Taiwanese Journal of Obstetrics & Gynecology 57 (2018) 200–204

Contents lists available at ScienceDirect

Taiwanese Journal of Obstetrics & Gynecology

journal homepage: www.tjog-online.com

Original Article

Vitamin C attenuates negative effects of vitrification on sperm parameters, chromatin quality, apoptosis and acrosome reaction in neat and prepared normozoospermic samples

Esmat Mangoli [a], Ali Reza Talebi [a, *], Morteza Anvari [b, c], Fatemeh Taheri [a], Mahboobeh Vatanparast [a], Tahereh Rahiminia [a], Akram Hosseini [a]

[a] Research and Clinical Center for Infertility, Shahid Sadoughi University of Medical Sciences, Yazd, Iran
[b] Department of Biology, Shahid Sadoughi University of Medical Sciences, Yazd, Iran
[c] Department of Anatomical Sciences, Shahid Sadoughi University of Medical Sciences, Yazd, Iran

ARTICLE INFO

Article history:
Accepted 5 October 2017

Keywords:
Vitrification
Spermatozoa
Vitamin C
Chromatin
Human sperm

ABSTRACT

Objective: Aim of this study was to evaluate the effects of vitamin C on sperm parameters, sperm chromatin quality and apoptosis resulted of vitrification in neat semen and prepared spermatozoa of normozoospermic samples.

Material and methods: Forty semen samples from normozoospermic men were included in this prospective study. Each sample was divided into five groups. Group I: control or fresh semen, group II: semen prepared by swim-up method and then vitrified, group III: neat semen was vitrified, group IV: vitamin C (600 μM) was added to prepared spermatozoa and then vitrified and group V: vitamin C (600 μM) was added to neat semen and then vitrified. After warming, sperm analysis was done accordingly. For evaluating the sperm chromatin/DNA integrity status and acrosome reaction, we used toluidine blue (TB), acridine orange (AO), terminal transferase mediated deoxyuridine triphosphate biotin end labeling (TUNEL) and double staining tests.

Results: All of the sperm parameters (count, motility, morphology and viability) had significant differences ($P < 0.05$) between different groups, especially in group IV. Data showed sperm chromatin damages and acrosome reaction abnormality increased resulted of vitrification, but, in the groups that added vitamin C (IV, V) rate of damages was decreased and this was notable in the group IV.

Conclusion: Vitamin C can attenuate the detrimental effects of vitrification on sperm parameters, chromatin quality and rate of apoptosis in both neat semen and prepared spermatozoa of normozoospermic samples.

Introduction

Cryopreservation of human spermatozoa has industrialized as an important part of assisted reproductive technology programs. Sperm cryopreservation has been commonly used for the fertility preservation before chemotherapy, radiotherapy, and/or surgery that may cause testicular failure [1]. Currently, there are three methods of cryopreservation namely: slow freezing, rapid freezing and vitrification. The first two techniques have been used for decades. However, they have some problems, such as requiring expensive equipment, are time and toil consuming and have limited efficiency. Qualities of the conserved spermatozoa are usually reduced after a thawing due to damage to the mitochondria, acrosome and tail of the spermatozoa. The normal freezing method and its variant require fewer tools, therefore, are not handy in several circumstances. To improve a quality of preserved spermatozoa, and to simplify the methods, several new approaches are being tested [2,3]. Vitrification is based on ultrarapid freezing. It is easy, safe and cost-effective [2]. The sperm preparation is done for vitrification that removes seminal plasma and consequently the principal source of antioxidant [4]. Vitrification brings lipid peroxidation in the sperm plasma membrane and increases the concentration of reactive oxygen species (ROS) in the sperm cytoplasm.

* Corresponding author. Research and Clinical Center for Infertility, Shahid Sadoughi University of Medical Sciences, Safaie, Buali Avenue, Yazd, Iran. Fax: +983538247087.
E-mail address: prof_talebi@Hotmail.com (A.R. Talebi).

https://doi.org/10.1016/j.tjog.2018.02.006

Additionally, freezing and thawing of spermatozoa might cause a rise in the generation of superoxide radicals which makes DNA damage [5].

Evaluation of sperm chromatin and apoptosis are used for investigation of male fertility, early embryonic growth and pregnancy outcomes [6,7]. It is well recognized that sperm nuclear compaction preserves sperm genome from external damages containing oxidative stress, temperature height and acid-induced DNA denaturation [8]. In contrast, high polyunsaturated fatty acid content in the sperm plasma membrane and a limited free radical scavenging system in the cytoplasm create the spermatozoa very susceptible to free radicals attack [9]. ROS have been suggested as a cryopathogenetic factor [10]. Antioxidant supplementation has been shown to increase the quality of cryopreserved spermatozoa in animal experiments [10]. However, there are limited data about the role of cryoprotectants on human spermatozoa [11].

Vitamin C or ascorbic acid is one of the important antioxidant factors in biological system which is a ROS scavenger with high potency [12]. There are some studies that added Vitamin C to culture medium of oocyte, sperm and embryo. They showed vitamin C supplementation improved cytoplasm maturation of oocyte, sperm quality and the developmental ability of embryos in human and animals. So, the addition of ascorbic acid to sperm washing medium increases sperm performance by scavenging ROS [12].

Therefore, in this study, we added vitamin C to neat semen and prepared spermatozoa to examine the effect of it on DNA integrity, apoptosis and acrosomal status of neat and prepared spermatozoa after vitrification in normozoospermic specimens.

Materials and methods

Samples

Forty normozoospermic semen samples (aged 25–45 years) were included in this experimental study. After 2–7 days of abstinence, the semen samples were obtained by masturbation. Each of liquefied samples was divided into five groups. The group I ($n = 40$) as the control or fresh semen; In group II ($n = 40$), the semen samples were processed by swim-up method and then vitrified. In group III ($n = 40$), the neat semen samples were vitrified and in group IV ($n = 40$), vitamin C (600 μM) according to [11,12] was added to processed samples and then vitrified. In group V ($n = 40$), vitamin C (600 μM) was added to the neat semen samples and then vitrified. All the patients signed the consent form for use of the surplus semen in this research. The ethics committee author's institution approved this study.

Close vitrification system

Vitrification was done according to Isachenko and et al. [13]. The concentration of sperm to vitrify in 100 μl was 3–5 million. In this procedure, the suspension was mixed with Ham's F10 medium accompanied with 5% HSA 0.5 mol/l sucrose (1:1) at room temperature (RT). 100 μl of the sperm suspension was transferred into a 0.25 ml sterile plastic straw placed into 0.5 ml straws and the end of straw was sealed. The straws were immersed directly and stored in liquid nitrogen.

Warming

In warming step, one end side of the straw was cut and emptied into a tube containing Ham's F10 with 5% HSA pre-warmed to 37 °C and pipetting was done to remove sucrose. The simple washed was used for sperm preparation and sperm analysis was performed according to WHO 2010 criteria [13].

Sperm analysis

Sperm parameters like sperm count (10^6/ml), motility, viability and normal morphology were assessed on behalf of 200 spermatozoa for each sample. Sperm count and motility were evaluated by means of Makler chamber using a light microscopy (Olympus Co., Tokyo, Japan). The viability and morphology were assessed by Eosin and Papanicolaou staining tests, respectively [14].

Sperm chromatin quality assessments

For sperm chromatin assessment, toluidine blue (TB), acridine orange (AO) and TUNEL assay were used. All of the dyes and substances were purchased from Sigma Aldrich Company (St Louis, MO, USA). The positive and negative control was confirmed, respectively, with and without acid denaturation [15].

Toluidine blue staining

TB is a metachromatic dye which controls both quality and quantity of sperm chromatin condensation via binding to phosphate groups of DNA strands [15]. Dried smears were fixed in fresh 96% ethanol and acetone (1:1, 4 °C, and 30 min) then incubated in 0.1 N hydrochloric acid (4 °C, 5 min). Then, the slides were washed three times with purified water for 2 min and finally stained with 0.05% TB in 50% citrate phosphate for 10 min. 200 spermatozoa were counted and scored as follows: S0: light blue (good chromatin); (S1) dark blue (mild abnormal chromatin); (S2) violet and purple (sever chromatin abnormality). The spermatozoa with score 0 were considered as normal cells (TB-) and spermatozoa with dark blue, violet and purple heads (scores 1 and 2) were reflected as abnormal cells (TB+) [15].

Acridine orange test

AO is a metachromatic fluorescence stain used to determine the rate of spermatozoa with DNA denaturation [16]. The air-dried smears were fixed in Carnoy's solution (methanol/glacial acetic acid, 3:1, 4 °C, 2 h). Slides were stained for 10 min by freshly prepared AO (0.19 mg/ml in McIlvain phosphate-citrate buffer (pH = 4). On the same day, smears were evaluated using a fluorescent microscope (Zeiss Co., Jena, Germany) with a 460-nm filter. 200 spermatozoa were counted and the rate of normal (green fluorescent) and abnormal cells (red fluorescent) were reported as the percentage [8].

TUNEL assay

In terminal deoxynucleotidyl transferase-mediated (TdT) deoxyuridine triphosphate (dUTP) nick end labeling assay (TUNEL) test, the ends of any single or double-stranded fragmented DNA, are tagged by labeled nucleotides (dUTP) in a reaction catalyzed by the enzyme TdT [15]. The smears were fixed in methanol (RT, 30 min) and then were washed in phosphate-buffered saline (PBS, pH 7.4). The permeabilization was done on ice through 0.1% Triton X-100 (Merck, Germany) and 0.1% sodium citrate for 2 min. After washing with PBS, 30 ml of TUNEL mixture (Roche, USA) was added to all sample and incubated for 60 min at 37 °C in a humid chamber in the darkness. Then, the slides were washed three times with PBS and evaluated with fluorescence microscope under 100× eyepiece magnification. The nuclei of sperm cells with fragmented DNA (TUNEL+) showing bright green color, whereas the nuclei of the normal cells (TUNEL-) were seen pale green. For each sample, 200 spermatozoa were assessed and the percent of TUNEL + spermatozoa were reported [15,17].

Detection of acrosome-reacted spermatozoa with double staining method

Spermatozoa were fixed in glutaraldehyde 3% (pH 7.4, 30 min, RT). Washing was done twice by centrifuging at (1000g, 3 min), the sperm pellet was suspended in 50 μl of distilled water and smeared samples were air-dried at RT. The staining was done by Bismarck brown (0.8% in deionized water, pH 1.8, 10 min, 40 °C) and rinsed with distilled water, then stained with Rose Bengal (0.8% in 0.1 M Tris buffer, pH 5.3, 25 min, RT). After the second washing, spermatozoa were dehydrated in 50, 95 and 100% ethanol and rinsed in xylene for clearing. Finally, 200 spermatozoa per slide were examined under light microscopy [18]. Red or pink staining of the acrosomal region indicated acrosome-intact spermatozoa, whereas lack of colored sperm cells was considered as acrosome-reacted [18].

Statistical analysis

The data were shown as mean ± SD and median (lower limit, upper limit). The parametric data analyzed by one-way ANOVA test and the Tukey post-test, whereas the nonparametric data was analyzed by Kruskal–Wallis test to evaluate the significant differences between different groups. The term 'statistically significant' was used to signify a two-sided P-value <0.05.

Results

Table 1 shows the sperm parameters in five groups. This table reveals that almost all of the sperm parameters had significant differences (P < 0.05) between different groups especially between group IV and V. The data showed that vitrification declined sperm parameters and vitamin C reversed the detrimental effects of it on sperm parameters. Table 2 shows the data on sperm chromatin condensation, DNA integrity and acrosomal status. There were statistically significant differences (P < 0.05) between different groups. Sperm chromatin damages and acrosome reaction status increased followed by vitrification, but, in Vit C groups (IV, V), the rates of damage showed to decrease and this was notable in the group IV.

Discussion

The results indicated that all of the sperm parameters showed significant decrease following vitrification in the experiment groups in comparison with the control group and vitamin C can

Table 1
The results of sperm analysis in different groups.

variables	Group I (n = 40)	Group II (n = 40)	Group III (n = 40)	Group IV (n = 40)	Group V (n = 40)	P-Value
Count(10^6)	117.3 ± 30.15 100(88–200)	63.9 ± 15.02 60(40–88)	62.37 ± 18.24 59(38–120)	66.62 ± 17.63 67(38–110)	59.52 ± 14.04 58(40–90)	0.000[a]
Progressive motility (%)	50.55 ± 9.07 48.5(38–68)	46.30 ± 8.72 48(28–60)	36.8 ± 7.06 38(24–54)	56.05 ± 7.62 54(42–68)	32.8 ± 6.04 30(20–48)	0.000[a,c,e,f,g,h,i,k] 0.101[b] 0.016[d] 0.150[j]
Non progressive motility (%)	25.45 ± 7.23 20(8–36)	20.5 ± 5.91 20(8–32)	24.4 ± 7.15 24(8–40)	21.45 ± 6.51 22(10–34)	23.8 ± 6.58 22(10–38)	0.017[a] 1.000[b] 0.070[c,f] 0.965[d,g,j] 0.171[e] 0.183[h] 0.285[i] 0.519[k]
Immotile (%)	29 ± 8.27 30(18–42)	33.45 ± 8.92 34(18–50)	38.8 ± 7.47 40(18–54)	22.75 ± 8.44 22(7–38)	43.4 ± 6.02 42(28–54)	0.000[a,c,e,g,h,i,k] 0.09[b] 0.005[d] 0.023[f] 0.073[j]
Viability (%)	73.72 ± 8.5 73.5(60–92)	71.3 ± 9.12 70(50–88)	64.8 ± 6.43 65(50–80)	78.8 ± 8.28 78(65–95)	60.7 ± 6.09 60(50–80)	0.000[a,c,e,f,g,h,i,k] 0.633[b] 0.032[d] 0.132[j]
Normal morphology (%)	7.07 ± 1.5 7(4–10)	7.45 ± 0.78 8(6–9)	6.82 ± 1.08 7(5–9)	7.67 ± 0.99 8(6–10)	6.62 ± 1.1 7(5–8)	0.000[a,i,k] 0.565[b] 0.870[c,g] 0.121[d] 0.378[e] 0.096[f] 0.010[h] 0.931[j]

Group I: Control (fresh semen), Group II: Freeze of prepared spermatozoa, Group III: Freeze of neat semen, Group IV: Freeze of prepared spermatozoa + vitC, Group V: Freeze of Neat semen + vitC.

[a] Difference between all groups.
[b] Difference between groups I and II.
[c] Difference between groups I and III.
[d] Difference between groups I and IV.
[e] Difference between groups I and V.
[f] Difference between groups II and III.
[g] Difference between groups II and IV.
[h] Difference between groups II and V.
[i] Difference between groups III and IV.
[j] Difference between groups III and V.
[k] Difference between groups IV C and V.

improve them. In line with our results, Khalili et al. showed a significant decrease in sperm parameters and a significant increase in sperm DNA fragmentation after vitrification in normal and abnormal semen samples. They suggested preparing semen samples before vitrification particularly for cases with male factor infertility [3]. On the other hand, Donnelly et al. examined pre- and post-cryopreservation DNA integrity of both neat and prepared semen. They reported that freezing sperm with seminal plasma improves post-thaw DNA integrity and these spermatozoa seem to be more resistant to freezing damage than prepared ones. This may be due to the presence of plentiful antioxidants in the seminal plasma [4]. The differences in results may be due to the different media and methods of cryopreservation.

The present study showed the addition of vitamin C to semen and prepared spermatozoa improved recovery rate of sperm parameters and DNA integrity following vitrification. Rossi et al. observed that the supplementation of SOD or catalase, individually, did not affect the sperm recovery parameters. But when they used both of them, a significant improvement in sperm motility was initiate; which is most likely as a result of their combination and parallel action on superoxide anion and hydrogen peroxide and the end of sperm membrane lipid peroxidation by ROS [19]. Kotdawala et al. also showed that addition of zinc to semen samples before cryopreservation helps in preventing the freeze-thaw-induced sperm DNA damage and loss of sperm function [20]. In contrast, Khodayari et al. reported that the addition of ebselen to cryopreservation medium did not improve post-thaw sperm parameters and DNA fragmentation [21].

However, the effects of cryopreservation on the fertilization capacity, sperm parameters are well documented [22–24], but, there is no agreement in the literature on the type and extent of sperm DNA damage after cryopreservation. Our data showed that vitrification causes an increase in sperm DNA fragmentation, apoptosis, chromatin abnormalities and acrosome reaction. Additionally, we showed that the vitamin C improves sperm chromatin condensation, reduces DNA fragmentation and apoptosis and also decreases spontaneous acrosome reaction in vitrified neat and prepared spermatozoa. Some studies presented a significant increase of sperm DNA integrity after cryopreservation/thawing cycles [3,4], but others opposed this results [25–27]. This difference between results could be explained by the fact that they had different sample size, different freezing procedures, different sperm DNA tests, and different semen preparation techniques before cryopreservation.

Reactive oxygen species (ROS) has been introduced as a key pathological factor disturbing human sperm DNA after cryopreservation [9,25,28]. Certain levels of free radicals are necessary for normal functions of sperm cells [12]. But, the generation of more ROS is accompanying with cell damage, including morphological defects, DNA fragmentation and lipid peroxidation [9].

In physiological situations, there are intra- and extra-cellular scavenger systems which prevent the potential toxic effect of ROS [19]. SOD and catalase in the seminal fluid are the most important elements in protective system. Moreover, it has been validated that the freezing and thawing processes cause the partial inactivation of scavenging enzymes [11,19]. Similar to our results, Li et al. indicated that cryopreservation produces a range of injuries to human spermatozoa and these cryodamage are due to ROS. Moreover, it is shown that both ascorbate and catalase supplementation in cryoprotectant media reduces ROS levels protecting human spermatozoa [11].

Ascorbic acid is an antioxidant that reacts with ROS and in that way keeps cell components against oxidative damages [29]. It may diminish ROS brought DNA fragmentation, recover inactive vitamin E, and reduce lipid peroxidation [29]. In fact, ascorbic acid acts as a ROS scavenger mainly through neutralizing the adverse effects of

Table 2
The results of sperm chromatin/DNA evaluation and acrosome status in different groups.

Variables	Group I (n = 40)	Group II (n = 40)	Group III (n = 40)	Group IV (n = 40)	Group V (n = 40)	P-Value
TB	12.55 ± 3.24 12(8–21)	26.67 ± 5.26 28.5(18–38)	22.02 ± 4.73 23(12–30)	15.3 ± 3.39 15(10–20)	21.67 ± 4.76 22(12–30)	0.000[a,b,c,e,f,g,h,i,k] 0.041[d] 0.996[j]
AO	12.25 ± 3.36 11.5(4–20)	25.9 ± 6.18 25.5(6–40)	23.7 ± 6.66 24(8–40)	12.97 ± 3.51 12(8–20)	23.37 ± 6.04 23(12–40)	0.000[a,b,c,e,g,i,k] 0.974[d] 0.357[f] 0.222[h] 0.999[j]
TUNEL	12.12 ± 3.36 11.5(4–20)	25.92 ± 6.86 25(16–42)	24.72 ± 6.24 25(12–38)	13.05 ± 3.5 14(6–20)	22.62 ± 5.87 22(12–38)	0.000[a,b,c,e,g,i,k] 0.939[d] 0.856[f] 0.051[h] 0.407[j]
AR	10.3 ± 3.08 10(4–16)	27.85 ± 6.73 28(18–45)	23 ± 7.16 21(10–38)	12.97 ± 3.46 13(8–20)	25.82 ± 7.7 25(10–42)	0.000[a,b,c,e,f,g,i,k] 0.266[d] 0.551[h] 0.215[j]

Group I: Control (fresh semen), Group II: Freeze of prepared spermatozoa, Group III: Freeze of neat semen, Group IV: Freeze of prepared spermatozoa + vitC, Group V: Freeze of Neat semen + vitC.
Toluidine Blue (TB), Acridine Orange (AO), terminal deoxy nucleotidyl transferase-mediated (TdT) deoxyuridine triphosphate (dUTP) nick end labeling assay (TUNEL), Acrosome Reaction(AR).

[a] Difference between all groups.
[b] Difference between groups I and II.
[c] Difference between groups I and III.
[d] Difference between groups I and IV.
[e] Difference between groups I and V.
[f] Difference between groups II and III.
[g] Difference between groups II and IV.
[h] Difference between groups II and V.
[i] Difference between groups III and IV.
[j] Difference between groups III and V.
[k] Difference between groups IV and V.

H_2O_2 and O^{2-} and also as the main antioxidant in seminal plasma of fertile men [12,30,31]. In this study, although, we saw that the addition of vitamin C to neat and prepared spermatozoa before vitrification, can diminish sperm cryodamages, but the protective effect was more significant in prepared spermatozoa than neat semen. Because semen has vitamin C and more addition of it may had detrimental effect on spermatozoa [32,33]. Since the use of antioxidants is like a double-edged sword. When the correct dose is not used, it might have very destructive effects [32].

In this work, we did not examine the amount of ROS production followed by vitrification; therefore, we recommend next study on ROS and vitrification and adding of antioxidants before and after vitrification in normal and abnormal semen samples.

In conclusion, for the first time, our data revealed that although the vitrification may have detrimental effects on sperm parameters and DNA integrity, but, the addition of vitamin C to spermatozoa before vitrification may have beneficial effects on recovery of sperm parameters and also prevents sperm chromatin abnormalities and apoptosis fallowing vitrification.

Conflicts of interest

Authors don't have any conflict of interest in this research.

Acknowledgments

This study was supported by a grant from Research and Clinical Center for Infertility, Shahid Sadoughi University of Medical Sciences, Yazd, Iran. The authors specially thank Professor Abbas Aflatoonian and his colleagues for their support during the course of this study.

References

[1] Di Santo M, Tarozzi N, Nadalini M, Borini A. Human sperm cryopreservation: update on techniques, effect on DNA integrity, and implications for ART. Adv Urol 2012;2012:854837.
[2] Agha-Rahimi A, Khalili MA, Nabi A, Ashourzadeh S. Vitrification is not superior to rapid freezing of normozoospermic spermatozoa: effects on sperm parameters, DNA fragmentation and hyaluronan binding. Reprod Biomed Online 2014;28(3):352–8.
[3] Khalili MA, Adib M, Halvaei I, Nabi A. Vitrification of neat semen alters sperm parameters and DNA integrity. Urol J 2014;11(2):1465–70.
[4] Donnelly ET, McClure N, Lewis SE. Cryopreservation of human semen and prepared sperm: effects on motility parameters and DNA integrity. Fertil Steril 2001;76(5):892–900.
[5] Agarwal A, Prabakaran SA, Said TM. Prevention of oxidative stress injury to sperm. J Androl 2005;26(6):654–60.
[6] Talebi AR, Fesahat F, Mangoli E, Ghasemzadeh J, Nayeri M, Sadeghian-Nodoshan F. Relationship between sperm protamine deficiency and apoptosis in couples with unexplained repeated spontaneous abortions. Int J Reprod Biomed 2016;14(3):199–204.
[7] Talebi AR, Khalili MA, Vahidi S, Ghasemzadeh J, Tabibnejad N. Sperm chromatin condensation, DNA integrity, and apoptosis in men with spinal cord injury. Int J Reprod Biomed 2013;36(2):140–6.
[8] Pourentezari M, Talebi AR, Mangoli E, Anvari M, Rahimipour M. Additional deleterious effects of alcohol consumption on sperm parameters and DNA integrity in diabetic mice. Andrologia 2016;48(5):564–9.
[9] Chi HJ, Kim JH, Ryu CS, Lee JY, Park JS, Chung DY, et al. Protective effect of antioxidant supplementation in sperm-preparation medium against oxidative stress in human spermatozoa. Hum Reprod 2008;23(5):1023–8.
[10] Grossfeld R, Sieg B, Struckmann C, Frenzel A, Maxwell WM, Rath D. New aspects of boar semen freezing strategies. Theriogenology 2008;70(8):1225–33.
[11] Li Z, Lin Q, Liu R, Xiao W, Liu W. Protective effects of ascorbate and catalase on human spermatozoa during cryopreservation. J Androl 2010;31(5):437–44.
[12] Fanaei H, Khayat S, Halvaei I, Ramezani V, Azizi Y, Kasaeian A. Effects of ascorbic acid on sperm motility, viability, acrosome reaction and DNA integrity in teratozoospermic samples. Iran J Reprod Med 2014;12(2):103–10.
[13] Isachenko V, Maettner R, Petrunkina AM, Mallmann P, Rahimi G, Sterzik K, et al. Cryoprotectant-free vitrification of human spermatozoa in large (up to 0.5 mL) volume: a novel technology. Clin Lab 2011;57(9–10):643–50.
[14] Mangoli E, Talebi AR, Anvari M, Pourentezari M. Effects of experimentally-induced diabetes on sperm parameters and chromatin quality in mice. Iran J Reprod Med 2013;11(1):53–60.
[15] Talebi AR, Mangoli E, Nahangi H, Anvari M, Pourentezari M, Halvaei I. Vitamin C attenuates detrimental effects of diabetes mellitus on sperm parameters, chromatin quality and rate of apoptosis in mice. Eur J Obstet Gynecol Reprod Biol 2014;181:32–6.
[16] Taghizabet N, Mangoli E, Anbari F, Masoodi SA, Talebi AR, Mazrooei M. The effect of heracleum persicum (Golpar) oil and alcoholic extracts on sperm parameters and chromatin quality in mice. Int J Reprod Biomed 2016;14(6):365–70.
[17] Rahimipour M, Talebi AR, Anvari M, Sarcheshmeh AA, Omidi M. Effects of different doses of ethanol on sperm parameters, chromatin structure and apoptosis in adult mice. Eur J Obstet Gynecol Reprod Biol 2013;170(2):423–8.
[18] Jankovicova J, Antalikova J, Simon M, Horovska L. Acrosomal and viability status of bovine spermatozoa evaluated by two staining methods. Acta Vet Hung 2008;56(1):133–8.
[19] Rossi T, Mazzilli F, Delfino M, Dondero F. Improved human sperm recovery using superoxide dismutase and catalase supplementation in semen cryopreservation procedure. Cell Tissue Bank 2001;2(1):9–13.
[20] Kotdawala AP, Kumar S, Salian SR, Thankachan P, Govindraj K, Kumar P, et al. Addition of zinc to human ejaculate prior to cryopreservation prevents freeze-thaw-induced DNA damage and preserves sperm function. J Assist Reprod Genet 2012;29(12):1447–53.
[21] Khodayari Naeini Z, Hassani Bafrani H, Nikzad H. Evaluation of ebselen supplementation on cryopreservation medium in human semen. Iran J Reprod Med 2014;12(4):249–56.
[22] Askari HA, Check JH, Peymer N, Bollendorf A. Effect of natural antioxidants tocopherol and ascorbic acids in maintenance of sperm activity during freeze-thaw process. Arch Androl 1994;33(1):11–5.
[23] Critser JK, Huse-Benda AR, Aaker DV, Arneson BW, Ball GD. Cryopreservation of human spermatozoa. III. The effect of cryoprotectants on motility. Fertil Steril 1988;50(2):314–20.
[24] Yogev L, Gamzu R, Paz G, Kleiman S, Botchan A, Hauser R, et al. Pre-freezing sperm preparation does not impair thawed spermatozoa binding to the zona pellucida. Hum Reprod 1999;14(1):114–7.
[25] Isachenko E, Isachenko V, Katkov II, Rahimi G, Schondorf T, Mallmann P, et al. DNA integrity and motility of human spermatozoa after standard slow freezing versus cryoprotectant-free vitrification. Human Reprod 2004;19(4):932–9.
[26] Ribas-Maynou J, Fernandez-Encinas A, Garcia-Peiro A, Prada E, Abad C, Amengual MJ, et al. Human semen cryopreservation: a sperm DNA fragmentation study with alkaline and neutral Comet assay. Andrology 2014;2(1):83–7.
[27] Duru NK, Morshedi MS, Schuffner A, Oehninger S. Cryopreservation-Thawing of fractionated human spermatozoa is associated with membrane phosphatidylserine externalization and not DNA fragmentation. J Androl 2001;22(4):646–51.
[28] Baumber J, Ball BA, Linfor JJ, Meyers SA. Reactive oxygen species and cryopreservation promote DNA fragmentation in equine spermatozoa. J Androl 2003;24(4):621–8.
[29] Colagar AH, Marzony ET. Ascorbic Acid in human seminal plasma: determination and its relationship to sperm quality. J Clin Biochem Nutr 2009;45(2):144–9.
[30] Branco CS, Garcez ME, Pasqualotto FF, Erdtman B, Salvador M. Resveratrol and ascorbic acid prevent DNA damage induced by cryopreservation in human semen. Cryobiology 2010;60(2):235–7.
[31] Fernandes GS, Fernandez CD, Campos KE, Damasceno DC, Anselmo-Franci JA, Kempinas WD. Vitamin C partially attenuates male reproductive deficits in hyperglycemic rats. Reprod Biol Endocrinol: RB&E 2011;9:100.
[32] Tarin JJ, Brines J, Cano A. Antioxidants may protect against infertility. Hum Reprod 1998;13(6):1415–6.
[33] Lewis SE, Sterling ES, Young IS, Thompson W. Comparison of individual antioxidants of sperm and seminal plasma in fertile and infertile men. Fertil Steril 1997;67(1):142–7.