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Brief Report

Combined Effect of Trolox and EDTA on Frozen-Thawed Sperm Quality

Sara Keshtgar[1], PhD;
Farideh Iravanpour[2], MS;
Behrooz Gharesi-Fard[3,4], PhD;
Marjaneh Kazerooni[5], MS

[1]Department of Physiology, School of Medicine, Shiraz University of Medical Sciences, Shiraz, Iran;
[2]Student Research Committee, Shiraz University of Medical Sciences, Shiraz, Iran;
[3]Department of Immunology, School of Medicine, Shiraz University of Medical Sciences, Shiraz, Iran;
[4]Infertility Research Center, Shiraz University of Medical Sciences, Shiraz, Iran;
[5]Shiraz Infertility Center, Shiraz, Iran

Correspondence:
Sara Keshtgar, PhD;
Department of Physiology, School of Medicine, Zand St, Postal code: 71348-45794, Shiraz, Iran
Tel/Fax: +98 71 32302026
Email: Keshtgar@sums.ac.ir
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Abstract

The freezing and thawing process not only is associated with serious damage to sperm such as damage to the plasma membrane and the acrosomal membrane but also changes the membrane permeability to some ions including calcium. Also, the generation of oxygen free radicals is increased during the freezing-thawing process. The purpose of this study was to evaluate of the effects of Trolox as an antioxidant and edetic acid (EDTA) as a calcium chelator on frozen-thawed (FT) sperm and compare these effects with those on fresh sperm. This study was done on these men of 25 healthy men, who referred to Shiraz Infertility Centerbetween2012 and2013. Normal samples were transferred to the ReproductivePhysiology Laboratory, Department of Physiology,Shiraz University of Medical Sciences, Shiraz. The samples were divided into two groups randomly: fresh and FT sperm groups. Each group was divided into five subgroups: control group, the solvent group (0.1%dimethyl sulfoxide [DMSO]), Trolox group (200µM), EDTA group (1.1mM), and Trolox+EDTA group. The percentages of motility, viability, and acrosome-reacted sperm were tested. The percentages of motility and viability in the FT sperm were lower than those in the fresh sperm. The progressive motility of the FT sperm was improved nonsignificantly with Trolox+EDTA. However, the effect of Trolox+EDTA on the progressive motility of the FT sperm was much more than that on the fresh sperm. The fewest acrosome-reacted sperm were observed in the EDTA-containingFT sperm. Antioxidant supplementation or omission of extracellular calcium may partly improve motility and also reduce acrosomal damage in FT sperm.

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Keywords ● Freezing ● 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid ● Edetic acid ● Sperm motility ● Acrosome reaction

What's Known

- Cryopreservation of sperms is associated with serious damage to these cells such as damage to plasma and acrosomal membrane and changes inmembrane permeability to some ions, including calcium. Generation of oxygen-free radicals increases during the freezing-thawing process.
- These events lead to a decrement in sperm motility and viability.

What's New

- We, for the first time, investigated the effects of Trolox as an antioxidant and Edetic Acid (EDTA) as a calcium chelator on fresh and frozen-thawed sperm.
- Combination of Trolox and EDTA partly improved frozen-thawed sperm motility and reduced the spontaneous acrosomal reaction of sperm.

Introduction

Frozen-thawed (FT) sperm have been used for artificial insemination for more than 5 decades.[1] Nowadays, the storage of frozen sperm is done in infertility clinics for various reasons, including storage and maintenance of the donor's sperm for future use and preservation of fertility following chemotherapy, radiotherapy, or various surgical procedures such as vasectomy.[2-4] Sometimes, sperm are used for artificial insemination with the husband's semen in procedures such as intracytoplasmic

sperm injection, intrauterine insemination, and *in vitro* fertilization.[2,3] Despite the tremendous advances in cryopreservation methods during recent years, research has demonstrated that the long-term storage of semen in a frozen state leads to severe damage to sperm.[5-7] Some studies have indicated a decrease in the motility and viability of such sperm[8,9] and changes in sperm morphology such as changes in the plasma membrane and acrosomal changes[8] after thawing. It has also been reported that the freezing process increases the rate of DNA damage and DNA fragmentation, decreases the percentage of non-capacitated sperm, and increases the rate of acrosome reaction.[9] Likewise, investigations on the effect of the sperm freezing and thawing process suggest that the percentage of the production of oxygen free radicals is increased during incubation.[10,11] It has also been shown that a reduction in the content of glutathione, which plays a role in the antioxidant defense system of sperm, is induced by the freezing and thawing process. Also, in this report it was demonstrated that the addition of 1mM of glutathione to freezing medium was able to reduce free radicals and increase the motility and viability of post-thaw sperm.[11] Moreover, the addition of reduced glutathione to the fertilization medium significantly increased the rate of the *in vitro* fertilization of eggs by FT sperm.[12] The evaluation of intracellular antioxidants showed that the levels of antioxidants such as superoxide dismutase and glutathione were much lower in FT sperm than in fresh sperm.[13] A variety of antioxidants such as Trolox, catalase, alpha-tocopherol, and ascorbic acid have been used to improve FT sperm status in many experiments.[7,14-21]

Thermal shock, formation of intracellular ice crystals, cellular dehydration, increased concentration of salts, and osmotic shock may occur during the freezing and thawing of human spermatozoa.[5] Moreover, cryopreservation leads to structural adverse changes in the plasma membrane.[5-7,14,18] Changes in the membrane permeability to some ions such as calcium during the freezing and thawing process have also been reported.[6,22]

Calcium plays an essential role in sperm motility and also in the membrane fusion process during acrosome reaction. Binding to the zona pellucid or progesterone leads to an increase in the intracellular calcium concentration of sperm due to the opening of the calcium channel and also the releasing of calcium from intracellular stores.[23,24] The rise in the intracellular calcium concentration causes the activation of phosphatidylinositol 4,5-bisphosphate (PIP_2)-specific phospholipase Cand hydrolysis of PIP_2 and production of two second messengers: diacylglycerol and inositol 1,4,5-triphosphate(IP_3).[23] IP_3 stimulates the release of calcium from intracellular stores, and diacylglycerol activates cytoplasmic protein kinase and phospholipase A_2.[23,25] These events once again cause a rise in the intracellular calcium concentration. Eventually, a further elevation in $[ca^{2+}]_i$, which occurs prior to acrosome reaction, together with hydrolysis of PIP_2 and resulting products from phospholipase A_2 activation, leads to actin depolymerization and membrane fusion, the final event of acrosome reaction.[23,25]

On the other hand, the influx of extracellular calcium into fresh sperm leads to an increase in the production of free radicals in the cell.[26-28] Likewise, some studies have demonstrated that fresh sperm incubation in media containing calcium ionophore A23187 results in a significant increase in the production of reactive oxygen species.[26,28-30] Based on this finding, calcium chelators such as edetic acid(EDTA) have been used in some studies to improve sperm function. However, EDTA has spermicidal activity and this effect is time-dependent and dose-dependent.[22,31]

Considering the increase in intracellular calcium due to cell membrane damage in FT sperm[6,32] and with respect to the beneficial effects of antioxidants on these sperm,[7,16,17,20] we sought to examine the simultaneous effect of Trolox as an antioxidant and EDTA as a calcium chelator on FT sperm. The combination effect of an antioxidant with EDTA on the quality of fresh and FT sperm has not been investigated previously, and the results of this study may suggest a simple method to augment the quality of FT sperm.

Patients and Methods

Sample Collection and Semen Analysis

Semen samples were obtained from male volunteers (between 20 and 40 years old) who referred to the Shiraz Infertility Center to undergo routine semen analysis between 2012 and 2013. Samples were collected after 3 to 5 days of sexual abstinence. Semen volume, appearance, pH, and viscosity as well as sperm concentration, motility, and morphology were evaluated after liquefaction based on the guidelines of the World Health Organization (WHO).[2] The experiments were carried out randomly on 25 completely normal samples (table 1). These samples were transferred to the Reproductive Physiology Laboratory of the Physiology Department, Shiraz University of Medical Sciences, Shiraz,

Table 1: Analysis of the selected semen samples

Semen characteristics	Mean±standard error	
	Fresh group (n, 13)	Frozen-thawed group (n, 12)
Semen volume (mL)	4.0±0.4	3.2±0.3
pH	7.8±0.07	7.8±0.07
Sperm concentration ($\times 10^6$/mL)	107.6±12.6	125±12
Total sperm count ($\times 10^6$)	438.6±74.7	374.5±38.7
Progressive motility (%)	41.8±3.1	40.7±3.4
Nonprogressive motility (%)	14.8±1.8	12.9±1.6
Immotile sperm (%)	43.4±3.2	46.4±2.3
Abnormal sperm morphology (%)	52.1±1.4	48.7±2.1
Viability (%)	57.1±1.2	57.9±1.9
Viscosity	Normal	Normal

Iran. This research was approved by the local Research Ethics Committee of Shiraz University of Medical Sciences (code # EC-91-6159).

Sperm Prepration and Treatment

The samples were randomly divided into two groups (freshsperm group, n, 13; FTspermgroup, n, 12). The semen samples of the two groups were washed with Ham's F-10 medium (Sigma, N6633), centrifuged at 1,200 rpm for 10 minutes, and the supernatant was removed. Half a milliliter of Ham's F-10 was added, and the samples were incubated at 37°C under 5% CO_2. After 60 minutes of incubation, the supernatant containing motile sperm was collected. Sperm concentration, motility, and viability were evaluated. Thirteen samples were considered as the fresh sperm group and 12 samples as the FTsperm group. A CryoSperm medium (ORIGIO11010010) was added to the samples very slowly, which was considered as the FTsperm group. The samples were loaded into straws after 10 minutes and kept at -70°C for 30 minutes and thereafter in liquid nitrogen for at least 1 month. Thawing was done at room temperature as previously described.[2,33]

The fresh and FT sperm underwent experimental procedures as follows. Each sample was divided into five groups, containing 10×10^6 sperm/mL. One group was designated the control group, and the incubation medium of the other groups contained 0.1% dimethyl sulfoxide (DMSO) (Sigma, D2650), 200 µM of Trolox (Sigma, 238813), 1.1 mM of EDTA (Sigma, E6758), and 1.1 mM of EDTA + 200 µM Trolox, respectively. The samples were then incubated for 60 minutes at 37°C under 5% CO_2.

Asessment of Sperm Motility and Viability

Sperm motility was assessed at 400X magnification using phase-contrast microscopy (Olympus, BX51, Tokyo, Japan). Video-recording equipment was also used for the quality control of manual motility assessment procedures according to the WHO's guideline.[2] The percentages of the progressive and nonprogressive motility and immotile sperm were assessed.

Sperm viability was evaluated by Eosin Y staining. At least 200 sperm were examined in each evaluation.

Evaluation of Acrosomal Status

Acrosomal status was evaluated with fluorescein isothiocyanate-conjugated Pisumsativum (FITC-PSA) staining according to the procedure previously reported.[34] Briefly, 10µL of the sample was smeared on glass slides and fixed by methanol for 30 seconds at room temperature. The slides were frozen at -20°C until staining. These slides were then incubated for 30 minutes with 50 µg/mL of FITC–PSA (Sigma, L0770) and, thereafter, washed with double-distilled water. Acrosomal status was assessed using fluorescence microscopy at 1000X magnification (Olympus, BX51, Tokyo, Japan). Sperm with green fluorescence in the acrosomal region were scored as acrosome intact, whereas those with no fluorescence in the acrosomal region or only fluorescence of the equatorial segment were considered acrosome-reacted.

Statistical Analysis

The statistical analyses were performed using Statistical Package for the Social Sciences (SPSS), version 16.0, for Windows. The evaluation of the differences between the experimental groups was performed using the Mann–Whitney U test. All the data are presented as mean values± standard error (SE). A P value <0.05 was considered statistically significant.

Results

At the commencement of the experiment, the total motility of the sperm chosen for the fresh and FT sperm groups was 88.7±1.8 and 83.9±1.3, respectively. The sperm viability of the sperm chosen for the fresh and FT sperm groups was 90.9±1.4 and 88.2±1.4, respectively.

The percentages of the sperm with progressive motility and the total motility of the fresh and FT sperm, after treatments, are reported in table 2. The freezing procedures decreased sperm motility significantly. The motility of the FT sperm treated with Trolox+EDTA was increased nonsignificantly. None of the

treatments significantly affected the motility of the experimental groups.

The changes in the percentage of the motile sperm in each subgroup were calculated relative to their control group (table 2). This comparison showed that EDTA, especially in combination with Trolox, had the best effect on progressive motility in the FT sperm.

In the samples selected for freezing, 88% of the cells were viable but the percentage of the live sperm was decreased significantly to 44% after thawing. The incubation of the sperm in the medium containing EDTA and Trolox did not cause any significant changes in viability (table 3).

Acrosome loss was significantly greater in the FT sperm than in the fresh ones (table 4). Spontaneous acrosomal reaction in the fresh sperm was not affected by EDTA or Trolox; however, EDTA decreased acrosome loss in the FT sperm.

Discussion

The long-term storage of sperm necessitates that they be maintained in a frozen state. This technique confers hope to individuals who previously had no chance of fertility.[4] Much effort has been made to improve freezing and thawing methods, and these methods have quickly evolved during the last years. Despite these improvements and the fact that we employed one of the best available standard media and methods suggested in the literature, sperm cryopreservation caused a severe decrease in sperm quality (motility and viability). Moreover, acrosome reaction was observed in almost 80% of these sperm. Such sperm are not capable of fertilizing eggs.

In the present study, 1.1 µM of EDTA was used to investigate the effect of the decrement of extracellular calcium on sperm motility. Our findings revealed no effect of EDTA on sperm motility either singularly or in combination with Trolox. In physiological conditions, specific stimuli such as progesterone and zona pellucida cause the opening of membrane calcium channels,[23,24] and we suggest that the impact of extracellular calcium depletion on cellular function is observed just when the calcium channels are open. Although it has been reported that EDTA treatment(10 and 100 µM/mL) improves the progressive motility of sperm,[35] in the current study, the use of EDTA in a higher concentration (more than 1.1 µM) resulted in a severe decline in sperm viability (data are not shown). Many reports have mentioned that the use of Trolox (40,60, and 120 µM) in a freezing extender augments the motility of FT sperm.[7,17] Moreover, the addition of Trolox (100 and 200 µM) or alpha-tocopherol to freezing extenders of human[19] and bearsperm[18,20] improved motility in thawed sperm. In the current study, the addition of 200 µM of Trolox to an FT sperm medium led to no change in the motility of FT sperm. We suggest that sperm are damaged during the freezing procedure and that Trolox exerts a protective effect if added to the freezing medium.

It was reported that the use of sperm-freezing extenders or thawing solutions supplemented by

Table 2: Motility of the fresh and FT sperm in different experimental subgroups after 1 hour's incubation andchanges (%) in progressive motility relative to the control group under treatment with Trolox and EDTA

Group Motility(%)	Mean±standard error				
	Control	DMSO	Trolox	EDTA	Trolox+EDTA
Fresh (N, 13)					
Progressive motility	77.0±2.9	75.3±3.6	78.6±2.7	76.3±2.6	76.3±2.0
Total motility	92.8±1.1	91.8±1.5	93.5±1.3	91.7±0.9	90.1±1.3
Change in progressive motility (%)	100	98.4±3.9	102.6±2.9	100.4±4.4	100.3±4.2
FT (N, 12)					
Progressive motility	16.5±2.2[a]	14.3±2.8[a]	17.8±2.6[a]	21.4±2.8[a]	23.4±2.3[a]
Total motility	38.4±3.9[a]	39.8±5.3[a]	42.4±4.6[a]	41.3±3.5[a]	44.6±3[a]
Change in progressive motility (%)	100	108.6±28.2	115.6±16.2	157±29.6	184.5±37[a]

[a]Significant difference relative to fresh sperm under similar treatment (P<0.005); FT: Frozen-thawed; EDTA: Edetic acid; DMSO: Dimethyl sulfoxide

Table 3: Viability of the fresh and FT sperm under treatment with EDTA and Trolox

Subgroups Live sperm (%)	Mean±standard error				
	Control	DMSO	Trolox	EDTA	Trolox+EDTA
Fresh sperm (N, 13)	91.8±1.2	91±1.3	94.4±1	91±0.8	90.1±1.4
FT sperm (N, 12)	44.0±3.4[a]	47.6±2.6[a]	44.4±4.7[a]	45.9±4.0[a]	47.4±3.3[a]

[a]Significant difference relative to fresh sperm under similar treatment (P<0.005); FT: Frozen-thawed; EDTA: Edetic acid; DMSO: Dimethyl sulfoxide

Table 4: Acrosomal reaction in the fresh and FT sperm under treatment with EDTA and Troloxt

Groups Acrosome reaction (%)	Mean±standard error (N, 5)			
	Control	Trolox	EDTA	Trolox+ EDTA
Fresh	8.3±1.9	5.7±0.6	5.4±0.6	5.6±1.4
FT	83.4±3.2[ab]	80.08±3.3[ab]	64.6±2.7[a]	63.9±4[a]

[a]Significant difference relative to fresh sperm under similar treatment (P<0.005); [b]Significant difference relative to EDTA-containing groups (P<0.02); FT: Frozen-thawed; EDTA: Edetic acid

EDTA resulted in the improved motility of thawed sperm.[22,31] The addition of 6mM of EGTA + 6 mM of EDTA to the thawing solution enhanced sperm motility.[22] However, it was mentioned that the treatment of post-thaw sperm with any dose of ethylene glycol tetra acetic acid (EGTA) in the absence of EDTA had limited positive effects.[22] In the present study, the addition of Trolox and EDTA to the FT sperm medium (after thawing) either separately or concomitantly did not cause any significant changes in sperm motility; nevertheless, the sperm motility in the group containing Trolox+EDTA was better than that in the other groups. To compare the effects of Trolox and EDTA on progressive motility between fresh and FT sperm, we considered the percentage of progressive motility in the control group 100% and evaluated the other groups with respect to it (table 2). Our results indicated that although the addition of Trolox and EDTA to the fresh sperm medium did not cause any changes in sperm motility, the use of Trolox+EDTA caused a significant increase in the motility of the FT sperm. As has been previously mentioned, calcium channels in fresh sperm do not open without stimulation.[23,24] In this condition, the addition of a calcium chelator or antioxidant would cause no changes in sperm. Nonetheless, FT sperm membrane undergoes impairment,[7,8,36] causing an over rate of calcium influx.[6,22,32] As was mentioned earlier, the influx of extracellular calcium into fresh sperm leads to a rise in the production of free radicals in the cell. Consequently, the increased intracellular calcium either directly or due to the production of extra reactive oxygen species leads to a reduction in sperm motility. Thus, the concomitant addition of Trolox and EDTA to a thawed sperm medium can improve sperm motility. Also other studies on FT sperm have indicated that the fraction of viable cells declines and that sperm undergo early capacitation.[22] Trolox and EDTA could neither in fresh sperm nor in FT sperm significantly influence sperm viability. We suggest that the adverse effects on sperm viability occur during the freezing process. Hence, using antioxidant supplementation or extracellular calcium depletion after thawing would not work.

Although some investigators have reported that the use of EDTA has no effect on acrosome reaction,[35] there are studies indicating that EDTA supplementation reduces acrosome reaction through the calcium omission of the sperm medium.[31,37] However, in the present research, EDTA supplementation had no effect on the acrosomal status of the fresh sperm. Perhaps the most interesting observation in this study was that the lowest rate of acrosome reaction was observed in the groups containing EDTA (with or without Trolox), and this decline was significant compared to all the groups lacking EDTA. EDTA considerably reduced the rate of acrosome reaction through the omission of extracellular calcium. The difference in the effect of EDTA on the fresh and FT sperm may have been due to the membrane impairment in the FT sperm.

The effect of other antioxidants and calcium chelators should be examined to achieve the best incubation extender for FT sperm.

Conclusion

The findings of this study indicated that the freezing and thawing process of sperm caused severe irreversible damage to the sperm and that the antioxidant supplementation or omission of extracellular calcium might partly improve motility and reduce acrosomal damage.

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Conflict of Interest: None declared.

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