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Research Article

THE ANTIOXIDANT EFFECT OF ASTAXANTHIN ON QUANTITATIVE AND QUALITATIVE PARAMETERS OF BULL SPERM

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ABSTRACT
Astaxanthin is a pigment of the carotenoid family, which is known as an antioxidant besides an anti-cancer, anti-diabetic, and anti-inflammatory factor. The aim of the present research was to assess the astaxanthin effect on some bull sperm quantitative and qualitative parameters. The semen samples were collected from three bulls with the use of the artificial vagina and were diluted with extender after the initial evaluation. The diluted semen was primarily divided into four equal aliquots (1mL) and astaxanthin was added to each aliquot so that the final concentration of astaxanthin in diluted sperm was 0.5, 1 and 2 micromolar respectively, and then the volume of each aliquots was reached to 4 mL with extender. Also one aliquot without astaxanthin were considered as control. The volume of control was reached to 4 mL only with extender. Eight samples from each group were introduced in 0.5mL straws and subsequently were frozen. After two weeks the frozen straws were thawed for a period of 30 seconds at 37 ˚C and then were subjected to the case study. The parameters *viz.* concentration, progressive motility percent and immotile sperms percent were assed using CASA computer software and MDA concentration was measured with the TBARS measurement method. The data were analyzed *via* the ANOVA procedure - SAS software. The results showed that in the diluted semen with astaxanthin concentration of 0.5 and 1, the progressive motility percent was increased significantly ($P<0.001$) and led to a reduction of immotile sperms percent ($P<0.05$). Moreover, these astaxanthin concentrations significantly reduced MDA level. Nevertheless, 2 micromolar astaxanthin concentration not only had no effect on the improvement of sperm parameters, but also led to damage of the sperm cell. In general, the results showed that with the use of the suitable concentrations (0.5 and 1 micromolar), this antioxidant prevents the production of Reactive Oxygen Species (ROS) and even sperm membrane lipid peroxidation during the freezing-thawing process.

Keywords: *Astaxanthin, Antioxidant, Bull Sperm, Semen Freezing, Reactive Oxygen Species, Malondialdehyde*

INTRODUCTION
Today, considering the ever-growing expansion of reproductive technologies such as artificial insemination and experimental fertility for long preservation of different cells and tissues (sperm, ovum and embryo) the freezing process is used (Esteves *et al*., 1996). Semen freezing is a suitable method to preserve and expand invaluable genetic resources that lead to the improvisation of the breed improvement programs with the use of superior male sperm insemination (Holt *et al*., 1997). The mammals sperm membrane contains much quantity of unsaturated fatty acids that have high sensitivity to peroxidation reactions, the freezing process leads to peroxidation of the sperm membrane lipids (Maxwell *et al.*, 1996) where this event can damage the natural performance and membrane structure of sperm (Hammerstedt *et al.*, 1990) and lead to motility reduction and sperm fertility ability (Maxwell *et al*., 1996). The research results showed that antioxidants protect the sperm plasma membrane during the freezing process against oxidative reactions. The research results showed that antioxidants protect the sperm plasma membrane during the freezing process against oxidative reactions. In fact, with a decrease in free radical formation they change the cell condition in a manner that sperm motility is preserved (Stanic *et al*., 2002; Gonagle *et al*., 2000; Zini *et al*., 2000). Semen consists of different antioxidants such as ascorbic acid, alpha-tocopherol, superoxidase dismutase and catalase (Sikka *et al*., 1995; Sikka 2001; Sharma *et al*., 1996;

Saleh *et al*., 2002). These antioxidants have different physiological roles and acts as an inhibitor of peroxidation process. In fact, the performance of these antioxidants can destruct the produced free radicals during peroxidation. But this sperm antioxidant capacity is not enough for the prevention of lipid peroxidation during the freezing-thawing process (Szczesniak *et al*., 2006). As far as antioxidant effect on sperm parameters after freezing is concerned, so far several researches have been carried out. For an example, addition of alpha-tocopherol to semen significantly increases the sperm qualitative parameters such as its motility (Bringer *et al*., 2005). Astaxanthin (CAS number: 472-61-7, 3-3'-dihydroxy-beta,beta-carotene-4,4'-dione) is the commonest carotenoid pigment in the marine organisms, which is produced by algae, bacteria and fungi (Fassett *et al*., 2011; Kidd *et al*., 2011) and is naturally found in skin, muscle and eggs of animals such as crab, creel, lobster, shrimp, salmon and rainbow trout besides it is found in flamingo and quail feathers (Bhosale *et al*., 2007; Kidd *et al*., 2011).

Astaxanthin with different biological activities *viz.* eliminator of free radicals and Reactive Oxygen Species (ROS), a multiplier of body immunity response, anti-cancer and anti-inflammatory factor, is recognized as a potent antioxidant (Iwamoto *et al*., 2000; McNulty *et al*., 2007; Kidd *et al*., 2011) and has important applications in different fields like poultry farming, fisheries, medicines, pharmaceutical and food industry. The most essential antioxidant activity of astaxanthin is protection of cellular organelles against damage and destruction of free radicals and lipid peroxidation (LPO) (Goto *et al*., 2001).

Malondialdehyde (MDA) is one of the final products obtained from lipids peroxidation. Today, mostly MDA concentration measurement is used to determine the LPO level in the cell membrane (Girotti *et al*., 1991).

The daily consumption of astaxanthin (16mg) in infertile men reduced ROS production in the sperm and led to sperms progressive motility increase and fertilization improvement as well (Comhaire *et al*., 2005; Elgarem *et al*., 2002). Nil research was specifically traced in the influence of astaxanthin on preservation of bull sperm parameters and several vague points are present in this regard. Therefore, in the present study the different concentrations of astaxanthin on the MDA level besides some of the bull sperm parameters after freezing-thawing process were investigated.

MATERIALS AND METHODS

In this study three Holstein bulls (average 3 years and weight 800 kilograms) present in the Genetic Material Production Centre of Jahed Livestock Inputs Company (Nahadehaye Dami Jahed Co.) located in Karaj was used. Semen was collected once (in the morning) with the use of artificial vagina and later its characteristics was primarily assessed *via* computer software CASA (Computer Assisted Semen Analysis).

Based on the information *viz.* semen volume, sperms motility percent and population besides count of the selective sperms for each straw (minimum 20 million sperms), the volume of desired extender was calculated and semen was diluted with AndroMed® semen extender (Minitube, Germany)**.**

For the preparation of stock solution (400µg/mL), 4mg astaxanthin (Sigma Aldrich, A9335) was dissolved in 0.5mL Dimethyl sulfoxide (DMSO) and with the use of AndroMed® semen extender its volume was raised to 10 mL. The diluted semen was divided into 4 equal aliquots (1mL) and then to each of these 4 aliquots, 0, 30, 60, and 120 microliter of astaxanthin stock solution was added, respectively. Then with using of extender, the volume of them was reached to 4 mL. Thus, the concentration of astaxanthin in each of 4 mL aliquots, was equal to 0, 0.5, 1, and 2µM, respectively. From each aliquot, 8 straws (0.5 mL) was prepared and packaged where 4 straws were used for the first experiment and subsequent 4 straws for the second experiment. Later, the straws were transferred to refrigerator, 4 ˚C for 4 hours, after that the straws were introduced in liquid Nitrogen based controlled rate freezer (EF600M 100, Grant Instruments, UK) for a period of 10 minutes and reached to a temperature of -140 ˚C.

Eventually, the straws were transferred to a liquid nitrogen tank (temperature -196 ˚C). After two weeks, the frozen straws were thawed in the water bath at 37 ˚C for a period of 30 seconds. In the first experiment, with the use of CASA system equipped by phase-contrast microscope, the parameters *viz.* concentration, progressive motility percent and immotile sperms percent were assessed. The assessment

of each sample was repeated five times *via* CASA where on an average 140-170 sperms in each microscopic field of view were evaluated. In the second experiment, the Malondialdehyde concentration was measured according to the TBARS (Thiobarbituric Acid Reactive Substances) estimation method.
The first and second experiment data were analyzed in the generalized randomized block designs and completely randomized design respectively, and both the experiments were analyzed using the ANOVA procedure in the Statistical software (SAS) and the comparison of averages was carried out with a Tukey range test.
The mathematical model of the first experiment was as follows:
$X_{ijk} = \mu + T_i + B_j + e_{ijk}$

The mathematical model of the second experiment was as follows:
$X_{ijk} = \mu + T_i + e_{ijk}$

In these models, X_{ijk} was observation level, μ average, T_i astaxanthin addition effect, B_j Block effect (where in this experiment was bull) and e_{ijk} is the experimental error.

RESULTS AND DISCUSSION

Results

In the present research, different astaxanthin concentrations addition effect to diluted semen on bull sperm parameters after freezing-thawing process was assessed. The average of sperm parameters is presented in Table 1. The progressive motility percent in the first group (0.5 micromolar astaxanthin) in comparison to the other two groups (1 and 2 micromolar astaxanthin) and control group (without astaxanthin) showed a significant increase, in a manner that in the first and second groups the sperms progressive motility percent in comparison to control group had a significant increase, but in the third group in comparison to control group the sperms progressive motility was significantly reduced ($P<0.001$).
Immotile sperms percent in the first group (0.5 micromolar astaxanthin) in comparison to the other two groups (1 and 2 micromolar astaxanthin) and control group (without astaxanthin) had a significant reduction, but in the third group in comparison to other three groups the immotile sperms percent was significantly increased ($P<0.05$). The MDA concentration in the first group (0.5 micromolar astaxanthin) in comparison to the other two groups (1 and 2 micromolar astaxanthin) and control group (without astaxanthin) had a significant increase in a manner that in the first and second groups, the concentration of MDA in comparison to control group had a significant increase, but the third group in comparison to the other 3 groups, the MDA concentration had significantly reduced ($P<0.001$).

Table 1: Average of sperm parameters in different astaxanthin concentrations

Parameters	Astaxanthin concentration				SEM	Significant level
	Control	0.5 µM	1 µM	2 µM		
Concentration ($x10^9$ /mL)	0.69	0.59	0.73	0.278	0.3	Ns
Progressive motility (%)	54.5^{c}	63.9^{a}	58.06^{b}	44.2^{d}	1.096	***
Immotile (%)	26.15^{ab}	21.26^{b}	30.3^{ab}	34.41^{a}	4.222	*
MDA concentration (nmole/mL)	220.35^{b}	76.19^{d}	142.3^{c}	241^{a}	3.16	***

** Averages difference at the 5% level is significant.*
**** Averages difference at the 0.001 level is significant.*
Ns Averages difference is not significant.

The averages that do not have common letter have significant difference.

Discussion

In recent years, the application of antioxidants to remove damages incurred to sperm has been considered by several researches. Astaxanthin is a carotenoid pigment with extraordinarily potent antioxidant quality, soluble in fat and located in cells membrane perishes the free radicals and resultantly reduces the lipid peroxidation process (Ambati *et al.*, 2014).

Elgarem and coworkers (2002) assessed the effect of astaxanthin as a food supplement (16mg in a day) for a period of 12 months on infertile men. It was observed that the group which had received astaxanthin in comparison to control group, the ROS activity in semen reduced, besides morphology and sperm motility improved. In fact, astaxanthin improved the quality of sperm and eventually led to fertilization output. Few years later, Comhaire *et al*., (2005) carried out same research with more details and the results showed that the group that received astaxanthin in comparison to control group showed significant ROS reduction but sperms progressive motility increased.

In general, the said researches addressed the positive effects of astaxanthin on sperm parameters and fertility where the present research results are in concurrence with it. Besides, Gabriella *et al.*, (2013), assessed the effect of astaxanthin on the sperm capacitation, but their assessed parameters differed with the present research parameters. The potential astaxanthin effects were clearly exhibited in the maintenance and preservation of sperm which was in compliance with the former research where astaxanthin was prescribed as a food supplement to men.

In the present research, the effect of different astaxanthin concentrations on some bull sperm parameters was assessed. In the first experiment, the sperm parameters *viz.* concentration, progressive motility percent and immotile sperms percent were evaluated and in the second experiment, MDA concentration was measured to determine LPO.

Since in particular nil research was found on the effect of astaxanthin addition to livestock semen and only was prescribed in food capsules for infertile men, therefore the results of this research were in addition compared to researches on the other antioxidants.

In the present research, sperm progressive motility percent in the first and second groups (0.5 and 1µM astaxanthin) to which low concentrations of astaxanthin were added had significantly increased in comparison to control group.

The sperm progressive motility percent is an important parameter in fertility since its increase leads to increased speed of a sperm to reach an ovum and the fertilization percent improves, however the immotile sperms percent in the groups to which a little astaxanthin concentration was added significantly reduced in comparison to control group as well as third group (2µM).

In the third group, due to the presence of high astaxanthin concentration, the sperm progressive motility was significantly reduced, besides immotile sperms percent in this group in comparison to control group increased. Immotile sperms percent led to a reduction in the fertilization output.

The results of this research showed that MDA concentrations in first and second group (0.5 and 1µM) in comparison to control group had significantly reduced, but in the third group (2µM) its concentration had a significant increase in comparison to control group. This indicates that the suitable concentration of astaxanthin prevented LPO process but its high concentration not only failed to reduce LPO process but also created oxidative destructive effects which are in concurrence with the research results of Andreea *et al.*, (2010). In several studies it is depicted that increasing of free radicals and end products of LPO in the environment, can reduces sperm motility significantly, which is in concurrence with the present research results. Ball *et al.* (2002) did not observed any association between motility level and semen MDA concentration which conflicted present research results. In the present research a significant reverse relationship was observed between the MDA level and sperms motility percent. Although some studies exhibit positive effect of antioxidants, but other studies reject it. This could be due to the use of very low or high antioxidants concentration. Therefore, probably the special concentration of astaxanthin has benefit effects on sperm (Agarwal *et al.*, 2004). Thus, determination of their dosage is very important

(Arthur *et al.,* 1994) and even utilization of an unsuitable concentration has a reverse effect (Agarwal *et al*., 2004).

In the present research it was observed that astaxanthin effect on sperm parameters in relation to its concentration was different in a manner that its higher concentration had oxidative effects, but its low concentration has an anti-oxidative effect. This dose-dependent effect of antioxidants (in this study) is in concurrence with research results of Brezezinska and coworkers (1995). It can be said that the usage of antioxidants is not always accompanied with improve sperms motility, so that 2 µM astaxanthin not only did not exhibit any positive effect but also led to damage of sperms. In general, the results of this research showed that the use of a suitable astaxanthin concentration besides inheriting antioxidant properties in desirable level does not cause any damage to sperms. So, considering the results of present research and validity of astaxanthin antioxidant property, it can be safely used in semen extenders. Of course, confirmation of these findings needs further investigations with other related concentrations.

REFRENCES

Agarwal A, Nallella KP, Allamaneni SR and Said TM (2004). Role of antioxidant in treatment of male infertility. An overview of the literature. *Reproductive BioMedicine Online* **8** 616-627.

Ambati RR, Phang SM, Ravi S and Aswathanarayana RG (2014). Astaxanthin: Source, Extraction, Stability, Biological Activities and Its Commercial Applications. *Marine Drugs Review* **12** 128–152.

Andreea A, Stela Z, Coprean D and Dorina N (2010). The effect of antioxidant on the cytological parameters of cryopreserved buck semen. *Romanian Biotechnological Letters* **15** 26-32.

Arthur JR (1994). New metabolic roles for Selenium. *Proceedings Of The Nutrition Society* **53** 615-624.

Ball BA and Vo A (2002). Detection of lipid peroxidation in equine spermatozoa based upon the lipophilic fluorescent dye C1l-BODIPY581/591. *Journal of Andrology* **23** 259-269.

Bhosale P, Serban B, Zhaoda Y and Bernstein PS (2007). Identification and metabolic transformations of carotenoids in ocular tissues of the Japanese quail *Coturnix japonica*. *Biochemistry* **46** 9050-9057.

Breinger E, Beorlegui N, Flaherty C and Beconi M (2005). Alpha-tocopherol improves biochemical and dynamic parameters in cryopreserved boar semen. *Theriogenology* **63** 2126-2135.

Brezezinska E, Slebodzinski A, Pietras B and Wieczork G (1995). Antioxidant effect of vitamin E and glutatione on lipid peroxidation in boar seminal plasma. *Biological Trace Element Research* **47** 69-74.

Comhaire FH, El Garem Y, Mahmoud A and Schoonjans F (2005). Combined conventional/antioxidant "Astaxanthin" treatment for male infertility: a double blind, randomized trial. *Asian Journal of Andrology* 7 257-262.

Elgarem Y, Lignell A and Comhaire FH (2002). Supplementation with Astaxanthin (Astacarox) improves semen quality in infertile men. *Proceedings of the 13th International Carotenoid Symposium.* Honolulu, Hawaii 180-197.

Esteves SC, Sharma RK, Thomas AJ and Agarwal A (1996). Suitability of the hypo-osmotic swelling test for assessing the viability of cryopreserved sperm. *Fertility and Sterility* **66** 798-804.

Fassett RG and Combes JS (2011). Astaxanthin: A potential therapeutic agent in cardiovascular disease. *Marine Drugs* **9** 447–465.

Gabriella D, Ivana K, Anna MB, Alessandra, A, Guido A, Guglielmo B, Eugenio R, Decio A, Giulio C and Luciana B (2013). Effect of Astaxanthin on Human Sperm Capacitation. *Marine Drugs* **11** 1909-1919.

Girotti MJ, khan N and Mclellan BA (1991). Early measurement of systemic lipid peroxidation products in the plasma of major blunt trauma patients. *Journal of Trauma* **31** 32-35.

Gonagle L, Goldstein M, Feldschuh J and Foote R (2002). The influence of cryoprotective media and processing procedure motility and migration of freeze-thawed human sperm. *Asian Journal of Andrology* **4** 137-141.

Goto S, Kogure K and Abe K (2001). Efficient radical trapping at the surface and incide the phospholipids membrane is responsible for highly potent antioxidative activity of the carotenoid astaxanthin, *Biochimica et Biophysica Acta* **1521** 251-258.

Hammerstedt RH, Graham JK and Nolan JP (1990). Cryopreservation of mammalian sperm: what we ask them to survive, *Journal of Andrology* **11** 73-88.
Holt WV (1997). Alternative strategies for the long-term preservation of spermatozoa. *Reproduction, Fertility and Development* **9** 309-319
Iwamoto T, Hosoda K, Hirano R, Kuvato H, Matsumoto A, Miki W, Kamiyama M, Itakuva H, Yamamto S and Kondo K (2000). Inhibition of low-density lipoprotein oxidation by astaxanthin. *Journal of Atherosclerosis and Thrombosis* **7** 216-222.
Kidd P (2011). Astaxanthin, cell membrane nutrient with diverse clinical benefits and anti-aging potential. *Alternative Medicine Review* **16** 355–364.
Maxwell WMC and Watson PF (1996). Recent progress in the preservation of ram semen. *Animal Reproduction Science* **42** 55-65.
Mcnulty HP, Byun J, Lockwood SF, Jacob RF and Mason RP (2007). Differential effects of carotenoids on lipid peroxidation due to membrane interactions: X-ray diffraction analysis. *Biochimica et Biophysica Acta* **1768** 167-174.
Saleh RA and Agrawal A (2002). Oxidative stress and male infertility, from research bench to clinical practice. *Journal of Andrology* **23** 737-752.
Sikka SC, Rajasekaran M and Hellstrom WJ (1995). Role of oxidative stress and antioxidants in male infertility. *Journal of Andrology* **16** 464-468.
Sikka SC (2001). Relative impact of oxidative stress on male reproductive function. *Current Medicinal Chemistry* **8** 851-862.
Sharma RK and Agrawal A (1996). Role of reactive oxygen species in male infertility. *Urology* **48** 835-850.
Stanic P, Tandra M, Sonicki A and Sumunic V (2000). Comparision of protective techniques for cryopreservation of human semen, *Journal of Obstetrics & Gynecology and Reproductive Biology* **91** 65-70.
Szczesniak–Fabianczyk B, Bochenek M, Smorag Z and Ryszkaf f (2006). Effect of antioxidants added to boar semen extender on the semen survival time and sperm chromatine structure. *Reproductive Biology* **3** 81-87.
Zini A, Garrels K and Phang D (2000). Antioxidant activity in the semen of fertile and infertile men. *Urology* **55** 922-926.