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THE ROLE OF ANTIOXIDANTS AND BIOLOGICALLY ACTIVE SUBSTANCES ON THE MOTILITY AND SPEED PARAMETERS OF BUFFALO BULL SPERMATOZOA AFTER CRYOPRESERVATION

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Abstract

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After cryopreservation, the reactive oxygen species induce damage on various levels of the membrane and intracellular structures, which reduces sperm motility and causes DNA integrity damage. With the present study we have investigated the role of combined enzymatic and non-enzymatic antioxidants and biologically active substances on the spermatological status of thawed buffalo bull spermatozoa. The results show that the supplementation of caffeine to the thawing medium in doses of 5 mg/ml is appropriate for the biotechnology of freezing in pellets. After thawing of the straws, the presence of the antioxidant mix (L-Glutathione, N-Acetyl Cysteine, vitamin E, vitamin C, calcium, zinc and selenium) in doses of 1 mg/ml preserves the sperm velocity parameters and the percentage of spermatozoa with progressive motility at significantly higher values, compared to the controls and the other samples ($p<0.001$). We propose an optimized practical method of cryopreservation of buffalo bull semen through utilization of biologically active substances.

Key words: buffalo bulls; sperm; SCA analyze; antioxidants; cryopreservation

Abbreviations: BAS – biologically active substances; ROS - reactive oxygen species; GPx - glutathione peroxidase; SOD - superoxide dismutase; CAT - catalase; LPO - lipid peroxidation; NAC - N-Acetyl Cysteine; ATP - adenosine triphosphate; AOM - antioxidant mix; VCL- velocity curvilinear; VSL– velocity straight line; VAP– velocity average path

Introduction

Under the conditions of natural fertilization, the sperm cells are predominantly within an anaerobic environment. Thereby the potential damage from ROS is being reduced. Contrariwise, during the freezing process the spermatozoa are being exposed to visible light and oxygen which leads to ROS generation. This is the reason for the increase of membrane and intracellular structures impairment, motility reduction and DNA integrity damage (Baumber et al., 2003; Bilodeau et al., 2011). The sperm has a natural antioxidant defense system, which includes GPx, SOD, CAT, and some other natural antioxidants (Kobayashi et al., 1991; Peña et al., 2003; Silva et al., 2011; Partyka et al., 2012). It also possesses some low molecular antioxidants such as ascorbic acid (vitamin C) and α-tocopherol (Aitken and Baker, 2004). However, these substances cannot always compensate for the adverse impact of ROS; therefore the biological potency of the spermatozoa is being negatively affected by the process of cryopreservation. It is well known that low temperatures lead to an increase in the intensity of LPO (Chatterjee, 2001).

One study was made by supplementing NAC to the protective medium. In the presence of sulfur from NAC, the free radicals are being neutralized by direct chemical interactions

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with it (Cocco et al., 2005; Perumal et al., 2011). NAC acts as a precursor of the intracellular biosynthesis of cysteine and glutathione and, being a stimulant of the cytosolic enzymes, it participates in the metabolism of glutathione. Thereafter SOD, which is a biological antioxidant, purifies (neutralizes) ROS such as superoxide anions and hydroxyl radicals, resulting in control of the oxidative stress in mammalian sperm. There has been numerous studies concerning a cryoprotective medium supplemented with cysteine, which has been shown to improve the functions of thawed sperm in dogs (Michael et al., 2007), cats (Thuwanut et al., 2008), rams (Bucak et al., 2007; Silva et al. 2011), bulls (Tuncer et al., 2010) and muscovy ducks (Gerzilov, 2010; Gerzilov et al., 2011).

The spermatozoon membranes are rich in unsaturated fatty acids and do not have a cytoplasmic antioxidant-containing component, which makes them particularly susceptible to LPO in the presence of ROS, with a subsequent loss of membrane integrity, impaired cell function and reduced motility (Lenzi et al., 2002; Bucak et al., 2007).

It is known that further addition of SOD to the medium sometimes results in contradictory effects. Some authors have reported a protective effect of SOD on the sperm (Kobayashi et al., 1991; Berlinguer et al., 2003), while an increase in DNA fragmentation in the sperm has been reported by others (Baumber et al., 2005).

It has been reported that vitamin C has antioxidant activity on bull sperm (Hong Hu Jian et al., 2010). Similar investigations have been conducted regarding the first priority role of Vitamin E as an LPO protective antioxidant. Being the principle membrane-bound antioxidant molecule, vitamin E protects the cytosol from free radicals. In this regard, it has been shown that the supplementation of Vitamin E to frozen bovine spermatozoa helps in retaining their fertilization ability up to 70% (Dalvit et al., 1998).

Another antioxidant substance is selenium. It is known that Selenium serves as a component of the cytoplasmic GPx, which reduces peroxides. Selenium, supplemented to bovine seminal plasma prior to freezing, has been reported to increase the percentage of motile spermatozoa in the sperm (Siegel et al., 1980), but its effect on their viability has not yet been proven. It has been suggested that selenium also affects the metabolism of the sperm. Also, vitamin E and selenium have a synergistic effect. Together they provide with antioxidant protection, they delay cell aging and protect against cell malignant transformation (Rao et al., 2013).

Of the non-enzymatic antioxidants, calcium positively influences the viability and the ability for acrosomal reaction of bovine and caprine spermatozoa (Pereira et al., 2000). Furthermore, zinc acts as a regulator of the enzyme activity in the semen. Intracellularly, zinc is closely associated with sulfhydryl groups and disulfide bonds. It is concentrated in the area of the spermatozoon tail. Zinc controls sperm motility through monitoring the energy utilization by the cellular ATP system (Hidiroglou and Knipfel, 1984).

There is still not enough data regarding the role of the compound enzymatic and non-enzymatic antioxidants (such as L-Glutathione and NAC) in combination with vitamin E, vitamin C, calcium, zinc and selenium (AOM) on the spermatological status of the thawed sperm from buffalo bulls. Therefore, the goal of the present study is the comparative assessment of AOM efficacy, in comparison to the effect of vitamin C and caffeine on the motility, survival rate and speed parameters of buffalo bull spermatozoa, analyzed with a Sperm Computer Analyzer(SCA, Microptic SL, Spain).

Materials and Methods

In the present study we used buffalo bull semen, frozen into pellets and straws, property of Executive Agency for Selection and Reproduction in Animal Breeding - Sofia and Sliven. 8 ejaculates per freezing technology were examined.

Chemicals used

Freezing medium - Triladil (Sigma SL, USA); Thawing medium - 2.8% sodium citrate; Vitamin C (Sigma SL, USA); AOM - L-Glutathione, NAC, vitamin E, vitamin C, calcium, selenium, and zinc; Caffeine (Sigma SL, USA).

Thawing of pellets

For the purpose of this experiment each pellet was thawed in a water bath at 60°C for 5 seconds, using 0.5 ml of 2.8% sodium citrate medium. After thawing, the samples were stored in a thermostat at 37°C. From each sample, 100 µl of cell suspension were placed in 4 Eppendorf tubes, tempered at 37°C in thermostate. Different supplementations were added to each tube, as follows:

- 1st tube (control) – 100 µl 2.8% sodium citrate;
- 2nd tube - 100 µl of medium containing 1 mg/ml vitamin C;
- 3rd tube - 100 µl of medium containing 1 mg/ml AOM;
- 4th tube - 100 µl of medium containing 5 mg/ml caffeine.

Thawing of straws

According to the standard procedure each straw was thawed using a water bath at 37°C. After thawing, different supplementations were added to 4 Eppendorf tubes, containing 50 µl of cell suspension, as follows:

- 1st tube (control) – 50 µl 2.8% sodium citrate;
- 2nd tube - 50 µl of medium containing 1 mg/ml vitamin C;
- 3rd tube - 50 µl of medium containing 1 mg/ml AOM;
- 4th tube - 50 µl of medium containing 5 mg/ml caffeine.

Samples analysis

All semen samples were investigated for survival rate at 37°C for 6 h. Spermatological parameters were analyzed by SCA at the beginning of the experiment and on every hour until the 6th hour after thawing. The software "Motility & Concentration" (Microptic, Spain) was used for the analysis. SCA test was performed using "Leja 20" chambers with 2 µl drop volume. A minimum of 1000 spermatozoa per sample were analyzed.

The following velocity parameters were determined: VCL – curvilinear velocity; VSL – straight-line velocity; VAP – average path velocity.

Results

The results demonstrate that each of the tested substances affects sperm motility and velocity parameters to varying degrees. When comparing sperm motility and survivability results between the two freezing biotechnologies, the presence of caffeine induces a significant increase on the initial sperm motility in both pellets and straws. When freezing in the form of pellets, the positive effect of caffeine is significantly more pronounced on the 3rd and 5th hour after thawing (Figure 1). It was demonstrated that on the 5th hour after the thermal resistance test the percentage of spermatozoa with intact motility reaches 15.75 ± 2.46, compared with the control samples where the percentage was only at 11.02 ± 1.52 ($p<0.05$).

The results of the sperm velocity parameters test show that VCL, VSL and VAP are significantly higher when caffeine is used compared to the controls and the other biological substances (Figure 2). The effect of caffeine on the values of VCL is particularly pronounced at the 10th minute of its addition.

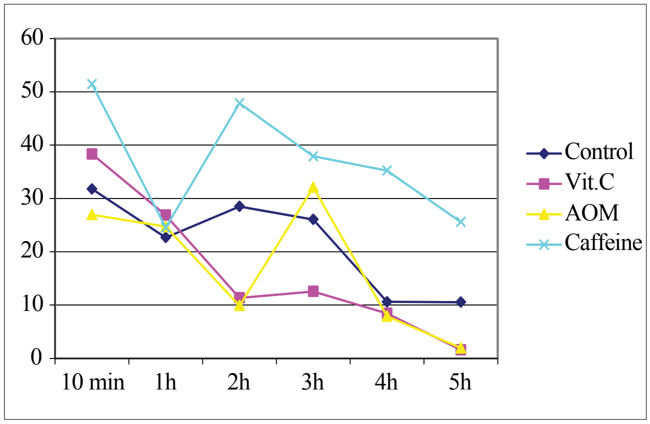

Fig. 1. Comparative analysis of the spermatozoa with progressive motility, frozen in pellets (n= 8)

The supplementation of AOM leads to similar results, but the VCL has high values over time for 5 hours. We consider that the documentation of this over time effect of AOM is of crucial importance. These are the spermatozoa with the highest velocity rates, which defines them as vital and with best chance of fertilization (Figure 2).

The presence of AOM, using the straws biotechnology, shows similar strongly positive and well pronounced protective effect on sperm motility and velocity parameters in times more, as compared with the controls (Figure 3).

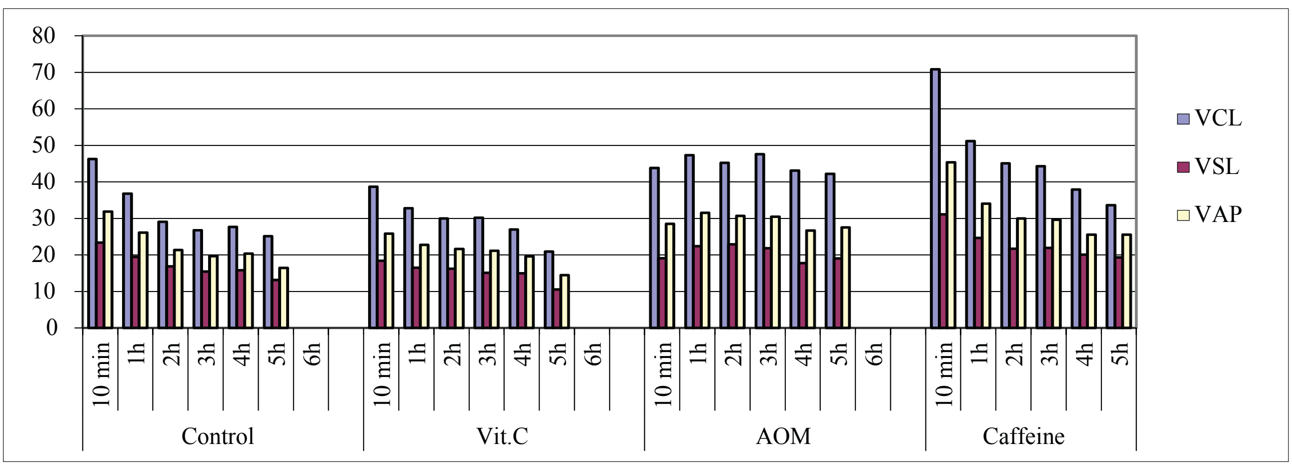

Fig. 2. Analysis of the biological role of antioxidants and BAS on the velocity parameters of buffalo bull semen, frozen in pellets (n= 8)

Sperm progressive motility is evident up to 6 hours after thawing within the range of 11.20 ± 2.02% as compared to the control samples, with only 0.5 ± 0.02% with high degree of statistical significance ($p < 0.001$).

The AOM supplementation induces pronounced progressive motility and higher values of VCL, VSL and VAP over time, compared to controls (Figure 4).

Discussion

Our study shows that caffeine neutralizes the negative impact of cryopreservation on sperm motility and increases the initial motility and velocity parameters (VCL, VSL, VAP) when freezing in pellets and straws. It has been reported that the mechanism of action of caffeine is related to its inhibitory effect on cyclic nucleotide phosphodiesterase. Thus, caffeine affects the cell respiration process and sperm motility (Garbers et al., 1971).

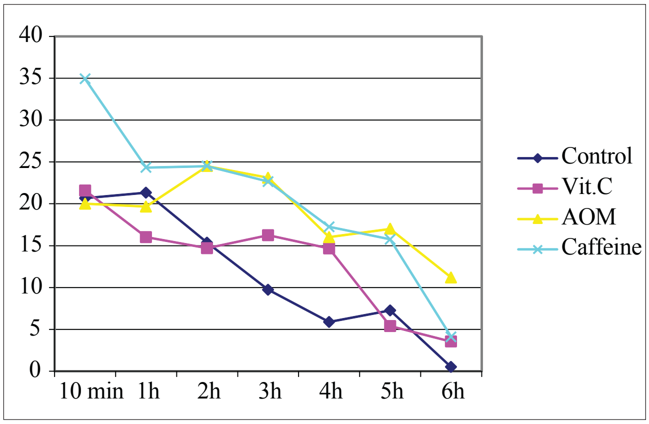

Fig. 3. Comparative analysis of the role of vitamin c, AOM, and caffeine on the progressive motility spermatozoa, frozen in straws (n= 8)

Also our results demonstrate that the AOM supplementation probably induces neutralization of some detrimental metabolic substrates, because the presence of AOM increases sperm motility to significantly higher levels and stabilizes the values of VCL over time within 5 hours range. We suppose that AOM exerts its influence by moderating or inhibiting the oxidation process that accompanies sperm metabolism. In this way, the sperm membrane phospholipids are being maintained in a stable condition and their sensitivity to peroxidation is being decreased. Thus an adequate antioxidant status is been created. It is the antioxidant substances, contained in AOM that ensure the complex protection against peroxidation and free radicals generation. In this way, AOM neutralizes the toxic products liberated by the sperm cells metabolism through biochemical interactions with them (Calamera et al., 2001).

The balance between free radicals production and antioxidant protection is of crucial importance for the sperm fertility. With the present study, it has been demonstrated that AOM not only protects the initial motility of the sperm, but also affects all investigated spermatological parameters. The sperm velocity parameters and progressive motility have significantly higher values, compared to the other samples. It is known that during the cryopreservation the oxidative stress levels and ROS production are increased. The presence of a suitable antioxidant in the medium ensures a good protection of the spermatozoa.

The supplementation of AOM contributes to the preservation of the energy potential of the sperm, which is im-

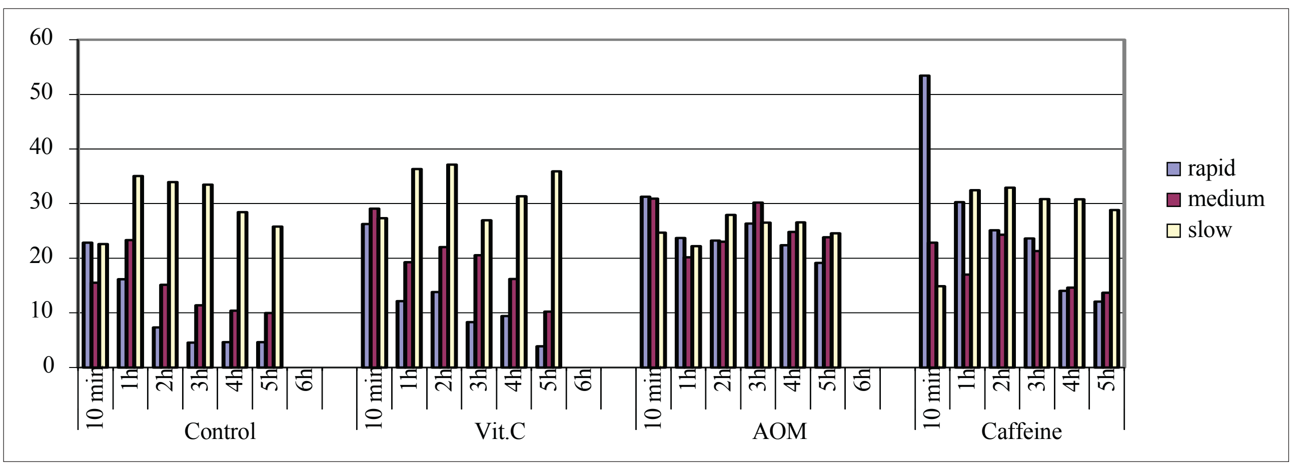

Fig. 4. Analysis of the biological role of antioxidants and BAS on sperm velocity parameters of buffalo bull semen, frozen in straws (n= 8)

portant for the fertilization ability. ROS negatively affect the motility of male sperm cells, with the damage being most commonly associated with the process of capacitation (O'Flaherty et al., 1997). It has been reported that the negative effects caused by the ROS production could be reduced by inclusion of antioxidants (Beconi et al., 1993; Maxwell and Stojanov, 1996).

With the present study, we propose a method for optimization of the cryopreservation process of buffalo bull semen by using biologically active substances. When using the pellets freezing biotechnology on buffalo bull semen, very good results are achieved by supplementing caffeine to the thawing medium in doses of 5 mg/ml. When freezing in straws, adding the AOM (L-Glutathione, NAC, vitamin E, vitamin C, calcium, selenium and zinc) in doses of 1 mg/ml guarantees good results and may be used in the practice of artificial insemination in buffalo bulls.

Conclusion

Considering the results of the present study, a method for optimization of the cryopreservation process of buffalo bull semen by using biologically active substances is being proposed.

When using the pellets freezing biotechnology on buffalo bull semen, very good results are achieved by supplementing caffeine to the thawing medium in doses of 5 mg/ml.

When freezing in straws, adding the AOM (L-Glutathione, NAC, vitamin E, vitamin C, calcium, selenium and zinc) in doses of 1 mg/ml guarantees good results and may be used in the artificial insemination practice.

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