IJMS
Vol 45, No 3, May 2020

Original Article

The Effects of EGTA on the Quality of Fresh and Cryopreserved-Thawed Human Spermatozoa

Bahareh Ebrahimi, MSc;
Sara Keshtgar, PhD

Department of Physiology, School of Medicine, Shiraz University of Medical Sciences, Shiraz, Iran

Correspondence:
Sara Keshtgar, PhD;
Department of Physiology, School of Medicine, Shiraz University of Medical Sciences, Zand Blvd, Postal Code: 71348-45794, Shiraz, Iran
Tel/Fax: +98 71 32302026
Email: keshtgar@sums.ac.ir
Received: 31 October 2018
Revised: 24 November 2018
Accepted: 02 December 2018

What's Known

- Despite the considerable development made in the sperm cryopreservation techniques, it has numerous harmful effects on sperm quality. The exact mechanism of this damage is not clear; however, the reduction in the sperm motility, increase in the mortality rate, and production of reactive oxygen species are the common damages incurred during cryopreservation and calcium overload conditions.

What's New

- Intracellular calcium content decreased after sperm cryopreservation, and it was found that the cryoinjury was not related to calcium overload. Calcium chelation with EGTA confirms this conclusion. EGTA showed no protective effect on fresh or cryopreserved human sperm. Adenosine triphosphate was decreased, while reactive oxygen species production increased in the EGTA-treated sperm.

Abstract

Background: Sperm cryopreservation-thawing process has damaging effects on the structure and function of sperm, namely cryoinjury. Calcium overload has been reported as a postulated mechanism for sperm damage during the first steps after thawing. This study was designed to assess the intracellular calcium (Ca^{2+}_i) after cryopreservation and to clarify the role of a calcium chelator ethylene glycol-bis (2-aminoethyl ether)-N, N, N′, N′-tetraacetic acid (EGTA) on human sperm quality.

Methods: Forty semen samples were obtained from fertile men (March 2017 to 2018). The samples were randomly divided into fresh (F) and cryopreserved-thawed (CT) groups. The F and CT samples were divided into control and 1 mM EGTA-treated groups. Sperm kinematics and membrane integrity were assessed. The reactive oxygen species (ROS) and adenosine triphosphate (ATP) were measured by luminescent methods. Ca^{2+}_i, apoptotic rate, and mitochondrial membrane potential (MMP) were evaluated using flow cytometric methods. Data were compared using SPSS software, version 16.0 by ANOVA and Kruskal-Wallis test. $P<0.05$ was considered as significant.

Results: Cryopreservation decreased sperm motility, viability, membrane integrity, Ca^{2+}_i, MMP, and induced cell apoptosis and ROS production. EGTA could not protect the cryopreserved sperm from cryoinjury. It was found to have destructive effects on fresh sperm motility and viability (P=0.009) relative to cryopreserved sperm. ATP was reduced (P=0.02) and ROS production (P=0.0001) was increased in the EGTA-treated F and CT sperms.

Conclusion: Despite Ca^{2+}_i reduction by EGTA, it had no protective effects on fresh or cryopreserved sperm. We concluded that sperm cryoinjury was not dependent on calcium overload, and it was suggested that cryoinjury was mainly related to cell membranes damage.

Please cite this article as: Ebrahimi B, Keshtgar S. The Effects of EGTA on the Quality of Fresh and Cryopreserved-Thawed Human Spermatozoa. Iran J Med Sci. 2020;45(3):188-198. doi: 10.30476/ijms.2019.45787.

Keywords ● Spermatozoa ● Cryopreservation ● Calcium ● Egtazic acid

Introduction

Calcium ion plays an important role in sperm motility, acrosome reaction, and fertilization.[1-3] Sperm exposure to progesterone or zona pellucid proteins induces hyperactivation, which is accompanied by intracellular Ca^{2+} (Ca^{2+}_i) elevation.[4] An increase in Ca^{2+}_i occurs due to the entrance of calcium through its channels and/or its release from intracellular stores.[5, 6] Ca^{2+}_i elevation affects mitochondria and produces more adenosine triphosphate (ATP).[7] It also activates many signaling cascades and increases reactive oxygen species (ROS) production by mitochondria and membrane oxidizing enzymes.[8, 9] In addition to the beneficial effects of calcium, its overload can potentiate a pathological condition by producing excessive amounts of ROS and oxidative stress, reducing ATP synthesis, and forming permeability transition (PT) pores in the mitochondrial inner membrane.[10, 11] PT pores are permeable to solutes of <1500 Dalton.[12] Mitochondrial swelling and its outer membrane rupture are the consequences of PT pore opening. Furthermore, calcium overload causes cytochrome C release from mitochondria, which is a key event in apoptosis.[13] Some researchers, such as Treulen, reported that PT pore formation and cell apoptosis were associated with an increment in Ca^{2+}_i, mitochondrial membrane potential (MMP) dissipation, ATP level reduction, ROS production, and deterioration of plasma membrane integrity.[14]

Sperm cryopreservation is used in infertility treatment clinics as a therapeutic option for various conditions, including storage and maintenance of the donor's sperm for future usage, preservation of fertility following chemotherapy, radiotherapy, or various surgical procedures such as vasectomy. Despite the considerable development made, cryopreservation has numerous harmful effects on the structure and function of sperm, namely cryoinjury.[15] A reduction in sperm motility and viability, acrosomal loss, the increase in ROS production, damage to the mitochondria, and DNA fragmentation were observed in semen samples that thawed after cryopreservation.[16, 17] Cryoinjury changes the membrane permeability to some ions such as calcium,[14, 18] which leads to their entrance. Although some studies have revealed other aspects of this issue, they have shown that after thawing, Ca^{2+}_i decreases significantly.[19, 20] Calcium overload has been reported as a postulated mechanism for sperm damage.[14, 18] As a result, and due to these harmful effects, some researchers believe that using calcium chelators would be effective in maintaining sperm viability and function.[21] Calcium chelation by agents such as ethylene glycol-bis (2-aminoethyl ether)-N, N, N′, N′-tetraacetic acid (EGTA) or ethylenediaminetetraacetic acid (EDTA) inhibited the zona pellucida-induced acrosome reaction and apoptosis, and increased sperm motility.[22, 23] It has been shown that adding both EDTA and EGTA to freeze-drying medium protected rabbits' sperm DNA.[24] Adding EGTA and EDTA to cryopreservation medium improved the developmental ability of oocytes and decreased boar sperm DNA fragmentation.[25] The protective effects of EDTA on cryopreserved human sperm have also been reported.[26]

There are several studies with conflicting results, suggesting that the use of calcium chelators cannot be effective since spermatozoa require calcium boost to succeed in reproduction.[27, 28] Studies have shown that calcium chelating by these chelators could cause harmful effects, reduce sperm motility, disrupt sperm capacitation, decrease tyrosine phosphorylation, and block Ca^{2+}_i stores release.[14, 28, 29] A decrement on boar sperm motility was reported after the use of 6 mmol/L EDTA.[25]

The incubation of bovine cryopreserved sperm with EDTA containing medium could not prevent the mitochondrial permeability damage.[14] In addition, EDTA has time and dose-dependent spermicidal activity.[24, 30]

EGTA is known as a specific calcium chelator, which has a lower affinity for other cations, whereas EDTA function is not specific for calcium and chelates several other metal ions such as Mg, Mn, Co, Zn, Pb, Cu, and Fe. It has a potential inhibitory activity on metal cation-dependent enzymes.

With regard to these controversies and to elucidate the exact mechanism of EGTA function on fresh and cryopreserved-thawed human sperm, we assessed the sperm kinematics, Ca^{2+}_i, apoptosis rate, ROS production, membrane integrity, mitochondrial membrane potential, and ATP content in the EGTA-incubated fresh and thawed sperms.

Materials and Methods

Samples Preparation and Cryopreservation

Semen samples were obtained randomly from 20-40-year-old fertile men who had referred to Shiraz Infertility Center from March 2017 to 2018 to undergo a routine semen analysis. The local Ethics Committee of Shiraz University of Medical Sciences approved this research (IR.sums.REC.1391-6159). All donors had signed a written informed consent. They were healthy, not drug addict or alcohol and dietary supplement consumers. A routine semen analysis was performed and oligozoospermic, azoospermic, teratozoospermic, and leukocytospermia samples, as well assemen with abnormal appearance, pH, and viscosity, were excluded from the study based on the guidelines from World Health Organization, 2010.[31] The semen characteristics of fertile men were considered as: semen volume, more than 1.5 mL; total sperm number, at least 39 million per ejaculate; sperm concentration, at least 15 million per mL; vitality, more than 58% live; progressive motility, at least 32%; total (progressive and non-progressive) motility, at least 40%; and morphologically normal forms, 4.0%.[31] After the usual routine semen analysis, the samples were transferred to our laboratory in less than one hour.

Forty samples were washed with Ham's F-10 medium (N6633, Sigma, Germany) and incubated at 37 °C, 5% CO_2, for one hour. The swim-upped sperm were counted and sperm viability and kinematics were assessed. All procedures were done according to the guidelines by World Health Organization, 2010.[31] The samples were diluted to 10×10^6 sperm/ml and, randomly, divided into fresh (F) and cryopreserved-thawed (CT) groups.

For sperm cryopreservation, the samples were mixed (1:1) gradually with cryoprotective medium (11010010, ORIGIO, Denmark) for 30 minutes, loaded into a cryotube, placed on nitrogen vapor for 30 minutes, then, plunged into liquid nitrogen for at least one month. Thawing procedure was done at 37 °C.

The F and CT samples were divided into control and EGTA-treated subgroups. The sperm were incubated with Ham's F10 and 1 mM EGTA (E3889, Sigma, Germany), in control and EGTA subgroups, respectively. In CT group, EGTA was added to the cryoprotective medium. All assessments were performed after 30 minutes of incubation in F group and after 30 minutes of thawing in washed CT sperm group.

Assessing Sperm Kinematics

Sperm kinematics was recorded by a Video Test (VT) sperm analyzer 3.1 software (VT sperm 3.1, Russia), using an Olympus CX41 (nimax GmbH, Germany) microscope. The percentage of progressive, non-progressive, immotile sperm, and motility parameters, such as straight-line velocity (VSL, µm/s), average path velocity (VAP, µm/s), and curvilinear velocity (VCL, µm/s) of at least 200 sperms were evaluated for all samples.[31]

Assessing Membrane Integrity

Hypo-osmotic swelling test (HOS test) was used to assess membrane integrity.[31] Hypo-osmotic medium contained 0.735 g of sodium citrate dehydrate (W302600, Sigma, Germany) and 1.351 g of D-fructose (F0127, Sigma, Germany) in 100 mL of purified water. This solution was mixed 1:1 with sperm samples. Spermatozoa with intact membranes were swollen within five minutes and the shape of all flagella were stabilized after 30 minutes. At least, 100 sperms were evaluated after five and 30 minutes.

Assessing ROS Production

ROS production was assessed using the chemiluminescent method.[31] Freshly prepared luminol (A8511, Sigma, Germany) with a concentration of 250 µM and 12 U/mL horseradish peroxidase (P6782, Sigma, Germany) were added to 300 µL of treated sperm in 96 black well plates. Luminol reacts with hydrogen peroxide and emits energy as a photon; horseradish peroxidase enhances the oxidation of luminal. Light emissions were recorded using a microplate reader with the ability to assess luminescence (Synergy HT, Bio Tek, Germany) every 10 seconds for 30 minutes and was reported as the relative light unit (RLU).

Assessing Apoptotic Rate

Apoptotic rate was assessed using an Annexin V–FITC, PI kit (phosphatidyl serine detection kit, IQ Products®, Netherlands). According to the manufacturer's instruction, the cells (1.0×10^6 cell/mL) were washed by cold calcium buffer. The suspensions (100µL) were incubated with 10 µL of FITC-conjugated Annexin-V for 20 minutes and 10 µL of propidium iodide (PI) for at least 10 minutes on ice in dark. At least, 50,000 sperm were analyzed using a BD FACSCaliberTM flow cytometer (BD Biosciences, USA). The data were analyzed using FlowJo® (version 10.4.1) software. The cells were classified into the following four categories according to staining with Annexin and/or PI (stained +, and unstained -): (Q1) necrotic sperm [Annexin-V (-)/PI (+)], (Q2) late apoptotic cells [Annexin-V (+)/PI (+)], (Q3) early apoptotic cells [Annexin-V (+)/PI (-)], and (Q4) viable cells [Annexin-V (-)/PI (-)].

Assessing Ca^{2+}_i

The suspension containing 1×10^6 sperm was loaded with 4 µM fluo-3/AM (73881, Sigma, Germany) and 0.08% pluronic acid F-127 (p2443, Sigma, Germany) for 30 minutes. The samples were washed and 5 µM PI (p4170, Sigma, Germany) was added to the medium. The incubation was done in darkness at 37 °C.[32] At least, 50,000 sperm were analyzed using a BD FACSCaliberTM flow cytometer and FlowJo® software. The cells were classified into a quadrant according to their Ca^{2+}_i content and viability. Q1-Q4 represent the dead sperm with low Ca^{2+}_i (Fluo3-AM$^-$, PI$^+$), dead sperm with high Ca^{2+}_i (Fluo3-AM$^+$, PI$^+$), live sperm with high Ca^{2+}_i (Fluo3-AM$^+$, PI$^-$), and live sperm with low Ca^{2+}_i (Fluo3-AM$^-$, PI$^-$), respectively. Mean Fluorescence intensity (MFI) of fluo-3/AM was calculated by the software.

Assessing MMP

MMP was assessed using MitoProbeTM JC1 assay kit (M34152, Molecular Probes, USA). According to the manufacturer's instruction, 1×10^6 sperm were suspended in 1 mL warm phosphate-buffered saline, and 2 µM tetraethylbenzimidazolylcarbocyanine iodide (JC-1) was added to the medium. After 30 minutes incubation in 5% CO_2 at 37 °C, the assessment was performed by a flow cytometer using 488 nm excitation with 530 nm and 585 nm bands pass emission filters. JC-1 dye exhibits potential-dependent accumulation in mitochondria. Its aggregation at high mitochondrial membrane potential yields a red to orange colored emission at 585 nm. At low mitochondrial membrane potential, JC-1 is predominantly a monomer that yields green fluorescence with the emission of 530 nm. Changing fluorescence from red to green means that the membrane potential has decreased. Fifty µM carbonyl cyanide 3-chlorophenylhydrazone (CCCP) was used as an MMP disrupter concurrently with JC-1 to confirm that JC-1 was sensitive to mitochondrial membrane changes (positive control). The cells, which were concurrently treated with JC-1 and CCCP showed green fluorescence.

Assessing Intracellular ATP

The ATP contents of spermatozoa were determined using the bioluminescence assay kit CLS II (Roche Diagnostics GmbH, Germany). According to the manufacturer's instruction, 25 µL of the samples (1×10^6 sperms per mL) were added to 225 µL boiling extraction medium, which consisted of 100 mM Tris–HCl and 4 mM EDTA (pH 7.75). After boiling for two minutes at 100 ºC, samples were centrifuged at 1,000 g for 60 seconds and their supernatant was frozen in -20 ºC until further assessment. In the assessment day, a serial dilution of an ATP standard solution (ranging between 10^{-5} to 10^{-10} M) was prepared, and 50 µL of luciferase reagent was added to 50 µL of the samples and standards. After a one-second delay, The luminescence was measured using a microplate reader (Synergy HT, Bio Tek, Germany) for 10 seconds. ATP standard curve was plotted and the ATP concentrations of all samples were calculated. The data were expressed as nM of ATP per 10^6 sperms.

Statistical Analysis

Statistical analyses were performed using SPSS software, version 16.0. Data normality was checked using Shapiro–Wilk test. Sperm motility, membrane integrity, and ROS production had a normal distribution, and their comparisons were done by ANOVA. Other data were statistically analyzed using non-parametric test (Kruskal Wallis). The data are presented as mean±SEM, and P<0.05 was considered statistically significant.

Results

Sperm Kinematics

Sperm progressive and non-progressive motility reduced significantly (P=0.0001) after thawing, and the percentage of immotile sperms increased (P=0.0001) (table 1). EGTA decreased the percentage of progressive motile sperms in F and CT samples, and this reduction was greater in F group (36.5%) than the CT group (16.9%). Velocities of motile sperms were recorded and analyzed. The results showed that the VSL and VAP reduced after thawing. EGTA decreased both VSL and VAP in F sperm (P=0.0001), whereas VCL reduced significantly in CT group (P=0.01) (table 1).

Membrane Integrity

Assessing sperm membrane integrity by HOS test showed severe damage to sperm membrane integrity after thawing, and using EGTA did not affect the cell membrane integrity (figure 1).

Table 1: Effect of EGTA on sperm kinematics of fresh and cryopreserved-thawed

Groups Mean±SEM	Progressive Motility (%)	Non-Progressive Motility (%)	Immotile (%)	VCL (µm/sec)	VSL (µm/sec)	VAP (µm/sec)
Fresh						
Control	72.84±0.6	6.12±0.92	21.04±1.2	117.93±6.9	87.11±3.5	101.48±3.9
EGTA	46.20±3.1*	3.55±0.62	49.23±3.5*	96.02±5.3	66.95±4.6*	72.30±5.0*
Cryopreserved-Thawed						
Control	36.97±0.6#	3.78±0.51	59.24±1.0#	107.02±5.4	66.68±4.3#	67.20±5.0#
EGTA	30.71±0.9*#	4.12±0.56	65.00±1.2#	81.49±2.3*	55.64±3.1#	57.05±3.1#

Curvilinear Velocity (VCL, µm/s), Straight Line Velocity (VSL, µm/s), Average Path Velocity (VAP, µm/s), and ethylene glycol-bis (2-aminoethyl ether)-N, N, N′, N′-tetraacetic acid (EGTA). ANOVA test was used to compare each variable between the experimental groups. *Significant difference with the related control group (P=0.05), #significant difference between fresh and cryopreserved-thawed sperm (P<0.0001). Results are expressed as mean±SEM

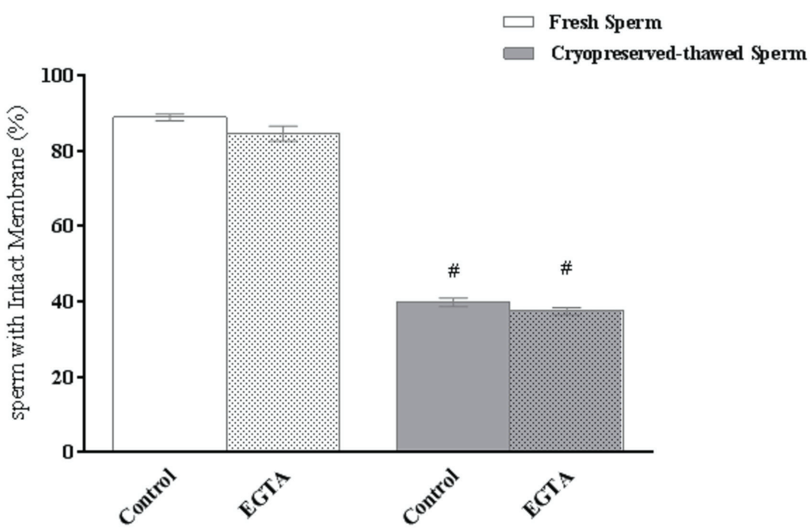

Figure 1: The graph represents the effect of EGTA on membrane integrity of fresh and cryopreserved-thawed sperm. ANOVA test was used to compare the membrane integrity between the experimental groups; #Significant difference between fresh and cryopreserved-thawed sperm *(*P<0.0001). Results are expressed as mean±SEM.

ROS Production

Chemiluminescence signals are indicators of ROS production and are recorded as RLU. ROS production increased after thawing and EGTA augmented the ROS generation in both CT and F groups (P=0.0001) (figure 2).

Sperm Apoptosis

Cryopreservation reduced the percentage of live cells and increased the apoptosis rate. EGTA decreased sperm viability in F group significantly (P=0.009), but it did not affect CT group apoptosis (figure 3).

Intracellular Calcium

Fluo3/Am and PI staining were used to assess Ca^{2+}_i in live and dead cells (table 2). Cryopreservation-thawing process significantly decreased the percentage of live cells with high Ca^{2+}_i (Fluo3-AM$^+$, PI$^-$) and increased the percentage of dead cells with low Ca^{2+}_i (Fluo3-AM$^-$, PI$^+$) (P=0.0001). MFI of Fluo-3 AM was lower in the CT group than the F group (table 2). EGTA reduced the count of live cells with high Ca^{2+}_i (Fluo3-AM$^+$, PI) and increased the live cells with low Ca^{2+}_i (Fluo3-AM$^-$, PI$^-$) in F group. EGTA decreased MFI in both F and CT groups.

Mitochondrial Membrane Potential

MMP was assessed using JC-1 staining via flow cytometric method (table 3). Red and green florescent intensity indicated the mitochondria with high and low membrane potential,

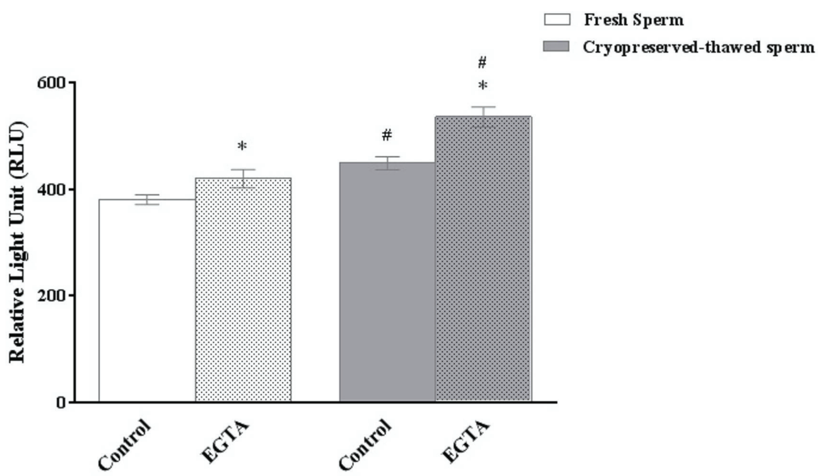

Figure 2: The graph represents the effect of EGTA on the ROS production offresh and cryopreserved-thawed sperm. ANOVA test was used to compare ROS production between the experimental groups; *Significant difference with the related control group (P<0.0001), #Significant difference between the fresh and cryopreserved-thawed sperm (P=0.01). Results are expressed as mean±SEM.

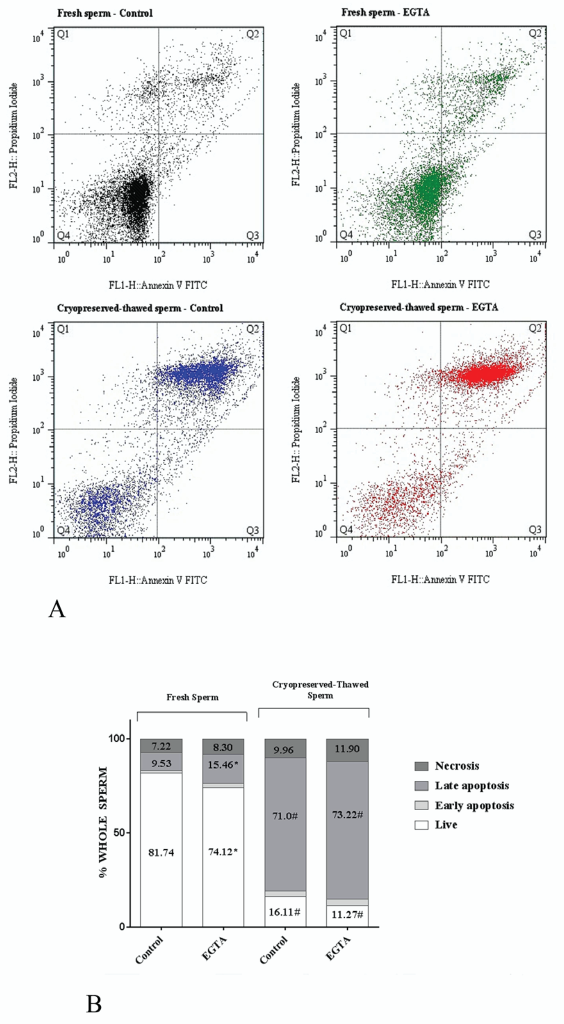

Figure 3: The graph represents the effect of EGTA on the apoptotic rate of fresh and cryopreserved-thawed sperm. Kruskal-Wallis test was used to compare the viability and mortality rate between experimental groups. Flow cytometric analysis of EGTA-treated and untreated fresh and cryopreserved-thawed sperm stained with AnnexinV-FITC and PI; (Q1) necrotic cells [Annexin-V (-)/PI (+)], (Q2) late apoptotic cells [Annexin-V (+)/PI (+)], (Q3) early apoptotic cells [Annexin-V (+)/PI (-)], and (Q4) viable cells [Annexin-V (-)/PI (-)]. (B) Stacked bar of sperm apoptosis in fresh and cryopreserved-thawed sperm and the effect of EGTA on them. *Significant difference with the related control group (P=0.05), #Significant difference between the fresh and cryopreserved-thawed sperm, (P=0.05). Results are expressed as mean±SEM.

respectively. Red/green ratio was decreased by 59.8% in CT group compared to F group. EGTA did not have any significant effect on MMP of both F and CT groups.

Intracellular ATP

ATP content did not change after the cryopreservation-thawing process, but adding EGTA reduced ATP in both F and CT sperm (P=0.02) (figure 4).

Discussion

A decrease in motility and an increase in cell ROS production, accompanied by Ca^{2+}_i reduction, were the damages caused by the cryopreservation-thawing procedure. Cryopreservation thawing also decreased sperm viability and disrupted MMP. The damaging effects of cryopreservation on sperm motility, viability, cell apoptosis, PT-pore formation, MMP disruption, antioxidants reduction, and ROS production are the well-known damages happening after the cryopreservation-thawing procedure,[16, 17] but the mechanism(s) of these destructive effects and their correlation with Ca^{2+}_i have not been conclusively investigated or proven yet. In the present study, in order to clarify the role of calcium in cryoinjury and its relation to mitochondrial function and finding the importance of membrane integrity, all aspects of sperm function were evaluated and considered together.

The elevation of Ca^{2+}_i after cryopreservation has been reported by some researchers.[14, 18] In contrast, some other studies have shown the reduction of Ca^{2+}_i after thawing.[20, 33] Kumaresan et al. showed that the proportion of sperm with high calcium decreased during the initial five minutes of incubation.[33] Based on the findings of these studies, it is possible that Ca^{2+}_i is increased promptly after a cryopreservation-thawing process, but this increase cannot be sustained more than a few minutes. Our study showed that the rate of live-high Ca^{2+}_i sperm was reduced after cryopreservation, the percentage of dead-low Ca^{2+}_i sperm increased,

Table 2: Effect of EGTA on intracellular calcium of fresh and cryopreserved-thawed sperm

Groups	Fluo3-AM$^-$, PI$^+$ (%)	Fluo3-AM$^+$, PI$^+$ (%)	Fluo3-AM$^+$, PI$^-$ (%)	Fluo3-AM$^-$, PI$^-$ (%)	MFI
Fresh					
Control	20.6 ±6.5	6.24±1.78	63.24±8.17	9.85±3.36	302.4±62.08
EGTA	30.96±7.6	1.66±.88	22.66±13.88*	44.72±12.23*	51.16±20.78*
Cryopreserved-Thawed					
Control	70.81±4.6#	3.76±1.19	13.87±3.95#	11.54±1.08	57.01±9.70#
EGTA	60.78±9.5 #	2.33±.56	14.32±2.38	22.58±9.78	42.18±7.97*

Dead cells with low Ca^{2+}_i (Fluo3-AM$^-$-PI$^+$), dead cells with high Ca^{2+}_i (Fluo3-AM$^+$-PI$^+$), live cells with high Ca^{2+}_i (Fluo3-AM$^+$- PI$^-$), live cells with low Ca^{2+}_i (Fluo3-AM$^-$-PI$^-$), mean fluorescent intensity (MFI), and ethylene glycol-bis (2-aminoethyl ether)-N, N, N′, N′-tetraacetic acid (EGTA). Kruskal Wallis test was used to compare intracellular calcium between the experimental groups. *Significant difference with the related control group (P=0.01), #Significant difference between the fresh and cryopreserved-thawed sperm (P<0.0001). Results are expressed as mean±SEM

Table 3: Effect of EGTA on the mitochondrial membrane potential of fresh and cryopreserved-thawed sperm

Groups	Aggregate JC-1 (Red, %)	Monomer JC-1 (Green, %)	Red/Green
Fresh			
Control	1124±187.4	650.0±76.0	1.79±0.26
EGTA	919.5±216.6	557.8±71.0	1.61±0.26
Cryopreserved-Thawed			
Control	709.0±186.5#	1080±122.6#	0.62±0.80#
EGTA	1112±153.3#	797.5±282.8#	0.69±0.13#

Ethylene glycol-bis (2-aminoethyl ether)-N, N, N′, N′-tetraacetic acid (EGTA). Kruskal Wallis test was used to compare mitochondrial membrane potential between the experimental groups. #Significant difference between the fresh and cryopreserved-thawed sperm (P=0.05). Results are expressed as mean±SEM

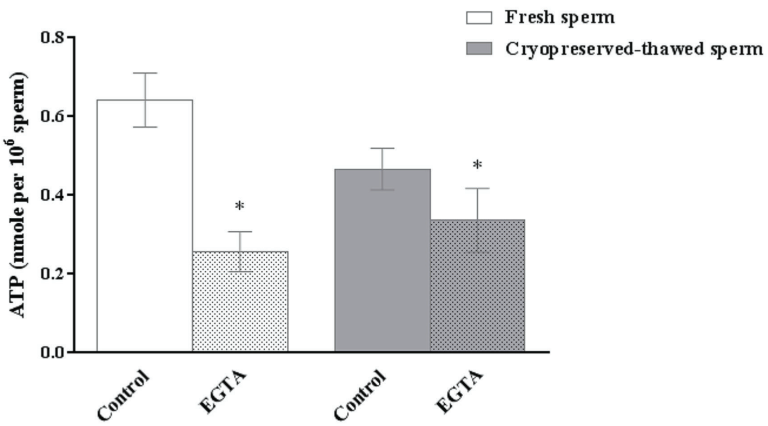

Figure 4: The graph represents the effect of EGTA on intracellular ATP of fresh and cryopreserved-thawed sperm. Kruskal Wallis test was used to compare intracellular ATP between the experimental groups. *Significant difference with related control group (P=0.01). Results are expressed as mean±SEM.

and significant damage to membrane integrity was observed (figure 2). It is possible that Ca^{2+}_i reduction was relative to calcium extrusion from the disintegrated cell membranes, induced by a cryopreservation-thawing process. We assessed the calcium levels after 30 minutes of incubation, while it might be expected that a time-dependent calcium measurement could have provided us with more precise information.

Extracellular calcium chelating with EGTA reduced the MFI of Fluo3-AM in F and CT groups. The percentage of live sperm, containing a higher level of calcium, was reduced significantly in EGTA-treated fresh sperm, but the same results were not obtained in thawed sperm. EGTA chelated the extracellular calcium; therefore, it was expected that calcium entrance would be inhibited and Ca^{2+}_i reduced in the fresh sperm. Using calcium-imaging method and tracing the calcium entrance could confirm our suggestion. However, in cryopreserved sperm, the cell membrane was severely damaged and calcium homeostasis was disrupted in a considerable number of sperms.

Cryopreservation reduced sperm motility significantly, and a similar reduction in motility was observed in fresh EGTA-treated sperm. The sperm motility was directly related to Ca^{2+}_i level;[34,35] furthermore, intact flagella membrane was required for normal motility.[36, 37] The inhibitory effect of EGTA on sperm motility has also been shown in other studies.[28, 38] It has been shown that EGTA decreased sperm motility time-dependently.[38] EGTA increased mouse sperm motility immediately after use, but the motility gradually decreased and, within 10 minutes, all sperms were immotile.[39] The observed significant reduction of sperm motility in groups containing EGTA could have been induced by Ca^{2+}_i level reduction. In addition, an intact membrane is also required for normal sperm motility.

Calcium overloads cause ROS production and PT pore generation, and inhibit ATP production.[7-9] Ca^{2+}_i entrance to the mitochondria activates the rate-limiting enzymes of the tricarboxylic acid cycle, increases oxidative phosphorylation, and enhances ATP synthesis. Oxidative and nitrosative radicals arise from the respiratory chain and might lead to mitochondrial damage if the cell encounters calcium overload condition. Oxidative or nitrosative stress promotes the opening of PT pore, causes ATP depletion and apoptosis. The release of cytochrome C from the intermembrane space of mitochondria is a major trigger to activate the caspase cascade, initiating the programmed apoptotic cell death.[40, 41] In some studies, a calcium chelator was used for the preservation of sperm quality after cryopreservation thawing and freeze-drying procedures.[25, 42] To the best of our knowledge, no study has investigated the role of EGTA on ROS production or apoptosis of human sperm. This study showed that ROS production increased in CT group. EGTA did not reduce ROS, but it elevated ROS levels. ROS increased after thawing, and EGTA elevated the ROS level in thawing sperm as well as F sperm. The mitochondrial membrane disintegration and the leakage of the electron from the respiratory mitochondrial chain can be a reason for the excessive ROS generation after thawing.[43] To clarify the mitochondrial activity, we assessed the MMP and cellular content of ATP. We showed that the MMP in CT group was impaired, and EGTA did not affect MMP in F and CT sperm groups. This observation elucidates the destructive effect of cryopreservation on mitochondria.[14]

Cellular ATP content was reduced after EGTA treatment in both F and CT groups. Removed calcium from extracellular fluid produced a fast Na^+-dependent human sperm depolarization. It seems that in the absence of calcium, sodium enters the cell through calcium channel, which could stimulate the Na^+-K^+ ATPase and increase cellular ATP consumption.[44]

The apoptotic rate increased after thawing, and EGTA could not inhibit this process. Although calcium overload has harmful effects on cells, sperm cryoinjury is mainly related to cell membrane disintegration. Most studies that used EGTA and EDTA in cryopreservation or freeze-drying media focused on their protective function on DNA and nucleases activity; however, there are some studies which have shown the inhibitory effect of EGTA and EDTA on viability.[14, 25, 38]

This study had some limitations. As previously described, using calcium-imaging methods and live calcium recording could have provided us with a better explanation for the role of calcium on sperm function in fresh and cryopreserved-thawed conditions. Furthermore, we did not assess the apoptotic indicators such as caspases activities. Actually, Annexin V only detects the exposed phosphatidylserine, which is translocated to the outer layer of the cell membrane. To come to a sound judgment about cell apoptosis, measuring apoptotic and anti-apoptotic factors seems to be necessary.

Conclusion

Cryopreservation reduced sperm motility and viability. It also increased ROS production, induced cell apoptosis disrupted MMP, and reduced Ca^{2+}_i. According to our findings, EGTA did not have protective effects on human cryopreserved sperm. Therefore, we concluded that sperm cryoinjury was mainly related to the damage made to the membranes of intracellular organelles and the cell membrane. Thus, it could be suggested that improving the freezing and thawing methods and developing the new cryoprotective medium could reduce the sperm cryoinjury.

Acknowledgment

This work was supported by the Vice-Chancellor of Research Affairs, Shiraz University of Medical Sciences. The manuscript is extracted from the PhD thesis of Bahareh Ebrahimi (Grant # 94-7618). The authors would like to thank the staff of the Shiraz Fertility Center for their helpful assistant in sample collection and Mr. H. Argasi at the Research Consultation Center of Shiraz University of Medical Sciences for his invaluable assistance in editing this manuscript.

Conflict of Interest: None declared.

References

1 Visconti PE. Understanding the molecular basis of sperm capacitation through kinase design. Proc Natl Acad Sci U S A. 2009;106:667-8. doi: 10.1073/pnas.0811895106. PubMed PMID: 19144927; PubMed Central PMCID: PMCPMC2630107.

2 Rahman MS, Kwon WS, Pang MG. Calcium influx and male fertility in the context of the sperm proteome: an update. Biomed Res Int. 2014;2014:841615. doi: 10.1155/2014/841615. PubMed PMID: 24877140; PubMed Central PMCID: PMCPMC4022195.

3 Li K, Xue Y, Chen A, Jiang Y, Xie H, Shi Q, et al. Heat shock protein 90 has roles in intracellular calcium homeostasis, protein tyrosine phosphorylation regulation, and progesterone-responsive sperm function in human sperm. PLoS One. 2014;9:e115841. doi: 10.1371/journal.pone.0115841. PubMed PMID: 25541943; PubMed Central PMCID: PMCPMC4277372.

4 Sumigama S, Mansell S, Miller M, Lishko PV, Cherr GN, Meyers SA, et al. Progesterone Accelerates the Completion of Sperm Capacitation and Activates CatSper Channel in Spermatozoa from the Rhesus Macaque. Biol Reprod. 2015;93:130. doi:

10.1095/biolreprod.115.129783. PubMed PMID: 26490839; PubMed Central PMCID: PMCPMC4712691.
5 Lishko PV, Kirichok Y. The role of Hv1 and CatSper channels in sperm activation. J Physiol. 2010;588:4667-72. doi: 10.1113/jphysiol.2010.194142. PubMed PMID: 20679352; PubMed Central PMCID: PMCPMC3010136.
6 Tamburrino L, Marchiani S, Minetti F, Forti G, Muratori M, Baldi E. The CatSper calcium channel in human sperm: relation with motility and involvement in progesterone-induced acrosome reaction. Hum Reprod. 2014;29:418-28. doi: 10.1093/humrep/det454. PubMed PMID: 24430778.
7 Torres-Flores V, Picazo-Juarez G, Hernandez-Rueda Y, Darszon A, Gonzalez-Martinez MT. Sodium influx induced by external calcium chelation decreases human sperm motility. Hum Reprod. 2011;26:2626-35. doi: 10.1093/humrep/der237. PubMed PMID: 21810864; PubMed Central PMCID: PMCPMC3174032.
8 Li D, Cao W. Role of intracellular calcium and NADPH oxidase NOX5-S in acid-induced DNA damage in Barrett's cells and Barrett's esophageal adenocarcinoma cells. Am J Physiol Gastrointest Liver Physiol. 2014;306:G863-72. doi: 10.1152/ajpgi.00321.2013. PubMed PMID: 24699332; PubMed Central PMCID: PMCPMC4024726.
9 Guthrie HD, Welch GR, Theisen DD, Woods LC, 3rd. Effects of hypothermic storage on intracellular calcium, reactive oxygen species formation, mitochondrial function, motility, and plasma membrane integrity in striped bass (Morone saxatilis) sperm. Theriogenology. 2011;75:951-61. doi: 10.1016/j.theriogenology.2010.10.037. PubMed PMID: 21247623.
10 Hajnoczky G, Csordas G, Das S, Garcia-Perez C, Saotome M, Sinha Roy S, et al. Mitochondrial calcium signalling and cell death: approaches for assessing the role of mitochondrial Ca2+ uptake in apoptosis. Cell Calcium. 2006;40:553-60. doi: 10.1016/j.ceca.2006.08.016. PubMed PMID: 17074387; PubMed Central PMCID: PMCPMC2692319.
11 Giorgi C, Marchi S, Pinton P. The machineries, regulation and cellular functions of mitochondrial calcium. Nat Rev Mol Cell Biol. 2018;19:713-30. doi: 10.1038/s41580-018-0052-8. PubMed PMID: 30143745.
12 Bernardi P, Forte M. The mitochondrial permeability transition pore. Novartis Found Symp. 2007;287:157-64. doi: 10.1002/9780470725207.ch11. PubMed PMID: 18074637.
13 Peng TI, Jou MJ. Oxidative stress caused by mitochondrial calcium overload. Ann N Y Acad Sci. 2010;1201:183-8. doi: 10.1111/j.1749-6632.2010.05634.x. PubMed PMID: 20649555.
14 Treulen F, Arias ME, Aguila L, Uribe P, Felmer R. Cryopreservation induces mitochondrial permeability transition in a bovine sperm model. Cryobiology. 2018;83:65-74. doi: 10.1016/j.cryobiol.2018.06.001. PubMed PMID: 29864412.
15 Ozkavukcu S, Erdemli E, Isik A, Oztuna D, Karahuseyinoglu S. Effects of cryopreservation on sperm parameters and ultrastructural morphology of human spermatozoa. J Assist Reprod Genet. 2008;25:403-11. doi: 10.1007/s10815-008-9232-3. PubMed PMID: 18704674; PubMed Central PMCID: PMCPMC2582121.
16 Saeednia S, Bahadoran H, Amidi F, Asadi MH. Impact of Cryopreservation Process on Viability, Nitric Oxide and DNA Apoptosis in Fertile Human Spermatozoa. Anatomical Sciences Journal. 2013;10:17-23.
17 Zhang W, Li F, Cao H, Li C, Du C, Yao L, et al. Protective effects of l-carnitine on astheno- and normozoospermic human semen samples during cryopreservation. Zygote. 2016;24:293-300. doi: 10.1017/S0967199415000180. PubMed PMID: 26081351.
18 Albrizio M, Moramarco AM, Nicassio M, Micera E, Zarrilli A, Lacalandra GM. Localization and functional modification of L-type voltage-gated calcium channels in equine spermatozoa from fresh and frozen semen. Theriogenology. 2015;83:421-9. doi: 10.1016/j.theriogenology.2014.10.005. PubMed PMID: 25459425.
19 Kumaresan A, Siqueira AP, Hossain MS, Johannisson A, Eriksson I, Wallgren M, et al. Quantification of kinetic changes in protein tyrosine phosphorylation and cytosolic Ca(2)(+) concentration in boar spermatozoa during cryopreservation. Reprod Fertil Dev. 2012;24:531-42. doi: 10.1071/RD11074. PubMed PMID: 22541541.
20 Wang S, Wang W, Xu Y, Tang M, Fang J, Sun H, et al. Proteomic characteristics of human sperm cryopreservation. Proteomics. 2014;14:298-310. doi: 10.1002/pmic.201300225. PubMed PMID: 24259508.
21 Xie Z, Zhang Y, Li A, Li P, Ji W, Huang D. Cd-induced apoptosis was mediated by the release of Ca2+ from intracellular Ca storage. Toxicol Lett. 2010;192:115-8. doi:

10.1016/j.toxlet.2009.10.011. PubMed PMID: 19853025.
22 Fong JC, Lin CH, Wei YH, Ho LT, Hong CY. Calcium buffering capacity of human seminal plasma: the role of EGTA in stimulating sperm motility. Chin J Physiol. 1986;29:7-12. PubMed PMID: 3093172.
23 Yeste M, Fernandez-Novell JM, Ramio-Lluch L, Estrada E, Rocha LG, Cebrian-Perez JA, et al. Intracellular calcium movements of boar spermatozoa during 'in vitro' capacitation and subsequent acrosome exocytosis follow a multiple-storage place, extracellular calcium-dependent model. Andrology. 2015;3:729-47. doi: 10.1111/andr.12054. PubMed PMID: 26097097.
24 Domingo P, Olaciregui M, Gonzalez N, De Blas I, Gil L. Long-term preservation of freeze-dried rabbit sperm by adding rosmarinic acid and different chelating agents. Cryobiology. 2018;81:174-7. doi: 10.1016/j.cryobiol.2018.01.004. PubMed PMID: 29366763.
25 Okazaki T, Yoshida S, Teshima H, Shimada M. The addition of calcium ion chelator, EGTA to thawing solution improves fertilizing ability in frozen-thawed boar sperm. Anim Sci J. 2011;82:412-9. doi: 10.1111/j.1740-0929.2010.00856.x. PubMed PMID: 21615834.
26 Keshtgar S, Iravanpour F, Gharesi-Fard B, Kazerooni M. Combined Effect of Trolox and EDTA on Frozen-Thawed Sperm Quality. Iran J Med Sci. 2016;41:230-7. PubMed PMID: 27217608; PubMed Central PMCID: PMCPMC4876302.
27 Bondarenko O, Dzyuba B, Rodina M, Cosson J. Role of Ca(2+) in the IVM of spermatozoa from the sterlet Acipenser ruthenus. Reprod Fertil Dev. 2017;29:1319-28. doi: 10.1071/RD16145. PubMed PMID: 27246727.
28 Alavi SM, Gela D, Rodina M, Linhart O. Roles of osmolality, calcium - Potassium antagonist and calcium in activation and flagellar beating pattern of sturgeon sperm. Comp Biochem Physiol A Mol Integr Physiol. 2011;160:166-74. doi: 10.1016/j.cbpa.2011.05.026. PubMed PMID: 21651987.
29 Wei C, Zhang Y, Li R, Wang S, Wang T, Liu J, et al. Terahertz irradiation-induced motility enhancement and intracellular calcium elevation in human sperm in vitro. Biomed Opt Express. 2018;9:3998-4008. doi: 10.1364/BOE.9.003998. PubMed PMID: 30615720; PubMed Central PMCID: PMCPMC6157776.
30 Lee CH, Anderson M, Chien YW. Characterization of in-vitro spermicidal activity of chelating agent against human sperm. J Pharm Sci. 1996;85:649-54. doi: 10.1021/js9501573. PubMed PMID: 8773964.
31 Organization WH. WHO laboratory manual for the examination and processing of human semen. Geneva: World Health Organization; 2010.
32 Ghanbari H, Keshtgar S, Kazeroni M. Inhibition of the CatSper Channel and NOX5 Enzyme Activity Affects the Functions of the Progesterone-Stimulated Human Sperm. Iran J Med Sci. 2018;43:18-25. PubMed PMID: 29398748; PubMed Central PMCID: PMCPMC5775990.
33 Kumaresan A, Gonzalez R, Johannisson A, Berqvist AS. Dynamic quantification of intracellular calcium and protein tyrosine phosphorylation in cryopreserved boar spermatozoa during short-time incubation with oviductal fluid. Theriogenology. 2014;82:1145-53. doi: 10.1016/j.theriogenology.2014.07.029. PubMed PMID: 25175760.
34 Suarez SS. Control of hyperactivation in sperm. Hum Reprod Update. 2008;14:647-57. doi: 10.1093/humupd/dmn029. PubMed PMID: 18653675.
35 Alasmari W, Costello S, Correia J, Oxenham SK, Morris J, Fernandes L, et al. Ca2+ signals generated by CatSper and Ca2+ stores regulate different behaviors in human sperm. J Biol Chem. 2013;288:6248-58. doi: 10.1074/jbc.M112.439356. PubMed PMID: 23344959; PubMed Central PMCID: PMCPMC3585060.
36 Gagnon C, de Lamirande E. Controls of sperm motility. The sperm cell: production, maturation, fertilization, regeneration. 2006:108-33.
37 Piomboni P, Focarelli R, Stendardi A, Ferramosca A, Zara V. The role of mitochondria in energy production for human sperm motility. Int J Androl. 2012;35:109-24. doi: 10.1111/j.1365-2605.2011.01218.x. PubMed PMID: 21950496.
38 Kaneko T, Kimura S, Nakagata N. Offspring derived from oocytes injected with rat sperm, frozen or freeze-dried without cryoprotection. Theriogenology. 2007;68:1017-21. doi: 10.1016/j.theriogenology.2007.07.017. PubMed PMID: 17804050.
39 Kusakabe H, Szczygiel MA, Whittingham DG, Yanagimachi R. Maintenance of genetic integrity in frozen and freeze-dried mouse spermatozoa. Proc Natl Acad Sci U S A. 2001;98:13501-6. doi: 10.1073/pnas.241517598. PubMed PMID: 11707598; PubMed Central PMCID: PMCPMC61070.
40 Feissner RF, Skalska J, Gaum WE, Sheu SS. Crosstalk signaling between mitochondrial

Ca2+ and ROS. Front Biosci (Landmark Ed). 2009;14:1197-218. doi: 10.2741/3303. PubMed PMID: 19273125; PubMed Central PMCID: PMCPMC2683671.

41 Rasola A, Bernardi P. Mitochondrial permeability transition in Ca(2+)-dependent apoptosis and necrosis. Cell Calcium. 2011;50:222-33. doi: 10.1016/j.ceca.2011.04.007. PubMed PMID: 21601280.

42 Men NT, Kikuchi K, Nakai M, Fukuda A, Tanihara F, Noguchi J, et al. Effect of trehalose on DNA integrity of freeze-dried boar sperm, fertilization, and embryo development after intracytoplasmic sperm injection. Theriogenology. 2013;80:1033-44. doi: 10.1016/j.theriogenology.2013.08.001. PubMed PMID: 24041826.

43 Brookes PS, Yoon Y, Robotham JL, Anders MW, Sheu SS. Calcium, ATP, and ROS: a mitochondrial love-hate triangle. Am J Physiol Cell Physiol. 2004;287:C817-33. doi: 10.1152/ajpcell.00139.2004. PubMed PMID: 15355853.

44 Torres-Flores V, Garcia-Sanchez NL, Gonzalez-Martinez MT. Intracellular sodium increase induced by external calcium removal in human sperm. J Androl. 2008;29:63-9. doi: 10.2164/jandrol.107.003368. PubMed PMID: 17673433.