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DETERMINATION OF PHYSIOLOGICAL BIOMARKERS IN THE SEMEN AND PERIPHERAL BLOOD OF SAHIWAL BULLS OF TWO AGE GROUPS

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Aim of the present study was to investigate the status of different antioxidant systems in semen and peripheral blood of bulls of two age groups. For this purpose, different physiological biomarkers in the peripheral blood and seminal plasma of 3-5 years old and 8-10 years old Sahiwal bulls, kept at the Semen Production Unit (SPU), Qadirabad, Sahiwal were monitored. Six bulls with clinically normal reproductive tract were included in each group. Semen samples were collected from each bull weekly for four weeks, as per routine practice at the SPU, and examined for semen quality parameters. Then seminal plasma was separated by centrifugation and stored at -20ºC. On the day of semen collection, blood was also collected from each bull, serum was separated and stored at -20ºC. Serum and seminal plasma samples were thawed and analyzed for physiological biomarkers including total antioxidant status (TAS), total oxidant status (TOS), paraoxonase, arylesterase, malondialdehyde (MDA), ceruloplasmin and catalase. Results showed that mass activity, individual sperm motility and sperm concentration were significantly higher ($P<0.05$) in 3-5 years old bulls than those in 8-10 years old bulls. However, dead sperm percentage did not differ between 3-5 year (12.54±0.25%) and 8-10 year (12.75±0.33%) old bulls. Serum activities of paraoxonase, TAS and TOS were significantly higher ($P<0.05$) in 3-5 years old than 8-10 years old bulls, while reverse was true for serum ceruloplasmin activity. However, serum MDA, catalase and arylesterase activities did not differ significantly between bulls of two age groups. Seminal plasma showed higher activities of paraoxonase, arylesterase and ceruloplasmin in 8-10 years old bulls compared to bulls aged 3-5 years ($P<0.05$), while an opposite trend was seen for TAS and TOS. Moreover, bulls aged 3-5 years had higher activities of paraoxonse and arylesterase in the serum, while older bulls showed higher activities of paraoxonse, arylesterase, TAS, and ceruloplasmin in the seminal plasma. Activities of catalase and MDA were higher in the serum than in seminal plasma in bulls of both age groups ($P<0.05$). However, activities of TAS in bulls aged 3-5 years and TOS in older bulls did not differ between the two body fluids. In conclusion, semen quality in terms of mass activity, individual sperm motility and sperm concentration was better in 3-5 years old compared to 8-10 years old Sahiwal bulls. However, no specific trend for levels of various physiological biomarkers in relation to semen quality of these bulls could be demonstrated.
Keywords: Age, physiological biomarkers, semen, serum, Sahiwal bulls.

INTRODUCTION

Pakistan is blessed with some of the finest dairy breeds; namely Sahiwal and Red Sindhi of cattle and Nili Ravi and Kundi breeds of buffaloes, but their potentials are not yet fully exploited. The main avenue of livestock development through selective breeding is the selection of breeding bulls because a bull is considered as half of the herd. Evaluation of semen quality is an important key in selecting breeding bulls. Besides factors like season, nutrition and management, quality of semen ejaculate is also affected by age of the bull (Mathevon *et al.*, 1998). Semen quality is usually estimated in terms of ejaculatory volume, sperm concentration, sperm motility, percentages of live and morphologically normal sperm, seminal pH and optimum biochemical and metabolic features of individual sperm and seminal plasma (Hoque, 1998).

Oxidative stress is generally associated with altered physiological activities in the body. It occurs when an organism is unable to control and absorb the excessive free radical production in the body (Bansal and Bilaspuri, 2010). Sperm are highly susceptible to oxidative stress caused due to release of reactive oxygen species (ROS), which induce damage to sperms due to the presence of polyunsaturated fatty acids (PUFA) in the sperm plasma membrane (Alvarez and Storey, 1995). Function and integrity of sperm plasma membrane are impaired under peroxidation of PUFAs, which is an autocatalytic and self-propagating reaction. Reactive oxygen species cause lipid peroxidation cascade initiation by oxidative attack on sperm plasma membrane (Sharma and Agarwal, 1996).

However, different anti-oxidant systems are present in the seminal plasmato protect spermatozoa from the harmful effects of ROS. In the seminal plasma, catalase is one of the

important antioxidant enzymes that control concentration of ROS and protect the sperm from damage. Catalase is produced by the testis, epididymis and accessory reproductive organs and helps in maintaining sperm motility for a long time (Kawakami *et al.,* 2006). Antioxidants like paraoxonase-I (PON-I), arylesterase and ceruloplasmin are other major defenses of sperm in protecting from oxidants and ROS (Nguyen and Sok, 2003).

In male infertility treating plan, antioxidants play a key role in which the oxidative stress is minimized and sperm motility is enhanced (Bansal and Bilaspuri, 2010). Natural antioxidants neutralize the damage to cellular structures caused by ROS. Seminal plasma is the fluid part of semen, which is mostly contributed by accessory sex glands. Although most of the constituents of seminal plasma, including antioxidants, are mostly derived from blood, their levels may vary in these two body fluids. Moreover, activities of different antioxidants can vary with age of bulls.

Therefore, aim of the present study was to investigate the status of different antioxidant systems through monitoring different physiological biomarkers in the seminal plasma and peripheral blood of 3-5 years old and 8-10 years old Sahiwal bulls. Attempts were also made to determine any association of these physiological biomarkers with physical parameters of semen quality in these bulls.

MATERIALS AND METHODS

Experimental Animals: This study was conducted on 12 adult and clinically healthy Sahiwal bulls maintained at the Semen Production Unit, Qadirabad, District, Sahiwal, Pakistan. These bulls were kept in north-south directionally situated pens under natural climatic conditions during summer season (April and May). Green fodder at 10% of body weight, along-with 2-3 kg concentrate (Anmol Wanda, prepared by Livestock Production Research Institute, Okara, Pakistan) was provided to each bull per day. Detail about ingredients, TP and TDN of the Anmol Wanda are given in Table 1. Fresh and clean water was made available throughout 24 hours. Bulls were vaccinated against Foot and Mouth disease and haemorrhagic Septicaemia and dewormed as per scheduled practice at the farm.. Overgrown hooves were trimmedwhen necessary. Exercise was managed daily for about one hour for each bull. These bulls were divided into two age groups viz 3-5 years old and 8-10 years old, with six bulls in each group.

Collection and Evaluation of Semen: Semen from each bull was collected weekly for four weeks using an artificial vagina. Before collection, 1-2 false mounts were allowed for sexual stimulation. After collection, each ejaculate was kept in a water bath at 37ºC and manually evaluated for mass activity, sperm motility, sperm concentration and dead sperm percentage, using light microscope, as described previously (Hafez, 1987).Following evaluation, each ejaculate was centrifuged at 3000 rpm for 10 minutes, the seminal plasma was collected in small 1.5 ml eppendorfs and stored at -20ºC for determination of physiological biomarkers.

Table 1. Ingredients, TP and TDN of the Anmol Wanda fed to experimental bulls

Ingredients	Amount (%)	TP (%)	TDN (%)
Maize grain (crushed)	12.00	1.08	10.20
Rape seed meal	12.00	3.84	9.00
Cotton seed cakes	7.00	1.96	5.25
Gluten 30%	25.00	6.50	20.00
Wheat bran	30.50	4.27	21.35
Molasses (cane)	12.00	0.36	8.40
Mineral mixture + premix	1.50	-	-
Total	100.00	18.01	74.20

Blood Collection and Separation of Serum: On the day of semen collection, blood was also collected from jugular vein of each bull and allowed to clot. Then test tubes containing blood were centrifuged at 3000 rpm for 10 minutes, serum was separated and stored at -20ºC for estimation of serum physiological biomarkers.

Serum and Seminal plasma analysis: Serum and seminal plasma samples stored at -20ºCwere thawed and analyzed for various physiological biomarkers including paraoxonase, arylesterase, Total Oxidant Status (TOS), Total Anti-oxidant Status (TAS), catalase, malondialdehyde (MDA) and ceruloplasmin,

Total oxidant status: Total oxidant status in serum and seminal plasma was measured as per method described by Erel (2005), using spectrophotometer (BTS-330, Biosystems, Spain). Total oxidant status in the sample was expressed as µMol (H_2O_2 equivalent) per liter.

Total antioxidant status: Total antioxidant status was estimated following the method of Erel (2004). Absorbance of standards and samples was measured spectrophotometrically, using wavelength of 660 nm. The total antioxidant concentration was estimated from the standard curve and denoted as mMol (Trolex equivalent) per liter.

Malondialdehyde level: Determination of malondialdehyde (MDA) level in samples was carried out as described by Ohkawa *et al.* (1979). The absorbance of standards and samples was estimated at 532nm wavelength. Calculations were made by using 1, 1, 3, 3-tetramethoxypropane (TMP) as external standard for standard curve plotting and MDA level was determined in terms of lipid peroxides level as nMol/liter.

Arylesterase activity: Arylesterase activity was estimated by the procedure of Juretic *et al.* (2006). The absorbance was determined at 270nm wavelength, using the spectrophotometer and arylesterase activity was calculated by the following formula:

$$\text{Arylestrase (KU/L)} = \frac{\frac{Absorbance}{5}}{0.017} \times 50$$

Ceruloplasmin: Ceruloplasmin was estimated by the method of Schosinsky *et al.* (1974). The absorbance was determined at 540nm wavelength of spectrophotometer. Ceruloplasmin level was measured in terms of substrate consumption and denoted in IU per litre, as given below:

Ceruloplasmin = (absorbance of B-absorbance of A) x 6.25 x 10^2

Catalase activity: The catalase activity was estimated in U/L, as described by Cohen *et al.* (1970). Calculations were made by using different standard curves and intra-assay and inter-assay coefficients of variation were 8.8 and 8.4%, respectively.

Paraoxonase Activity: The paraoxonase activity in the serum and seminal plasma samples was determined according to the method of Macknessand Mackness. (2015). using paraoxonase as a substrate. The paraoxonase activity in the sample was expressed as U/L

Statistical Analysis: Mean values (±SE) were computed for various parameters of semen quality and physiological biomarkers for bulls of two age groups (Steel *et al.*, 1997). Data on semen quality parameterswere subjected to t-test for verification of significance of variation of mean values between groups. Data on physiological biomarkers in the serum and seminal plasma of bulls of two age groups were analyzed through two-way ANOVA, under completely randomized design. Computer software "Statistical Package for Social Sciences (SPSS, version 21, IBM, New York, USA) was used for this purpose.

RESULTS

Physical Parameters of Semen: Mean values ± (SE) of physical parameters of semen quality for ejaculates collected from young and old Sahiwal bulls are given in Table 2, Mean values for mass activity, individual sperm motility and sperm concentration were significantly higher (P<0.05) in 3-5 years old than those in 8-10 years old bulls. Older bulls showed non-significantly higher dead sperm percentage in their ejaculates than bulls aged 3-5 years.

Physiological biomarkers: In the present study, serum activities of paraoxonase, TAS and TOS were significantly higher (P<0.05) in 3-5 years old than 8-10 years old bulls, while reverse was true for serum ceruloplasmin activity. However, serum activities for arylesterase, catalase and MDA did not differ between bulls of the two age groups (Table 3). Seminal plasma showed higher activities of paraoxonase, arylesterase and ceruloplasmin in 8-10 years old bulls compared to those aged 3-5 years (P<0.05), while an opposite trend was seen for TAS and TOS. Seminal plasma activities of catalase and MDA did not differ statistically (P>0.05) between bulls of the two age groups.

A comparison of activities of physiological biomarkers

Table 2. Mean values (±SE) of the physical parameters of semen quality of Sahiwal bulls of two age groups

Age of bulls (years)	Mass activity (0-5)	Sperm motility (%)	Sperm concentration (10^6/ ml)	Dead sperm (%)
3-5	2.19±0.17^a	72.50±1.17^a	1118.83±89.10^a	12.54±0.25^a
8-10	1.73±0.12^b	68.96±1.16^b	985.75±85.58^b	12.75±0.33^a

Values with different superscripts within a column differ significantly from each other (P<0.05).

Table 3. Mean values (±SE) of the physiological biomarkers in serum and seminal plasma of Sahiwal bulls of two age groups

Physiological biomarkers	Age of bulls (years)	Serum	Seminal plasma
Total Oxidant Status (µMol/L)	3-5	2.40±0.168^{bA}	3.24±0.021^{aA}
	8-10	1.95±0.039^{aB}	1.74±0.050^{aB}
Malondialdehyde (nMol/L)	3-5	0.23±0.017^{aA}	0.06±0.004^{bA}
	8-10	0.19±0.009^{aA}	0.06±0.005^{bA}
Ceruloplasmin (IU/L)	3-5	11.16±0.24^{bB}	14.10±0.34^{aB}
	8-10	15.25±0.28^{bA}	34.31±0.41^{aA}
Catalase (U/L)	3-5	2540.42±53.92^{aA}	1389.94±45.44^{bA}
	8-10	2415.24±41.28^{aA}	1347.85±11.91^{bA}
Paraoxonase (U/L)	3-5	116.34±1.27^{aA}	91.25±1.96^{bB}
	8-10	97.05±1.78^{bB}	117.98±2.16^{aA}
Arylesterase (KU/L)	3-5	59.63±0.69^{aA}	31.93±1.60^{bB}
	8-10	58.22±3.67^{bA}	82.86±3.24^{aA}
Total Antioxidant Status (mMol/L)	3-5	1.56±0.022^{aA}	1.59±0.023^{aA}
	8-10	0.89±0.021^{bB}	1.07±0.010^{aB}

Values with different small superscripts within a row or capital superscripts within a column for each parameter differ significantly from each other (P<0.05).

between serum and seminal plasma revealed that bulls aged 3-5 years had higher activities of paraoxonse and arylesterase in the serum, while older bulls showed higher activities of paraoxonse, arylesterase, TAS, and ceruloplasmin in the seminal plasma. Activities of catalase and MDA were higher in the serum than in seminal plasma in bulls of both age groups ($P<0.05$). However, activities of TAS in bulls aged 3-5 years and TOS in older bulls did not differ between the two body fluids (Table 3).

DISCUSSION

Physical parameters of Semen: In this study, bulls aged 3-5 years showed better semen quality in terms of mass activity, individual sperm motility and sperm concentration compared to bulls aged 8-10 years. However, percentage of dead sperm did not differ between bulls of the two age groups. Bhakat*et al*. (2011) observed that sperm concentration per ejaculate increased as the age of bulls was increased up to 5 years, and decreased later. According to Lemma and Shemsu (2015), percentage of dead sperm was higher in pre-service young bulls compared to breeding bulls. Besides age, factors like season of the year, nutritional status and level of sexual stimulation of the bull before semen collection can also influence semen quality parameters and can be attributed to be a possible cause of variations in the results of the present and the previous studies.

In the present study, mean values for sperm motility and sperm concentration do not coincide apparently with mean mass activity values in bulls of both age groups. This might have been due to the fact that evaluation of semen was carried out subjectively using light microscope. In this procedure, accuracy of results mostly depends on expertise of the operator and can vary from person to person, or even those recorded by the same person at different occasions. Such discrepancy could have been overcome if semen was evaluated by using some computer assisted semen analysis (CASA) system, which could not be done in this study.

Physiological biomarkers: Oxidative stress is generally associated with production of reactive oxygen species (ROS), which have adverse effects on physiological functions in the body (Bansal and Bilaspuri, 2010). Reactive oxygen species cause degeneration of polyunsaturated fatty acids (PUFAs), forming malondialdehyde (MDA). The latter is a reactive aldehyde and exerts stressful effects on the cells. Thus, the level of MDA in the serum is an indicative of the level of oxidative stress in the body. In the present study, the level of MDA did not differ between 3-5 years and 8-10 years old bulls, both in the serum, as well as in the seminal plasma. However, total oxidant status was significantly higher in the 3-5 years old than in 8-10 years old bulls, both in the serum and seminal plasma. Perhaps MDA was not a contributory factor for the higher oxidant status in the serum and seminal plasma of bulls aged 3-5 years. Besides age, time of the year has also been shown to influence oxidative stress and antioxidant variables (Vince *et al*., 2018). Whether some other unknown physiological factors have been responsible for this higher oxidant status in the serum and seminal plasma of 3-5 years old bulls than 8-10 years old bulls is not clear. Surprisingly, both serum and seminal plasma showed higher level of ceruloplasmin in the older bulls than those aged 3-5 years. Ceruloplasmin is the major copper carrying protein and carries about 95% of copper in the blood (Hellman and Gitlin, 2002); copper ions catalyze the process of oxidation.

When the total antioxidant status was considered, it was also significantly higher ($P<0.05$) in young than the old bulls, both in the seminal plasma and the serum. Perhaps this higher total antioxidant status was necessary to minimize the effects of higher oxidant status recorded in the serum and seminal plasma of young than old bulls.

Paraoxonase is associated with high density lipoprotein and protects HDL, as well as LDL from oxidation, through ability of hydrolyzing some oxidized phospholipids and cholesteryl linoleate hydroperoxides (Aksoy *et al.*, 2008; Litvinov *et al.*, 2012; Aggarwal *et al.*, 2016). Similarly, arylesterase, which is a thiol enzyme, also has lipophilic anti-oxidant activity and decreases the oxidative stress (Erdem*et al.*, 2010). In the present study, serum level of paraoxonase was higher in 3-5 years old than 8-10 years old bulls. However, serum levels of other antioxidants like arylesterase and catalase did not differ between bulls of two age groups. This indicates that higher serum total antioxidant status in bulls aged 3-5 years was mainly due to higher paraoxonase activity in these bulls.

The comparison of levels of different physiological biomarkers between two body fluids revealed that in bulls aged 3-5 years, paraoxonase, arylesterase and TAS were higher in serum, while TOS was higher in the seminal plasma. In older bulls, paraoxonase, arylesterase and TAS were higher in the seminal plasma. Catalase and MDA activities were higher in the serum, while ceruloplasmin was higher in the seminal plasma in bulls of both age groups. Previous studies have shown that paraoxonase is primarily synthesized in the liver, from where it is secreted into the general circulation, where it is predominantly associated with HDL (Ceron*et al.*, 2014; Mackness and Mackness, 2015). The same may be true for other biomarkers. Thus, peripheral blood seems to be the main source of these biomarkers. However, physiological significance of higher level of certain biomarkers in the seminal plasma in relation to age of bulls remains unclear.

Since sperm are suspended in the seminal plasma, the activity of physiological biomarkers in the seminal plasma can influence semen quality parameters. The ceruloplasmin activity was found to be higher in the seminal plasma of 8-10 years old bulls compared to younger ones. Ceruloplasmin is known to carry 95% of copper in the blood (Hellman and Gitlin, 2002) and copper ions catalyze the process of oxidation. Thus, higher activity of ceruloplasmin in the seminal plasma of older bulls could have adversely affected

sperm quality of these bulls. According to Hulbert *et al.* (2007), in old group of bulls, the oxidative stress increased as a result of high ROS production. However, the value for TOS was higher in the seminal plasma of bulls aged 3-5 years, while the values of paraoxonase, arylesterase and TAS, which are known for their anti-oxidant properties, were higher in bulls aged 8-10 years. Unfortunately, correlation between various parameters of semen quality and levels of physiological biomarkers in the seminal plasma of bull of two age groups could not be computed in the present study. Thus, the possible influences of levels of physiological biomarkers in the seminal plasma on the semen quality parameters remain unclear.

***Conclusions*:** In conclusion, semen quality in terms ofmass activity, individual sperm motility and sperm concentration was better in 3-5 years old compared to 8-10 years old Sahiwal bulls. Concentration of most of the physiological biomarkers in the serum was higher in 3-5 years age groups except that of ceruloplasmin. However, no specific trend for seminal plasma levels of various physiological biomarkers in relation to semen quality of these bulls could be demonstrated.

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REFERENCES

Aggarwal, G., R. Prajapati, R.K. Tripathy, P. Bajaj, A.R.S. Iyengar, A.T. Sangamwar and A.H. Pande. 2016. Towards understanding the catalytic mechanism of human paroxonase-1: Site-specific mutagenesis at position 192. PLoS One.11:e0147999.

Aksoy, A.N., N. Ozturk, H. Aksoy and F. Akcay. 2008. Paraoxonase and arylesterase activities in patients with preeclampsia. Euro-Asian J. Med. 40:10-13.

Alvarez, J. and B. Storey. 1995. Differential incorporation of fatty acids into and peroxidative loss of fatty acids from phospholipids of human spermatozoa. Mol.Reprod. Dev. 42:334-346.

Bansal, A.K. and G.S. Bilaspuri. 2010. Impact of oxidative stress and antioxidants on semen functions. Vet. Med. Intern. Article ID 686137.pp.1-7.

Bhakat, M., T.K. Mohanty, V.S. Raina, A.K. Gupta, H.M. Khan, R.K. Mahapatra and M. Sarkar. 2011. Effect of age and season on semen quality parameters in Sahiwal bulls. Trop. Anim. Health Prod. 43:1161-1168.

Ceron, J.J., F. Tecles and A. Tvarijonaviciute. 2014. Serum paraoxonase-1 (PON-1) measurement: an update. BMC Vet. Res. 10:74.

Cohen, G., D. Dembiec and J. Marcus. 1970. Measurement of catalase activity in tissue extracts. Anal. Biochem. 34:30-38.

Erdem, F.H., S. Karatay, K. Yildirim and A. Kiziltunc. 2010. Evaluation of serum paraoxonase and arylesterase activities in ankylosing spondylitis patients. Clinics. 65:175-179.

Erel, O. 2004. A novel automated method to measure total antioxidant response against potent free radical reactions. Clin. Biochem. 37:112-119.

Erel, O. 2005. A new automated colorimetric method for measuring total oxidant status. Clin. Biochem. 38:1103-1111.

Hafez, E.S.E. 1987. Semen evaluation. In: Hafez E.S.E. (ed), Reproduction in Farm Animals. 5th Ed., Lea and Febiger, Philadelphia, USA.pp.365-375.

Hellman, N.E. and J.D. Gitlin. 2002. Ceruloplasmin metabolism and function. Ann. Rev. Nutr. 22:439-458.

Hoque, M.D. 1998. Phenotypic variation and repeatability of semen characteristics of purebred and cross bred bulls. M.Sc. diss., Bangladesh Agri. Univ., Mymensigh, Bangladesh.

Hulbert, A.J., R. Pamplona, R. Buffenstein and W.A. Buttemer. 2007. Life and death: metabolic rate, membrane composition and life span of animals. Physiol. Rev. 87:1175-1213.

Juretic, D., A. Motejlkova, B. Kunovic, B. Rekic, Z.F. Mestric, L. Vujic, R. Mesic, J.L. Bajalo and V.S. Rudolf. 2006. Paraoxonase/arylesterase in serum of patients with type II diabetes mellitus. Acta. Pharma. 56:59-68.

Kawakami, E., A. Takemura, M. Sakuma, M. Takano, T. Hirano, T. Hori and T. Tsutsui. 2006. Superoxide dismutase and catalase activities in the seminal plasma of normozoospermic and asthenozoospermic beagles. J. Vet. Med. Sci. 69:133-136.

Lemma, A. and T. Shemsu. 2015. Effect of age and breed on semen quality and breeding soundness examination of pre-service young bulls. J. Reprod. Infertil. 6:35-40.

Litvinov, D., H. Mahini and M. Garelnabi. 2012. Anti-oxidant and anti-inflammatory role of paraoxonase-1: implication in arteriosclerosis diseases. N. Am. J. Med. Sci. 4:523-532.

Mackness, M. and B. Mackness. 2015. Human paraoxonase-1 (PON1): Gene structure and expression, promiscuous activities and multiple physiological roles. Gene. 567:12-21.

Mathevon, M., M.M. Buhr and J.C.M. Dekkers. 1998. Environmental managemental and genetic factors affecting semen production in Holstein bulls. J. Dairy Sci. 81:3321-3330.

Nguyen, S.D. and D.E. Sok. 2003. Oxidative inactivation of paraoxonase-I, an antioxidant protein and its effect on antioxidant action. Free Radic. Res. 37:1319-1330.

Ohkawa, H., N. Ohishi and K. Yagi. 1979. Assay for lipid peroxides in animal tissues by thiobarbituric acid reaction. Analyt. Biochem. 95:351-358.

Schosinsky, K.H., H.P. Lehmann and M.F. Beeler. 1974. Measurement of ceruloplasmin from its oxidase activity in serum by use of o-dianisidine dihydrochloride. Clin. Chem. 20:1556-1563.

Sharma, R.K. and A. Agarwal. 1996. Role of reactive oxygen species in male infertility. Urology 48:835-850.

Steel, R.G.D., J.H. Torrie and D.A. Dickey. 1997. Principles and Procedures of Statistics: A biometrical approach, 3rd Ed. McGraw Hill Book Co., New York. pp. 633-666.

Vince, S., I.Z. Zaja, M. Samardzija, I.M. Balic, M. Vilic, D. Duricic, H. Valpotic, F. Markovic and S. Milinovic-Tur. 2018. Age related differences of semen quality, seminal plasma and spermatozoa antioxidative and oxidative stress variables in bulls during cold and warm periods of the year. Animal. 12:559-568.

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