Lycopene Offers Protection against Oxidative Damage in Frozen-Thawn Bovine Semen

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Abstract

Oxidative stress associated with spermatozoa cryopreservation may result in lipid peroxidation (LPO), DNA damage and apoptosis, leading to decreased sperm motility and fertilization ability. Lycopene, the most powerful singlet oxygen quencher of all carotenoids, may be a possible option for a more effective semen cryopreservation because of its antioxidant properties. This study focused to evaluate the effects of lycopene on selected oxidative stress parameters in cryopreserved bovine sperm. Ten bovine ejaculates were splitted into two aliquots and diluted with a commercial semen extender containing lycopene (1.5 mM/L) or no supplement (control), cooled to 4°C, frozen and kept in liquid nitrogen. Frozen straws were thawn in a water bath for subsequent experiments. Computer assisted semen analysis was used to evaluate spermatozoa motility, reactive oxygen species (ROS) generation was quantified using luminometry and LPO was assessed via the TBARS assay. Lycopene supplementation significantly ($P<0.001$) increased the spermatozoa motility and provided a significantly ($P<0.001$) higher protection against ROS overgeneration caused by semen freezing and thawing. It was determined that the presence of lycopene resulted in a significantly ($P<0.001$) lower LPO of the spermatozoa membranes. In conclusion, lycopene supplementation may be recommended to facilitate the improvement of semen preservation in bovine breeding industry.

Keywords: bulls, lipid peroxidation, lycopene, reactive oxygen species, spermatozoa

1. Introduction

The unique structure and function renders spermatozoa to be highly sensitive to the freeze-thawing process. Such instability caused by temperature changes leads to cold shock and oxidative stress (OS)-induced damage to the cell. The abundance of polyunsaturated fatty acids (PUFAs) in the membrane structures causes susceptibility of the male reproductive cell to lipid peroxidation (LPO), and reactive oxygen species (ROS) generated during such process lead to cellular damage during the long-term storage of semen [1, 2]. Semen contains a variety of antioxidants, which under normal circumstances provide protection against the toxic effects of ROS [3, 4]. Nevertheless, following the freeze-thawing process, this system fails to protect spermatozoa against oxidative damage [5, 6]. Because of these reasons, and in order to reduce the toxic effects of ROS during sperm cryopreservation, semen extenders are usually supplemented with various antioxidant compounds, which have the ability to improve the post-thaw semen parameters [3, 7]. Lycopene (LYC) is the most abundant carotenoid found in tomatoes, tomato products and red fruits, and is considered to be the most efficient antioxidant of all carotenoids [8]. LYC has been proposed to be involved in processes related to the quenching of singlet oxygen and trapping of peroxy radicals, and therefore contributes to the protection of cells and tissues against the harmful effects of OS. Previous studies have reported that LYC has the ability to improve sperm motility,

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membrane integrity [9, 10] and DNA damage [10, 11].
Based on these preliminary reports, we focused on evaluating the protective effect of lycopene on the sperm motility, ROS production and lipid peroxidation in cryopreserved bovine semen.

2. Materials and methods

Ejaculates from eight adult Simmental-Fleckvieh breeding bulls (Slovak Biological Services, Nitra, Slovakia) were used in the study. The bulls were of similar age and were kept under uniform feeding and housing conditions. 10 semen samples were collected from each bull on a regular collection schedule using an artificial vagina. Immediately after collection, sperm concentration and motility was estimated using phase-contrast microscopy (200 x). 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.
Each semen sample was divided into two equal fractions and diluted to a final concentraion of $11x10^6$ sperm/mL in an extender consisting of Triladyl (Minitüb GmbH, Tiefenbach, Germany), containing 20 % (w/v) fresh egg yolk, TRIS, Citric acid, Sugar, Buffers, Glycerol and antibiotics (Tylosin, Gentamicine, Spectinomycine and Lincomycine) and diluted with distilled water. In case of the experimental group, the extender additionally contained 1.5 mmol/L LYC. Diluted semen samples were loaded into 0.25-ml French straws and cooled down to 4 °C in 2 h and frozen at a pre-programmed rate in a digital freezing machine (Digitcool 5300 ZB 250; IMV). Subsequently, the straws were plunged into liquid nitrogen. At least after 24 h, frozen straws were thawed in a 37 °C water bath for 20 s immediately before use. Ten replicates from every sample were evaluated in each experiment.
Spermatozoa motion (%) was assessed using the computer-aided sperm analysis (CASA, Version 14.0 TOX IVOS II.; Hamilton-Thorne Biosciences, Beverly, MA, USA). 10 μL of each sample were placed into the Makler counting chamber (depth 10μm, 37 °C; Sefi Medical Instruments, Haifa, Israel) and immediately assessed. 10 microscopic fields were subjected to each analysis in order to include at least 300 cells.
ROS levels in samples were assessed by chemiluminescence assay using luminol (5-amino-2, 3- dihydro-1, 4-phthalazinedione; Sigma, St Louis, MO) as the probe [12]. The test samples consisted of luminol (10 μL, 5 mM) and 400 μL of control or experimental sample. Negative controls were prepared by replacing the sperm suspension with 400 μL of the extender. Positive control included 400 μL of the extender and 50 μL of hydrogen peroxide (30%; 8.8 M; Sigma-Aldrich, St. Louis, USA) in triplicates. Chemiluminescence was measured on a 48-well plate for 15 min using the Glomax Multi$^+$ Combined Spectro-Fluoro-Luminometer (Promega). The results were expressed as relative light units (RLU)/sec/10^6 sperm.
The extent of LPO by means of malondialdehyde (MDA) content was quantified with the help of the TBARS assay, modified for a 96-well plate and ELISA reader. The MDA-TBA product formed by the reaction of MDA and thiobarbituric acid (TBA; Sigma-Aldrich, St. Louis, USA) under high temperature (90–100°C) and acidic conditions was measured at 530–540 nm [13]. Semen extender was run as a negative control in order to assure the objectivity of the results. MDA concentration was expressed as μM/L.
Results are expressed as mean±SEM. Statistical analysis was carried out using the GraphPad Prism program (version 5.0 for Windows; GraphPad Software, La Jolla California USA, www.graphpad.com). Descriptive statistical characteristics were evaluated at first. Paired t-test was used for specific statistical evaluations, based on the assumption that values in each row represent paired observations. The level of significance was set at $P<0.001$, $P<0.01$, and $P<0.05$.

3. Results and discussion

Oxidative stress has become one of the leading causes related to the loss of viable spermatozoa during cryopreservation. ROS overgeneration is a prominent side effect of temperature fluctuations during freeze-thaw cycles, leading to major disruptions in the oxidative metabolism of the cell, accompanied by changes in the redox characteristics and stress response by the cell [1,

2, 5]. Oxidative stress, resulting from ROS overproduction leads to irreversible alterations of membrane structures via LPO, as well as oxidative degradation of proteins or DNA and activation of signaling pathways leading to apoptosis [14].

Based on previous evidence on the useful effects of lycopene on post-thawed sperm quality and antioxidant profile [9, 10, 15], this study was performed to investigate specific ROS-quenching and protective properties of lycopene in relation to the functional activity of frozen-thawed bovine spermatozoa.

Lycopene administration to the semen extender increased the spermatozoa motility in comparison with the control (P<0.001), following freezing and thawing (Table 1).

Table 1. The effect of lycopene on the selected quality parameters of cryopreserved bovine semen

Control group	Experimental group
Spermatozoa motility [%]	
50.80±1.43	32.40±1.20***
ROS production [RLU/sec/10^6 sperm**]**	
17.80±2.82	36.19±3.41***
MDA content [μM/L**]**	
16.43±0.56	27.45±0.99***

Mean ± SEM. *** (P<0.001)

Associations have been shown to exist between spermatozoa motion, membrane stability and high mitochondrial activity [16, 17]. In the present study, lycopene supplementation to the semen extender may act as an effective motion-promoting and membrane-protecting molecule, significantly improving spermatozoa motility following sperm cryopreservation. Our CASA data are in agreement with previous studies on ram [9], rabbit [18] and turkey semen [15], suggesting the maintenance of structural intergrity and/or functional activity of spermatozoa following cryopreservation using a low dose of lycopene.

Long-term sperm storage results in membrane deterioration due to specific membrane phase transitions occurring in the highly specialized regions of the plasma membrane and leads to alterations of the sperm function [19]. Increases in cryodamage and the innate sensitivity of sperm to OS after the freeze-thawing process, may result in a reduction of sperm motility, viability, membrane and DNA integrity as well as antioxidant activity during cryopreservation [7, 10].

Frozen-thawn semen is more susceptible to oxidative damage than fresh semen. The intracellular antioxidant capacity fails to provide a proper protection against oxidative damage following semen freezing and thawing [18]. Therefore, this study was designed to investigate whether lycopene could provide protection against the temperature shock and oxidative damage during cryopreservation of bovine semen.

Our luminometric assessment shows that lycopene administration provided a significantly (P<0.001) higher protection against ROS overgeneration caused by semen freezing and thawing when compared to the lycopene-free control (Table 1). At the same time, it was determined that the presence of lycopene in the semen extender resulted in a significantly (P<0.001) lower lipid peroxidation of the spermatozoa membranes in comparison to the control group (Table 1).

The findings obtained in the present study for ROS production and LPO are complementing reports evaluating bovine [10, 20] and ram [21] semen, indicating low levels of free radicals detected, however with no decrease observed in the LPO level in the presence of various antioxidants at a frozen state. Hence, we may argue that antioxidants are not an influential factor in the prevention of LPO following cryopreservation of mammalian sperm.

4. Conclusions

In conclusion, results of this study emphasize on the impact of lycopene against the cryoinjury of bovine spermatozoa in vitro. The data obtained suggest that lycopene supplementation to the semen extender offers protection for sperm motility, ROS-protecting profile and membrane stability. Thus, lycopene supplementation may be recommended to facilitate the improvement of semen preservation in bovine breeding industry.

Acknowledgements

This study was supported by the Scientific Grant Agency of the Ministry of Education of the Slovak Republic and of the Slovak Academy of Sciences VEGA Project no. 1/0857/14.

References

1. Holt, W.T., Basic aspects of frozen storage of semen. Animal Reproduction Science, 2000a, 62, 3-22.

2. Holt, W.T., Fundamental aspects of sperm cryobiology: the importance of species and individual differences. Theriogenology, 2000b, 53, 47-58.
3. Alvarez, J.G., Storey, B.T., Taurine, hypotaurine, epinephrine and albumin inhibit lipid peroxidation in rabbit spermatozoa and protect against loss of motility. Biology of Reproduction, 1983, 29, 548-555.
4. Nissen, H.P., Kreysel, H.W., Superoxide dismutase in human semen. Klinische Wochenschrift, 1983, 61, 63-65.
5. Chatterjee, S., De Lamirande, E., Gagnon, C., Cryopreservation alters membrane sulfhydryl status of bull spermatozoa: protection by oxidized glutathione. Molecular Reproduction and Development, 2001, 60, 498-506.
6. Gadea, J., Selles, E., Marco, M.A., Coy, P., Matas, C., Romar, R., Ruiz, S., Decrease in glutathione content in boar sperm after cryopreservation. Effect of the addition of reduced glutathione to the freezing and thawing extenders. Theriogenology, 2004, 62, 690-701.
7. Bucak, M.N., Tuncer, P.B., Sarıozkan, S., Baspınar, N., Taspınar, M., Coyan, K., Bilgili, A., Akalın, P.P., Buyukleblebici, S., Aydos, S., Ilgaz, S., Sunguroglu, A., Oztuna, D., Effects of antioxidants on post-thawed bovine sperm and oxidative stress parameters: antioxidants protect DNA integrity against cryodamage. Cryobiology, 2010, 61, 248-253.
8. Di Mascio, P., Kaiser, S., Sies, H., Lycopene as the most efficient biological carotenoid singlet oxygen quencher. Archives of Biochemistry and Biophysics, 1989, 274, 532-538.
9. Uysal, O., Bucak, M.N., Effect of oxidized glutathione, bovine serum albumin, cysteine and lycopene on the quality of frozen thawed ram semen. Acta Veterinaria Brno, 2007, 76, 383-390.
10. Bucak, M.N., Ataman, M.B., Baspinar, N., Uysal, O., Taspınar, M., Bilgili, A., Ozturk, C., Gungor, S., Inanc, M.E., Aka, E., Lycopene and resveratrol improve post-thaw bull sperm parameters: sperm motility, mitochondrial activity and DNA integrity. Andrologia, 2015, 47, 545–552.
11. Zini, A., San Gabriel, M., Libman, J., Lycopene supplementation in vitro can protect human sperm deoxyribonucleic acid from oxidative damage. Fertility and Sterility, 9, 1033-1036.
12. Kashou, A.H., Sharma, R., Agarwal, A., Assessment of oxidative stress in sperm and semen. Methods in Molecular Biology, 2013, 927, 351-361.
22.
13. Tvrda, E., Knazicka, Z., Lukac, N., Selected heavy metals versus antioxidant parameters in bull seminal plasma - a comparative study. Journal of Environmental Science and Health. Part A, Toxic/Hazardous Substances & Environmental Engineering, 2012, 47, 1261-1266.
14. Wang, X., Sharma, R.K., Sikka, S.C., Thomas, A.J. Jr., Falcone, T., Agarwal, A., Oxidative stress is associated with increased apoptosis leading to spermatozoa DNA damage in patients with male factor infertility. Fertility and Sterility, 2003, 80(3), 531-535.
15. Rosato, M.P., Centoducati, G., Santacroce, M.P., Iaffaldano, N., Effects of lycopene on in vitro quality and lipid peroxidation in refrigerated and cryopreserved turkey spermatozoa. British Poultry Science, 2012, 53(4), 545-552.
16. Kasai, T., Ogawa, K., Mizuno, K., Nagai, S., Uchida, Y., Ohta, S., Fujie, M., Suzuki, K., Hirata, S., Hoshi, K., Relationship between sperm mitochondrial membrane potential, sperm motility, and fertility potential. Asian Journal of Andrology, 2002, 4(2), 97-103.
17. Martinez-Pastor, F., Johannisson, A., Gil, J., Kaabi, M., Anel, L., Paz, P., Rodriguez-Martinez, H., Use of chromatin stability assay, mitochondrial stain JC-1, and fluorometric assessment of plasma membrane to evaluate frozen-thawed ram semen. Animal Reproduction Science, 2004, 84(1-2), 121-133.
18. Rosato, M.P., Di Iorio, M., Manchisi, A., Gambacorta, M., Petrosino, G., Centoducati, G., Santacroce, M.P., Iaffaldano, N., In vitro survival and lipid peroxidation status of rabbit spermatozoa after both chilled and frozen storage in lycopene enriched extenders. Livestock Science, 2012, 146, 199-202.
19. Maxwell, W.M.C., Watson, P.F., Recent progress in the preservation of ram semen. Animal Reproduction Science, 1996, 42, 55-65.
20. Bucak, M.N., Baspınar, N., Tuncer, P.B., Sarıozkan, .S, Akalın, P.P., Coyan, K., Effects of curcumin and dithioerythritol on frozen-thawed bovine semen. Andrologia, 2012, 44, 102-109.
21. Coyan, K., Baspınar, N., Bucak, M.N., Akalın, P.P., Effects of cysteine and ergothioneine on post-thawed Merino ram sperm and biochemical parameters. Cryobiology, 2011, 63, 1-6.