



Original Article

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

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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.© 2018 Taiwan Association of Obstetrics & Gynecology. Publishing services by Elsevier B.V. This is an open access article under the CC BY-NC-ND license (<http://creativecommons.org/licenses/by-nc-nd/4.0/>).

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.

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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 μ M) according to [11,12] was added to processed samples and then vitrified. In group V ($n = 40$), vitamin C (600 μ M) 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 μ l 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 μ l 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 McIlvaine 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) deoxy-uridine 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 \times 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,i} 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.

H₂O₂ and O²⁻ 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 following vitrification.

Conflicts of interest

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

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