17] Mitochondrial movement can result in re-arrangement of the spatial pattern of ATP production and Ca^{2+} buffering. The same molecular motors are present in the flagellum of the spermatozoa, and its movement is the result of forces generated between adjacent peripheral doublets of the axoneme. Parkinson (2009) stated that dynein arms of the doublet, which in the state of resting are bound to the adjacent doublet, unbind, elongate and then bind to a new site further along the filament.[18] The unbinding process, which is the adenosine triphosphate (ATP) using step, is then repeated, resulting in progressive bending of the flagellum.

Garcia and Graham (1989) working with bull spermatozoa observed that solutions containing Ca^{2+} and Mg^{2+} provided significantly less protection to the cells during freezing and thawing, while Kaludin and Dimitrova (1986) found a direct proportional correlation between Mg content of ram spermatozoa and the percent of spermatozoal motility and reverse proportional correlation existed between Ca content and the motility of seminal cells.[19,20] While, Kaya *et al*. (2002) by increasing ejaculation frequency observed a reduction in Ca and Mg content of seminal plasma in parallel with a decrease in sperm motility and concentration and the semen volume.[21]

Fakih *et al*. (1986) incubated human semen with 1 mmol calcium solution in vitro, and by multiple photography found that Ca increased the sperm motility and velocity.[22] These reports support the association of Ca and Mg content of seminal plasma and motility observed in this study.

The TAC of seminal plasma in this study was highly positively correlated with sperm motility, viability and seminal plasma Ca and Mg values. The mean TAC values in Ex group was highly correlated with sperm motility and viability, and correlated with seminal plasma Ca values, but in Go group, it was highly correlated with sperm viability only, and in Mo group, TAC was significantly correlated with seminal plasma Mg content. This means that TAC of seminal plasma in buffalo bulls is also an important factor for the sperm motility. Lindemann *et al*. (1988) in an investigation of the effectiveness of certain antioxidants in preserving the motility of bull sperms concluded that oxidation could be a factor in motility loss in living sperm.[23] Lapointe *et al*. (1998) reported on the presence of antioxidants in the region of the acrosomal cap of the spermatozoa.[6]

Zini *et al*. (2002) study showed that seminal plasma activity of SOD in infertile men is significantly greater than in fertile men while catalase activity is not different between these groups.[24] They also reported that catalase-like and SOD-like activities (two major antioxidant activities) are primarily derived from post-epididymal (i.e. seminal vesicle, prostate) secretions.

Ahotupa and Huhtaniemi (1992) reported a decrease of catalytic activity of antioxidants in the testis of experimentally cryptorchid rats.[25] Lapoite *et al*. (2000) reported that antioxidants had a significant positive effect on the maintenance of sperm motility in the bovine sperm.[26] Bilodeau *et al*. (2002) observed that in

vitro addition of antioxidants to the bovine semen samples overcame the loss of motility caused by 100 μM H_2O_2 and increased intracellular ATP level.[27] Baumber *et al*. (2003) observed that addition of antioxidants to the equine semen prevented the increase in live acrosome reacted sperms.[28] Verberckmoes *et al*. (2005) reported that addition of antioxidants to the semen diluents had no effect on sperm quality in the bovine.[29] Cordoba *et al*. (2006) observed that antioxidants failed to modify oxygen uptake and block capacitation in heparin-treated samples in the bovine.[30] Marti *et al*. (2007) reported that antioxidants activity was higher in the first ejaculate of rams in all months of the year and higher in non-breeding season[31], and, de Graaf *et al*. (2007) found that in vitro addition of antioxidants had no effect on the post thaw sperm motility in the ram.[32]

It can be concluded that the Ca and Mg content of seminal plasma in buffalo bulls play important roles in preserving sperm motility and viability, and seminal plasma TAC by protecting spermatozoa from damages caused by oxidative reactions affects the semen. This, in turn, would improve the quality of ejaculate leading to higher semen fertility.

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References

1. Endres DB, Rude RK. Mineral and bone metabolism. In: Burtis CA, Ashwood ER, Bruns DE, eds. Tietz text book of clinical chemistry and molecular diagnostics, 4th ed. St. Louis: Elsivier Saunders 2006;1823-1891.
2. Kaplan A, Jack R, Opheim KE, et al. Mineral and trace elements. In: Clinical chemistry, 4th ed. Baltimore: Williams and Wilkins 1995;351-356.
3. Ward DN, Bousfield GR, Moore KH. Gonadotropins. In: Cupps PT, ed. Reproduction in Domestic animals, 4th ed. San Diego: Academic Press 1991;69.
4. Henricks D M. Biochemistry and physiology of the gonadal hormones. In: Cupps PT ed. Reproduction in Domestic animals, 4th ed. San Diego: Academic Press 1991;100.
5. Zubkova EV, Robaire B. Effects of glutathione depletion on antioxidant enzymes in the epididymis, seminal vesicles, and liver, and on spermatozoa motility in the aging Brown Norway rat. Biol Reprod 2004;71:1002-1008.
6. Lapointe S, Sullivan R, Sirard MA. Binding of bovine oviductal fluid catalase to mammalian spermatozoa. Biol Reprod 1998;58:747-753.
7. Turner TT, Lysiak JJ. Oxidative stress: A common factor in testicular dysfunction. J Androl 2008;29(5):488.
8. Ax RL, Dally M, Didion BA, et al. Semen evaluation, In: Hafez B, Hafez ESE, eds. Reproduction in Farm Animals (7th ed). Philadelphia: Lippincott Williams & Wilkins 2000;365-375.
9. Barth AD. Evaluation of Potential Breeding Soundness of the Bull. In: Youngquist RS and Threlfall WR, eds. Current Therapy in Large Animal Theriogenology (2nd ed). St. Louis: WB Saunders 2007:235.
10. Sansone G, Nastri MJF, Fabbrocini A. Storage of buffalo (Bubalus bubalis) semen. Anim Reprod Sci 2000;62:55-76.
11. Cargle RG, Salisbury GW, Van Demark NI. Sodium, potassium, calcium and chloride distribution in bovine semen. J Dairy Sci 1958;9:1267-1272.
12. Cargle RG, Salisbury GW, Van Demark NI. Distribution of bulk and trace minerals in bull reproductive tract

fluids and semen. J Dairy Sci 1958;9:1273-1277.
13. Abdel-Rahman HA, El-Belely MS, Al-Qarawi AA, et al. Relationship between semen quality and mineral composition of semen in various ram breeds. Small Rumin Res 2000;38:45-49.
14. Pesch S, Bergmann M, Bostedt H. Determination of some enzymes and macro-and microelements in stallion seminal plasma and their correlations to semen quality. Theriogenol 2006;66(2):307-313.
15. Barrier-Battut I, Delajarraud H, Legrand E, et al. Calcium, magnesium, copper, and zinc in seminal plasma of fertile stallions, and their relationship with semen freezability. Theriogenol 2009;58:229-232.
16. TanakaY, Yoshihara K, Itaya A, et al. Mechanism of the inhibition of Ca^{2+},Mg^{2+}-dependent endonuclease of bull seminal plasma induced by ADP-ribosylation. J Biol Chem 1984;259(10):6579-85.
17. Beltran-Parrazal L, Lopez-Valdes HE, Brennan KC, et al. Mitochondrial transport in process of cortical neurons is independent of intracellular calcium. Am J Physiol Cell Physiol 2006;291, C1193-C1197.
18. Parkinson T. Normal reproduction in male animal. In: Noakes DE, Parkinson TJ, England GCW eds. Veterinary Reproduction and Obstetrics (9^{th} ed). Edinburgh: Saunders, Elsivier 2009; 681-704.
19. Garcia MA, Graham EF. Development of a buffer system for dialysis of bovine spermatozoa before freezing. III. Effect of different inorganic and organic salts on fresh and frozen-thawed semen. Theriogenol 1989;31(5):1039-48.
20. Kaludin I, Dimitrova I. Dependence between the magnesium and calcium content of the spermatozoa of ram and their motility. Vet Med Nayki, 1986; 23(2): 29-33.
21. Kaya A, Askoy M, Tekeli T. Influence of ejaculation frequency on sperm characteristics, ionic composition and enzymatic activity of seminal plasma in rams. Small Rumin Res 2002; 44:153-158.
22. Fakih H, MacLustky N, DeCherney A, et al. Enhancement of human sperm motility and velocity in vitro: effects of calcium and creatine phosphate. Fertl Steril, 1986; 46(5): 938-944.
23. Lindemann CB, O'Brien JA, Giblin FJ. An investigation of the effectiveness of certain antioxidants in preserving the motility of reactivated bull sperm models. Biol Reprod 1988;38:114-120.
24. Zini A, Fischer MA, Mak V, et al. Catalase-like and superoxide dismutase-like activities in human seminal plasma. Urol Res 2002; 30: 321 – 323.
25. Ahotupa M, Huhtaniemi I. Impaired detoxification of reactive oxygen and consequent oxidative stress in experimentally cryptorchid rat testis. Biol.Reprod 1992;46:1114-1118.
26. Lapointe S, Bilodeau J-F, Lemieux D, et al. Epithelial and stromal uterine cells cultured in vitro protect bovine sperm from hydrogen peroxide. Theriogenol 2000;54(3):355-369.
27. Bilodeau J-F, Blanchette S, Comier N, et al. Reactive oxygen species-mediated loss of bovine sperm motility in egg yolk Tris extender: protection by pyruvate, metal chelator and bovine liver or oviductal fluid catalase. Theriogenol 2002;57(3):1105-1122.
28. Baumber J, Sabeur K, Vo A, Ball BA. Reactive oxygen species promote tyrosine phosphorylation and capacitation in equine spermatozoa. Theriogenol 2003;60:1239-1247.
29. Verberckmoes S, Van Soom A, Dewulf J, et al. Comparison of three diluents for the storage of fresh bovine semen. Theriogenol 2005;63 (5):1354-1365.
30. Cordoba M, Mora N, Beconi MT. Respiratory burst and NAD(P)H oxidase activity are involved in capacitation of cryopreserved bovine spermatozoa. Theriogenol 2002;65 (4):882-892.

31. Marti E, Mara L, Marti JI, et al. Seasonal variation in antioxidant enzyme activity in ram seminal plasma. Theriogenol 2007;67 (9):1446-14454
32. de Graaf SP, Evans G, Gillan L, et al. The influence of antioxidant, cholesterol and seminal plasma on the in vitro quality of stored and non-stored ram spermatozoa. Theriogenol 2007;67(2):217-227.