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EFFECT OF VITAMINS ON THE QUALITY OF INSEMINATION DOSES OF BULLS

Faridullah Hashim[*1], *Eva Tvrdá, Hana Greifová, Norbert Lukáč*

Address(es): Ing. Faridullah Hashim,
[1]Slovak University of Agriculture in Nitra, Faculty of Biotechnology and Food Sciences, Department of Animal Physiology, Tr. A. Hlinku 2, 949 76 Nitra.

*Corresponding author: faridullah.h@gmail.com

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ABSTRACT

To preserve and protect spermatozoa against any possible oxidative damage the addition of natural antioxidants might be the ideal solution which has been investigated worldwide. The composition of the diluents is as follows: 50 µM/L vitamin C with 0.5% DMSO (dimethyl sulfoxide, Sigma, St. Louis, USA); 50 µM/L vitamin E with 0.5% ethanol; each added separately to the spermatozoa with the aim to determine its effect on post-thaw quality of spermatozoa. 20 samples from each control and experimental group were analysed. Semen was thawed at 37°C for 70 seconds. The sperm motility parameters were analysed immediately after thawing used Sperm Vision CASA (Computer Assisted Semen Analysis) system. The viability of the cells was evaluated by metabolic activity MTT assay and the nitroblue-tetrazolium (NBT) test was used to assess the intracellular formation of the superoxide radicals. Motility was observed with highest statistical differences ($P<0.001$) with vitamin E. Even though, the motility was significantly lower ($P>0.05$) with the samples containing vitamin C, however, higher percentage (54%) of motility was observed in comparison to the control group (51.61%). Based on MTT assay, viability was also improved with the supplementation of vitamin C with the highest significance ($P<0.001$) impact. Only vitamin E was observed significantly lower ($P>0.05$). however, had higher percentage of viable spermatozoa (105.8%) when compare to the control group (100.1%) which did not contain vitamin E. All of the mentioned substances used – vitamin C, vitamin E – prevented the intracellular overproduction of free radicals within the sperm mitochondrial membrane with the statistical significance ($P<0.01$), ($P<0.05$), respectively, resulted from NBT Test. Thus, the addition of vitamin C, vitamin E, in the semen extender could improve the frozen-thawed quality of bovine spermatozoa.

Keywords: Vitamin C, Vitamin E, motility, viability, oxidative stress

INTRODUCTION

Artificial insemination (AI) is one the most widespread biotechnological methods of animal reproduction through which rapid genetic improvement in livestock have been achieved. Artificial insemination techniques depend on the availability of high quality semen, whether fresh, diluted and stored, and/or frozen. Improving the quality of insemination doses are undoubtedly one of the most critical success factors of insemination. The investigation and handling of semen is considered a key and essential step for assessing fertility and the successful use of semen. Production-tested sires with high fertilizing capacity are essential in order to assure optimal reproductive efficiency for the livestock and artificial insemination industry.

Currently used and approved procedures for the preparation of insemination of farm animals use doses of biologically and chemically difficult identifiable components. The components, which are being used to extend and protect the semen in cryopreservation against any possible damage, are much complex and difficult to identify. The usage of such diluents with insemination will protect the farm animals from biological risk and diseases.

In order to maximize the number and quality of insemination doses prepared from fresh semen and to optimize fertility of thawed semen, AI centers need accurate and precise methods for determining sperm concentration and membrane integrity. If the sperm concentration estimation is not precise it affects production efficiency of breeding stations, product quality and fertility (**Anzer *et al.* 2009**).

In order to assess semen quality, evaluation of sperm concentration and motility has been used, however provides limited information about the potential fertility of sires (**Elliot, 1978; Correa *et al.*, 1997; Rodriguez-Martinez and Larsson, 1998; Zhang *et al.*, 1998; Brahmkshtri *et al.*, 1999**). Another criteria for estimation of semen quality is computerized analysis of motility, acrosome integrity and have been related to non-return rates of bulls, but correlations are not high or even consistent (**Budworth *et al.*, 1982; Kjaestad *et al.*, 1993; Januskauskas *et al.*, 2000; Arlindo, 2005**).

One of the most important achievements in dairy farming after the introduction of artificial insemination is the cryopreservation of bull semen, which has enabled the worldwide distribution and use of desired genetic lines at a reasonable cost (**Polge *et al.* 1949; Smith *et al.* 1950; Manjunath *et al.* 2002**), however, it is associated with increased reactive oxygen species (ROS) production and decreased antioxidant level. Indeed, the key structure affected by cryopreservation is the sperm plasma membrane. In general, oxidative stress is the imbalance between the productions of ROS and a biological systems ability to readily detoxify the reactive intermediates or easily repair the resulting damage. The main destructive aspect of OS is the production of reactive oxygen species (ROS) which contains free radicals and peroxides and is considered as the most dangerous factors affecting the semen quality (**Bansal and Bilaspuri, 2011**). Major ROS which are present in seminal plasma are superoxide anion, hydrogen peroxide and hydroxyl radicles (**Agarwal *et al.*, 2010**). In fact, the production of ROS is a normal physiological process of spermatozoa and is essential for fertilization, acrosome reaction, hyperactivation, motility, and capacitation (**Agarwal *et al.*, 2007**). However, the imbalance between ROS and antioxidants, which cannot scavenge the exceeded ROS, is harmful to spermatozoa and can cause male infertility (**Bansal and Bilaspuri, 2011**), as well as associated with many degenerative diseases such as atherosclerosis, cancer, trauma, stroke, asthma, hyperoxia, arthritis, age pigments, dermatitis, cataractogenesis, retinal damage, hepatitis and aging (**Pandey and Rizvi 2010**).

Sperms are highly sensitive to OS especially to lipid peroxidation because of their high concentration of unsaturated fatty acid in the plasma membrane. Actually, fatty acids are essential requirements for the male germ cell to maintain the normal sperm function (**Hekimoğlu *et al.*, 2009**), however, the imbalance – as mentioned above – causes damage to the sperm cell. Spermatozoa is being affected by OS in three main ways namely membrane lipid peroxidation, DNA damage and induction of apoptosis (**Durairajanayagam *et al.*, 2014**). In fact, OS targets all cellular components including lipids, proteins, nucleic acids, and sugars. The OS damage depends on nature and amount of ROS produced, the duration of ROS exposure as well as extra-cellular factors such as temperature,

oxygen tension and the composition of the surrounding environment (e.g. ions, proteins, and ROS scavengers) (**Agarwal *et al.* 2008**).

In general, antioxidants are compounds and reaction which dispose, scavenge, and suppress the formation of ROS, or oppose their actions. Antioxidants break the oxidative chain reaction, thus reduce the oxidative stress (**Bansal and Bilaspuri, 2011**). Natural antioxidant enzyme systems include catalase, glutathione peroxidase and superoxide dismutase (**Baker *et al.*, 1996; Agarwal *et al.*, 2010**). Actually, spermatozoa are protected by various antioxidants and enzymes with antioxidant quality, in the seminal plasma or in spermatozoa itself to prevent oxidative damage, however that's not enough to scavenge the excessive amount of ROS. The addition of antioxidants to cryopreservation extenders has positive effect and improve semen parameters such as sperm motility and membrane integrity after thawing (**Bansal and Bilaspuri, 2011**). Studies has also demonstrated that antioxidants protect spermatozoa from ROS produced by leukocytes, prevent DNA fragmentation, improve semen quality, reduce cryodamage to spermatozoa, block premature sperm maturation, provide an overall stimulation to the sperm cells and improve assisted reproductive techniques outcome (**Agarwal *et al.*, 2007; Tvrda *et al.*, 2013**). On the other hand, studies have also shown that the decreased activities of antioxidant enzymes are associated with various types of cancer such as prostate cancer, bladder cancer, breast cancer, hepatic cancer, multiple myeloma (**Khan *et al.* 2013**). The synthetic antioxidants are one of the solutions; however, the naturally occurring substances are preferable due its chemical diversity, structural complexity, availability, intrinsic biologic activity or lack of substantial toxic effects (**Tvrdá *et al.*, 2015a**).

MATERIAL AND METHODS

Bovine semen samples were obtained from 8 adult breeding bulls (Slovak Biological Services, Nitra, Slovak Republic). The samples had to accomplish the basic criteria given for the corresponding breed. The semen was obtained on a regular collection schedule using an artificial vagina. Only fresh semen with the required quality (minimum progressive motility 70 % and sperm concentration 1×10^9 sperm/mL) was used for the subsequent processing of samples. All semen samples met the criteria set by the common standards used for producing AI doses. The semen samples were then diluted to a final concentration ($11x10^6$ sperm/mL) in extender consisting of Triladyl, containing egg yolk, Tris, citric acid, sugar, buffers, glycerol, and antibiotics. Diluted semen samples were loaded into 0.25-ml French straws and cooled to 4°C and frozen at a pre- programmed rate in a digital freezing machine (Digitcool 5300 ZB 250; IMV). The straws were plunged into liquid nitrogen. Before use straws were thawed in 37°C water bath for 20 s. 50 µM/L vitamin E with 0.5% ethanol and 50 µM/L vitamin C (L-Ascorbic acid; Sigma-Aldrich; St. Louis, USA) with 0.5% DMSO (dimethyl sulfoxide, Sigma, St. Louis, USA) were added to the semen in order to investigate its effect on post-thaw quality of spermatozoa.

The motility analysis was carried out using a CASA (Computer Assisted Semen Analyzer) system – SpermVision™ program (MiniTűb, Tiefenbach, Germany) with the Olympus BX 51 microscope (Olympus, Japan). Each sample was placed into the Makler Counting Chamber (deph 10 µm, Sefi-Medical Instruments, Izrael) and the following parameters evaluated: the percentage of motile spermatozoa (motility > 5 µm/s; MOT), the percentage of progressively motile spermatozoa (motility > 20 µm/s; PROG), distance average path (DAP, µm), average path velocity (VAP, µm/s), linearity of track (LIN, %) and beat cross frequency (BCF, Hz). Twenty samples from each control and experimental group were analysed– each from experimental and control groups – of vitamin E and vitamin C. At least 1000 spermatozoa were analysed in each sample.

MTT (3[4,5-dimethylthiazol-2-y1]-2,5-diphenyltetrazolium bromide) is a yellow water-soluble tetrazolium salt. The succinate dehydrogenase system of the active mitochondria cleaves the tetrazolium ring and reduces it to water-insoluble purple formazan (**Slater *et al.*, 1963; Aziz, 2006**). Subsequently, the amount of formed formazan can be determined by spectrophotometer in order to estimate the number of mitochondria, from which we can estimate the number of living cells. MTT method is more efficient for processing of large number of samples, if only sperm count is required, it is better to use MTT rather than CASA (comparative investigation), as well as it is faster and less expensive (Aziz, 2006). Thus, the viability of the cells is evaluated by the above-mentioned method. This colorimetric assay measures the conversion of a yellow tetrazolium salt (MTT) to blue formazan particles by mitochondrial succinate dehydrogenase of intact mitochondria within living cells. Formazan was then measured spectrophotometrically. The MTT tetrazolium salt (Sigma, St. Louis, USA) was dissolved in PBS (Dulbecco´s Phosphate Buffer Saline, Sigma, St. Louis, USA) at 5 mg/mL. 20 µL of the solution was added to the cells (in 150 µL medium per well). After a 2 h incubation (shaker, 37 ºC, 95% air atmosphere, 5% CO2), the cells and the formazan crystals were dissolved in 80 µL of acidified (0.08 M HCl; Centralchem, Bratislava, Slovak Republic) isopropanol (Centralchem, Bratislava, Slovak Republic). The optical density was determined at a wavelength of 570 nm against 620 nm as reference by a microplate ELISA reader (Anthos MultiRead 400, Austria). The data are expressed in percentage of the control.

The nitroblue-tetrazolium (NBT) test was used to assess the intracellular formation of the superoxide radical. This assay was conducted by counting the cells containing blue NBT formazan deposits, which were formed by reduction of the membrane permeable, water-soluble, yellow-colored, nitroblue tetrazolium chloride (2,2′-bis(4Nitrophenyl)-5,5′-diphenyl-3,3′-(3,3′-dimethoxy-4,4′diphenylene) ditetrazoliumchloride; Sigma, St. Louis, USA) and superoxide radical. Formazan were measured spectrophotometrically. The NBT salt was dissolved in PBS (Dulbecco´s Phosphate Buffer Saline, Sigma, St. Louis, USA) containing 1.5% DMSO (dimethyl sulfoxide, Sigma, St. Louis, USA) to a final concentration of 1 mg/mL and added to the cells (10 µL per well). After 1 h of incubation (shaker, 37 ºC, 95% air atmosphere, 5% CO2), the cells were washed twice with PBS (phosphate buffer saline, Sigma, St. Louis, USA) and centrifuged at 300 x g for 10 min. Lastly, the cells and formazan crystals were dissolved in 2M KOH (potassium hydroxide; Centralchem, Bratislava, Slovak Republic) in DMSO. The optical density was determined at a wavelength of 570 nm against 620 nm as reference by a microplate ELISA reader (Anthos MultiRead 400, Austria). The data are expressed in percentage of the control.

Obtained data – from all the tests mentioned above – were statistically analysed using PC program GraphPad Prism 3.02 (GraphPad Software Incorporated, San Diego, California, USA). Descriptive statistical characteristics (mean, minimum, maximum, standard deviation and coefficient of variation) were evaluated. T-test and Wilcoxon matched pairs test were used for statistical evaluations. The level of significance was set at *** (P<0.001); ** (P<0.01) and * (P<0.05).

RESULTS AND DISCUSSION

The results have showed that the motility of the samples with the supplementation of vitamin C was with the mean value of 54% with S.D. of 7.896%, Min 39% and Max 69%, and CV of 14.62%, whereas, the control sample showed the mean value of 51.61% with the S.D. of 7.761%, Min 40% and Max 68%, and CV 15.04%. Which shows that the motility in comparison to the control group was significantly lower ($P>0.05$), however expressed higher percentage of motile spermatozoa (Table 1). The progressive motility indicated the mean value of 37.5% with the S.D. 4.934%, Min 28% and Max 48%, and CV 13.16%. On the other hand, progressive motility of the control group showed the mean value 33.17% with the S.D. 8.083%, Min 21% and Max of 50%, and CV of 24.37%. Thus, the progressive motility was significantly higher ($P<0.001$) in comparison to the control group (Table 4). Average path velocity (VAP) showed mean value of 71.95 µm/s with S.D. 3.198 µm/s and CV 4.45% and its related control group expressed mean value of 59.13 µm/s with S.D. 9.898 µm/s and CV 16.74%. Beat cross frequency (BCF) indicated mean value 27.33 Hz with the S.D. 1.784 Hz and CV 6.53% and the control group showed mean value 25.83 Hz with the S.D. 1.392 Hz and CV 5.39 %. Both VAP and BCF showed statistically higher significance ($P<0.001$) in comparison to its control groups. The other motility parameters such as LIN and STR have also indicated improved significance ($P<0.05$; Table 1).

Table 1 The effect of vitamin C supplementation on the motility of frozen-thawed bovine spermatozoa

Parameters	MOT		PROG		VAP		LIN		STR		BCF	
	Ctrl	Vit. C	Ctrl	Vit. C	Ctrl	Vit. C	Ctrl	Vit.C	Ctrl	Vit. C	Ctrl	Vit. C
x	51.61	54	33.17	37.5***	59.13	71.94***	46.6	47.59*	75.56	76.87*	25.83	27.33***
Min	40	39	21	28	43.5	64.1	42	43	68	65	23.5	23.8
Max	68	69	50	48	84.9	79.5	54	54	84	87	28.6	30.8
S.D.	7.761	7.896	8.083	4.934	9.898	3.198	2.866	2.985	3.202	3.292	1.392	1.784
CV (%)	15.04	14.62	24.37	13.16	16.74	4.45	6.15	6.27	4.24	4.28	5.39	6.53

x – mean; S.D. - standard deviation; CV – coefficient of variation; Ctrl – control group; *** ($P<0.001$); ** ($P<0.01$) and * ($P<0.05$).

The results from MTT test expressed – from the samples which included vitamin C – the mean value 166.2 % with the S.D. of 58.24 % and CV of 2.69 %. The control group – that did not contain vitamin C showed the mean value of 99.38 %, S.D. 19.79 % and CV 2.49 %. Thus, the MTT assay showed significantly (*P<0.001*) improved viability of spermatozoa with samples of vitamin C when compared to the control group (Table 2).

Table 2 The effect of vitamin C on viability of spermatozoa.

Parameters	MTT	
	Control group	Vitamin C
x	99.38	166.2***
Min	84.92	103.8
Max	141.9	352
S.D.	19.79	58.24
CV (%)	19.913	35.042

x – mean; S.D. - standard deviation; *** (*P<0.001*); ** (*P<0.01*) and * (*P<0.05*).

The NBT test has also indicated protection against oxidative damage with the samples of vitamin C with the significant impact (*P<0.01*). The NBT test has showed the mean value of 166.2 % with the S.D. of 58.24 % and CV 60.559 %. Whereas, the control group expressed the mean value 99.38 % with the S.D. 19.79 % and CV 92.706 % (Table 3).

Table 3 The effect of vitamin C on super oxide production of spermatozoa.

Parameters	NBT	
	Control group	Vitamin C
x	100.5	21.83**
Min	15.86	1.476
Max	289	46.1
S.D.	93.17	13.22
CV (%)	92.706	60.559

x – mean; S.D. - standard deviation; *** (*P<0.001*); ** (*P<0.01*) and * (*P<0.05*).

The results of motility of spermatozoa with the supplementation of vitamin E indicated mean value 63.44 % with the S.D. of 9.789 %, Min of 4 2% and Max 79 %, and CV of 24.71 %. On the other hand, the control group indicated mean value of 55.96 % with S.D. of 9.166 %, Min 42 % and Max 76 %, and CV of 16.38 %. The motility of the samples containing vitamin E expressed higher significance (*P<0.001*) in comparison to its related control group. The progressive motility showed mean value of 43.9 % with the S.D. 8.794 %, Min 31 % and Max 59 %, and CV 20.03 %. Whereas, the control group resulted the mean value 35.79 % with the S.D. of 8.843 %, Min also 16 % and Max 57%, and CV 24.71 %. The progressive motility has also indicated higher statistical significance (*P<0.001*) in comparison to the control group (Table 4). All other motility parameters such as Average path velocity (VAP), Linearity of track (LIN), Straightness of track (STR) and Beat cross frequency (BCF) were also observed with the higher significant differences (*P<0.001*). VAP of the samples with vitamin E had mean value of 77.86 µm/s with S.D. 9.369 µm/s and CV 12.03 %. The control group showed mean value 62.13 µm/s, with the S.D. of 6.869 µm/s and CV 11.06 %. The LIN showed mean value of 53.9 % with the S.D. of 3.293 % and CV 6.11 %. Whereas, its control group indicated mean value 44.59 % with S.D. of 2.678 % and CV 6.01 %. The STR resulted with the mean value of 80.26 % with S.D. 6.257 % and CV 7.8 %. To compare with its related control group expressed mean value of 74.63 % with S.D. 3.249 % and CV 4.35 %. BCF indicated mean value 27.44 Hz with the S.D. 2.533 Hz and CV 9.23 %. whereas, its control group showed mean value 25.68 Hz with the S.D. 1.196 Hz and CV 4.66 % (Table 4).

Table 4 The effect of vitamin E supplementation on the motility characteristics of frozen-thawed bovine spermatozoa

Parameters	MOT		PROG		VAP		LIN		STR		BCF	
	Ctrl	Vit. C	Ctrl	Vit. C	Ctrl	Vit. C	Ctrl	Vit.C	Ctrl	Vit. C	Ctrl	Vit. C
x	55.96	63.44***	35.79	43.9***	62.13	77.86***	44.59	53.9***	74.63	80.26***	25.68	27.44***
Min	42	42	16	31	55.1	55.1	36	43	66	67	23.8	20.2
Max	76	79	57	59	83	91.8	51	62	83	89	27.3	34.7
S.D.	9.166	9.789	8.843	8.794	6.869	9.369	2.678	3.293	3.249	6.257	1.196	2.533
CV (%)	16.38	15.43	24.71	20.03	11.06	12.03	6.01	6.11	4.35	7.8	4.66	9.23

x – mean; S.D. - standard deviation; CV – coefficient of variation; Ctrl – control group; *** (*P<0.001*); ** (*P<0.01*) and * (*P<0.05*).

The results from MTT assay – with samples containing vitamin E – indicated mean 105.8% with the S.D. of 43.83 %, as well as Min 53.83 % and Max 175.9 % and CV 41.427 %. Whereas, its control group showed mean value of 100.1 % with the S.D. 34.32 % and Min 60.19 % and Max 169.8 % and CV 34.286 %. Although, vitamin E is not statistically significant (*P>0.05*), however, higher number of spermatozoa was observed viable (Table 5).

Table 5 The effect of vitamin E on viability of spermatozoa.

Parameters	MTT	
	Control group	Vitamin E
x	100.1	105.8
Min	60.19	53.83
Max	169.8	175.9
S.D.	34.32	43.83
CV (%)	34.286	41.427

x – mean; S.D. - standard deviation; *** (*P<0.001*); ** (*P<0.01*) and * (*P<0.05*).

The NBT test – of the samples with vitamin E – showed the mean value of 62.61 % with the S.D. of 44.49 % and CV 71.059 %. Whereas, its related control group expressed mean value 101 % with S.D. 57.47 % and CV 56.9 %. Thus, the NBT test has also indicated protection against oxidative damage with the samples of vitamin E with the significant impact (*P<0.05*; Table 6).

Table 6 The effect of vitamin E on super oxide production of spermatozoa.

Parameters	NBT	
	Control group	Vitamin E
x	101	62.61*
Min	25.2	10
Max	225	165
S.D.	57.47	44.49
CV (%)	56.9	71.059

x – mean; S.D. - standard deviation; *** (*P<0.001*); ** (*P<0.01*) and * (*P<0.05*).

In the aerobic incubation, reactive oxygen species causes the sperm plasma membrane, which is rich in polyunsaturated fatty acids and is susceptible to peroxidative damage, loss membrane integrity, decreased sperm motility, and eventually loss in fertility (**Alvarez *et al.*, 1987**). Cryopreservation reduces the functionality of bull spermatozoa similar to other species (**Rasul *et al.*, 2001**). Thus, the present study was conducted with the supplementation of vitamin C

and vitamin E in the cryopreservation to improve post-thaw spermatozoa motility, viability and decreased the damaged caused by super oxide production. Similar results have been found by **Andrabi *et al.*, (2008)** on bull with the administration of vitamin C and vitamin E. **Breininger *et al.* (2005)** reported that the greater number of motile spermatozoa present in samples frozen with natural antioxidants would increase the fertilizing potential of post-thaw spermatozoa.

Our results showed improved motility with the samples with administration of vitamin C. In fact, all the motility parameters indicated higher percentage in comparison to the control group (Figure 1). However, motility itself was without statistical significance (*P>0.05*). According to **Verma and Kanwar (1998)** the doses dependent spermatozoa motility was the highest after 6 h incubation in 800 µM/L vitamin C. however, the motility was reduced with concentrations >1000 µM/L (**Verma and Kanwar, 1998**), which could be attributed to production ROS with the higher doses of vitamin C in presence of catalytic cations such as iron (**Agarwal *et al.*, 2004**). Therefore, the higher concentration of vitamin C reduces or has no protective effect against H_2O_2- induced peroxidative damage of motility (**Donnelly *et al.*, 1999; Agarwal *et al.*, 2004**).

Our results have also showed that vitamin C improved the protection against super oxide production of spermatozoa (Figure 6). Agarwal reported that Vitamin C is able to promote the release of transition metals such as iron and copper from proteins to redux cycle and consequently to form oxygen radicals, thus can act as on pro-oxidant when added without the addition any other antioxidant (**Agarwal *et al.*, 2004**). Concentration higher than 20 µM/L are not protective against H_2O_2 induced peroxidation damage of motility, in the contrary they increase the damage in normozoospermic and asthenozoospermic patient (**Agarwal *et al.*, 2004**). Agarwal has also stated that 10 µM/L of vitamin C along with TEST yolk buffer failed to reduce the loss of motility in cryopreserved semen samples and similar results were obtained with vitamin E at concentrations of 40 and 60 µM/L (**Agarwal *et al.*, 2004**). **Rolf *et al.* (1999)** reported based on a randomised double-blind study that vitamin C along with vitamin E failed to show improvement in semen parameters, sperm survival or pregnancy rates in couples with male factor infertility with the administration of high-dose the above mentioned both vitamin C and vitamin E for 56 days. However, **Agarwal *et al.* (2010)** on the contrary, has stated several other studies (**Giovenco *et al.*, 1987; Kessopoulou *et al.*, 1995; Moilanen and Hovatta, 1995; Rolf *et al.*, 1999**) to be effective and has also mentioned that further clinical studies should be carried out using selected patient with identified and clear DNA damage for whom antioxidant treatment could be beneficial.

Our results expressed that the viability of spermatozoa was improved with samples containing vitamin C (Figure 4). The oral administration of vitamin C in smokers had positive results as well (**Agarwal *et al.*, 2004**). When 200 and 1000 mg was administered, significant results (P<0.01, P<0.001, respectively) were noticed in serum and seminal plasma (**Agarwal *et al.*, 2004**). A greater significant improvement in concentration, morphology and viability was observed in 1000 mg treated group than 200 mg treated group, a significant positive correlation was observed between serum, seminal plasma vitamin C concentrations and sperm quality (**Agarwal *et al.*, 2004**).

Our results demonstrated that the motility was improved with the samples cultured with vitamin E after the deep freeze with the highest statistical significance (*P<0.001*; Figure 2). **Agarwal *et al.*, 2004** has reported that the addition of vitamin E (10 mM/L) along with cryoprotectants during cryopreservation is capable of preserving sperm motility more efficiently than cryoprotectant alone. Vitamin E may protect spermatozoa from oxidative damage, loss of motility and increase the sperm performance in the hamster egg penetration assay (**de Lamirande & Gagnon, 1992; Agarwal *et al.*, 2010**). **Tvrda *et al.* (2013)** showed that vitamin E increased the motility from 0 to 24 hours of incubation, as well as suggested that it prevents the rapid loss of motility which normally occurs during the incubation of spermatozoa and maintains the motility under oxidative stress conditions, thus, improves the percentage of motile and viable spermatozoa under *in vitro* conditions. **Aitken *et al.* (1989)** reported that variable concentrations of vitamin E (800 µM/L, 10 mM/L) proved to be protective against lipid peroxidation.

Our results have also demonstrated that vitamin E reduced damage caused by production of superoxide production (Figure 5). **Agarwal *et al*. (2010)** in another study reported based on results *in vitro* experiments that vitamin E may protect spermatozoa from oxidative damage, motility loss, and had also stated that randomized control trails of vitamin E to be effective in treating infertile males with high ROS levels. Another study assessed the use of vitamin E combination in the 27-infertile patience. 180 mg of vitamin E, NAC 600 mg/day or β-carotene 30mg/day were administered along with the conventional treatment and essential fatty acid for 6 months. Sperm concentration was significantly improved however, no change was noticed in morphology and motility in oligozoospermic patience (**Agarwal *et al.*, 2004**).

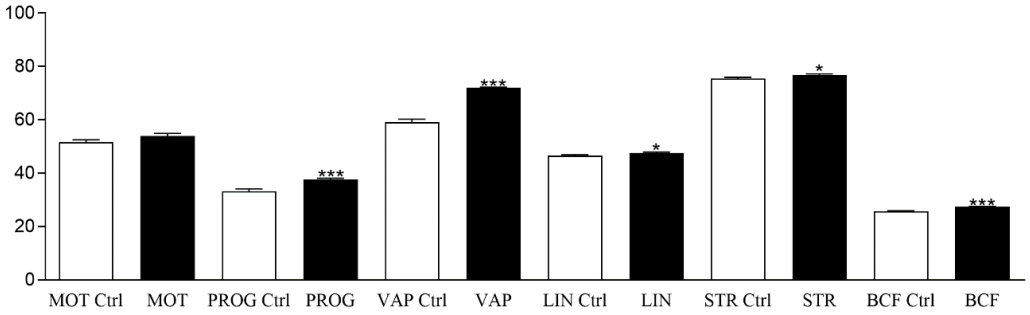

Figure 1 Motility parameters with the supplementation of Vitamin C. The level of significance was set at **** P<0.001; ** P<0.01; * P<0.05.*

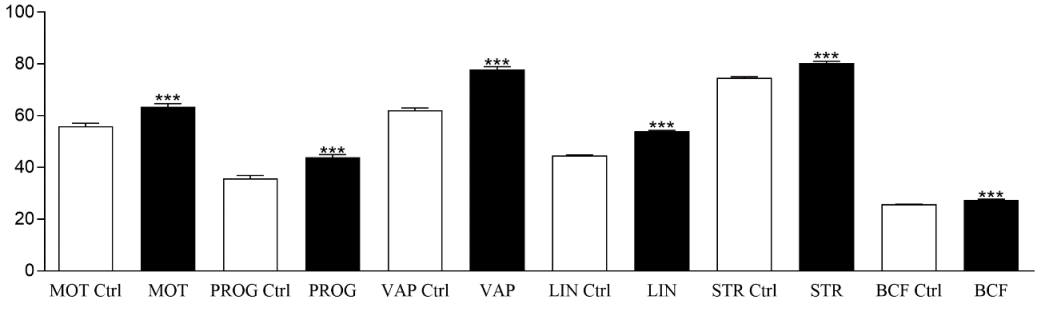

Figure 2 Motility parameters with the supplementation of Vitamin E. The level of significance was set at **** P<0.001; ** P<0.01; * P<0.05*.

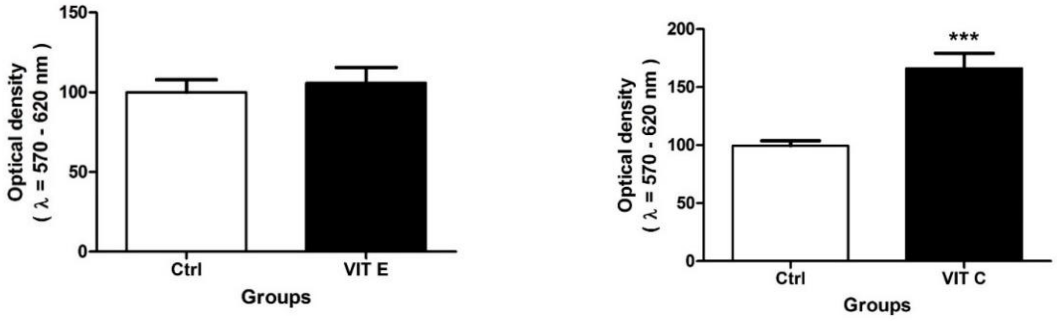

Figure 3,4 The effect of vitamin E and vitamin C – respectively – on the viability of spermatozoa by MTT assay. The level of significance was set at *** *P<0.001; ** P<0.01; * P<0.05.*

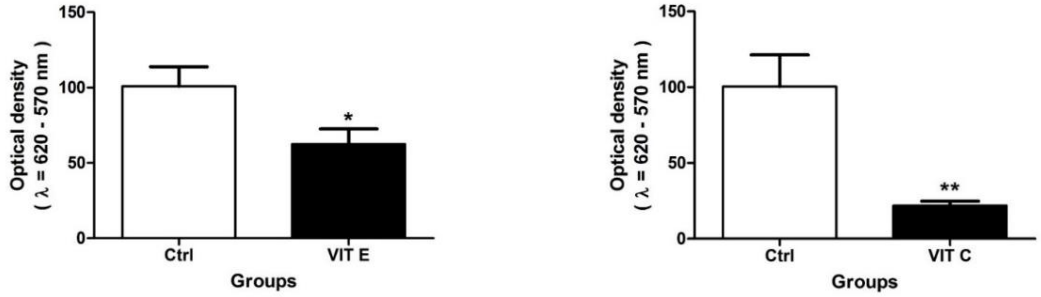

Figure 5,6 The effect of vitamin E and vitamin C – respectively – on the spermatozoa superoxide production. The level of significance was set at *** *P<0.001; ** P<0.01; * P<0.05.*

CONCLUSION

The natural antioxidant such as vitamin C and vitamin E were used to protect and improve the post-thaw quality of bovine insemination doses. Improving the quality of insemination doses are undoubtedly one of the most successful factors of insemination. Cryopreservation reduces the functionality of the bull spermatozoa. As well as bovine sperm – like any other species – is affected in aerobic incubation by reactive oxygen species (ROS) due to its polyunsaturated fatty acids on the sperm plasma membrane which is susceptible to peroxidative damage. The ROS reduces sperm motility, membrane integrity and eventually loss in fertility. Therefore, the above mentioned active substances were administered. Based on the comprehensive assessment of the identified laboratory analysis we have come to the following conclusion.

The highest significant differences of motility parameters were observed with vitamin E. Even though, the motility was significantly lower with the samples containing vitamin C. However, progressive motility, average path velocity (VAP) and beat cross frequency (BCF) were observed with the highest significance. The lowest motility was observed with vitamin C. Viability of spermatozoa had the highest significance impact with the supplementation of vitamin C. Only vitamin E was observed significantly lower, however, had higher percentage of viable spermatozoa when compare to the control group which did not contain vitamin E. The highest viable spermatozoa were detected with supplementation of vitamin C and the lowest was observed with the supplementation of vitamin E. The nitroblue-tetrazolium (NBT) test indicated that all substances used – vitamin C, vitamin E prevented the intracellular overproduction of free radicals within the sperm mitochondrial membrane with the statistical significance. The highest stimulating effect was observed with the supplementation of vitamin E and the lowest effect was detected with samples containing vitamin C.

Therefore, supplementing the above mentioned natural substances could be of scientific importance for extending the time of spermatozoa storage before further analysis of andrology experiments for instance artificial insemination or *in vitro* fertilization techniques. Moreover, further dosage and time dependant evaluation of similar natural antioxidant would be of great achievements. Hence, we can conclude from the present study that the post-thaw quality of bull spermatozoa could be improved with the supplementation of natural antioxidant namely vitamin C and vitamin E, which may increase the motility, viability and reduce the damage causes by super oxide production by quenching the free radicals which can be attributed to the antioxidant property of the selected mentioned substances.

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