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

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Received: 31 October 2018
Revised: 24 November 2018
Accepted: 02 December 2018

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$^{2+}$) after cryopreservation and to clarify the role of a calcium chelator ethylene glycol-bis (2-aminoethyl ether)-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$^{2+}$, 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$^{2+}$, 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 reduced (P=0.02) and ROS production (P=0.0001) increased in EGTA-treated F and CT sperm.

Conclusion: Despite Ca$^{2+}$ reduction by EGTA, it had no protective effects on fresh or cryopreserved human 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.

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$^{2+}$) elevation.4 An increase in Ca$^{2+}$, occurs due to the entrance of calcium through its channels...
and/or its release from intracellular stores.\textsuperscript{5, 6} \(Ca^{2+}\) elevation affects mitochondria and produces more Adenosine triphosphate (ATP).\textsuperscript{7} It also activates many signaling cascades and increases reactive oxygen species (ROS) production by mitochondria and membrane oxidize enzymes.\textsuperscript{8, 9} In addition to 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.\textsuperscript{10, 11} PT pores are permeable to solutes of <1500 Dalton.\textsuperscript{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.\textsuperscript{13} Some researchers, such as Treulen, reported that PT pore formation and cell apoptosis were associated with an increment in \(Ca^{2+}\), mitochondrial membrane potential (MMP) dissipation, ATP level reduction, ROS production, and deterioration of plasma membrane integrity.\textsuperscript{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.\textsuperscript{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.\textsuperscript{16, 17} Cryoinjury changes the membrane permeability to some ions such as calcium,\textsuperscript{14, 18} which leads to their entrance. Although some studies have revealed other aspects of this issue, they have shown that after thawing, \(Ca^{2+}\) decreases significantly.\textsuperscript{19, 20} Calcium overload has been reported as a postulated mechanism for sperm damage.\textsuperscript{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.\textsuperscript{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.\textsuperscript{22, 23} It has been shown that adding both EDTA and EGTA to freeze-dried medium protected rabbits’ sperm DNA.\textsuperscript{24} Adding EGTA and EDTA to cryopreservation medium improved the developmental ability of oocytes and decreased boar sperm DNA fragmentation.\textsuperscript{25} The protective effects of EDTA on cryopreserved human sperm have also been reported.\textsuperscript{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.\textsuperscript{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 \(Ca^{2+}\) stores release.\textsuperscript{14, 28, 29} A decrement on boar sperm motility was reported after the use of 6 mmol/L EDTA.\textsuperscript{25}

The incubation of bovine cryopreserved sperm with EDTA containing medium could not prevent the mitochondrial permeability damage.\textsuperscript{14} In addition, EDTA has time and dose-dependent spermicidal activity.\textsuperscript{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, \(Ca^{2+}\) content, apoptosis rate, ROS production, membrane integrity, mitochondrial membrane potential, and ATP content in EGTA-incubated fresh and thawed sperm.

**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. All donors had signed a written informed consent. They were healthy, not drug addict or alcohol and dietary supplements consumer. A routine semen analysis was performed and oligozoospermic, azoospermic, teratozoospermic, and leukocytospermia samples, as well semen with abnormal appearance, pH and viscosity, were excluded from the study based on guidelines from World Health Organization (WHO), 2010.\textsuperscript{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%. After the usual routine semen analysis, the samples were transferred to our laboratory in less than one hour. The local Ethics Committee of Shiraz University of Medical Sciences approved this research (IR.sums.REC.1391-6159).

Forty samples were washed with Ham's F-10 medium (N6633, Sigma, Germany) and incubated at 37 °C, 5% CO2, for one hour. The swim-upped sperm were counted and sperm viability and kinematics was assessed. All procedures were done according to the guidelines by WHO, 2010. The samples were diluted to 10×106 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, and 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 1mM 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.

Assessing Sperm Kinematics
Sperm kinematics was recorded by Video Test (VT) sperm analyzer 3.1 software (VT sperm 3.1, Russia), using an Olympus CX41 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 sperm, were evaluated for all samples.

Assessing Membrane Integrity
Hypo-osmotic swelling test (HOS test) was used to assess membrane integrity. 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 5 minutes and the shape of all flagella stabilized after 30 minutes. At least, 100 sperm were evaluated after 5 and 30 minutes.

Assessing ROS Production
ROS production was assessed using the chemiluminescent method. 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 luminol. 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 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 (1.0×106 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 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 Ca2+

The suspension containing 1×106 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. At least, 50,000 sperm were analyzed using a BD FACSCaliber™ flow cytometer and FlowJo® software. The cells were classified into a quadrant according to their Ca2+ content and viability. Q1-Q4 represent the dead sperm with low Ca2+ (Fluo3-AM-, PI+), dead sperm with high Ca2+ (Fluo3-AM+, PI+), live sperm with high Ca2+ (Fluo3-AM+, PI-), and live sperm with low Ca2+ (Fluo3-AM-, PI-), respectively. Mean Fluorescence intensity (MFI) of fluo-3/AM was
Effect of EGTA on human sperm quality

Assessing MMP
MMP was assessed using MitoProbe™ JC1 assay kit (M34152, Molecular Probes, USA). According to the manufacturer’s instruction, 1×10⁶ 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% CO₂ 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 JC1 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 (1×10⁶ sperm 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 2 minutes at 100 °C, samples were centrifuged at 1,000 g for 60 second and their supernatant was frozen in -20 °C until further assessment. In the assessment day, a serial dilution of ATP standard (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 ATP concentration of all samples was calculated. The data were expressed as nM of ATP per 10⁶ sperm.

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 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 values±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 sperm increased (P=0.0001) (table 1). EGTA decreased the percentage of progressive motile sperm in F and CT samples, and this reduction was greater in F group (36.5%) compared to the CT group (16.9%). Velocities of motile sperm 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 had no effect on cell membrane integrity (figure 1).

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

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

Curvilinear Velocity (VCL, μm/s), Straight Line Velocity (VSL, μm/s), Average Path Velocity (VAP, μm/s), and ethylene glycol-bis (2-aminohyethyl ether)-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.
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 percent 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\textsuperscript{2+} in live and dead cells (table 2). Cryopreservation-thawing process significantly decreased the percentage of live cells with high Ca\textsuperscript{2+} (Fluo3-AM\textsuperscript{+}, PI\textsuperscript{-}) and increased the percentage of dead cells with low Ca\textsuperscript{2+} (Fluo3-AM\textsuperscript{-}, PI\textsuperscript{+}) (P=0.0001). MFI of Fluo-3 AM was lower in the CT group in comparison with the F group (table 2). EGTA reduced the count of live cells with high Ca\textsuperscript{2+} (Fluo3-AM\textsuperscript{+}, PI) and increased the live cells with low Ca\textsuperscript{2+} (Fluo3-AM\textsuperscript{-}, PI\textsuperscript{-}) 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 fluorescent intensity indicated the mitochondria with high and low membrane potential,
respectively. Red/green ratio 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, accompany by Ca\(^{2+}\) reduction, were the damage 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 damage happening after the cryopreservation-thawing procedure,\(^{16,17}\) but the mechanism(s) of these destructive effects and their correlation with Ca\(^{2+}\) 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\(^{2+}\) after cryopreservation has been reported by some researchers.\(^{14,18}\) In contrast, some other studies have shown the reduction of Ca\(^{2+}\) after thawing.\(^{20,33}\) Kumaresan et al. showed that the proportion of sperm with high calcium decreased during the initial 5 minutes of incubation.\(^{33}\) Based on the findings of these studies, it is possible that Ca\(^{2+}\) increases 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\(^{2+}\) sperm was reduced after cryopreservation, the percentage of dead-low Ca\(^{2+}\) sperm increased.

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

<table>
<thead>
<tr>
<th>Groups</th>
<th>Fluo3-AM, PI (%)</th>
<th>Fluo3-AM*, PI (%)</th>
<th>Fluo3-AM*, PI (%)</th>
<th>Fluo3-AM*, PI (%)</th>
<th>MFI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fresh</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>20.6 ±6.5</td>
<td>6.24±1.78</td>
<td>63.24±8.17</td>
<td>9.85±3.36</td>
<td>302.4±62.08</td>
</tr>
<tr>
<td>EGTA</td>
<td>30.9±7.6</td>
<td>1.66±8.88</td>
<td>22.66±13.88*</td>
<td>44.72±12.23*</td>
<td>51.16±20.78*</td>
</tr>
<tr>
<td>Cryopreserved-Thawed</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>70.8±4.6#</td>
<td>3.76±1.19</td>
<td>13.87±3.95#</td>
<td>11.54±1.08</td>
<td>57.01±9.7#</td>
</tr>
<tr>
<td>EGTA</td>
<td>60.7±9.5 #</td>
<td>2.33±5.6</td>
<td>14.32±2.38</td>
<td>22.58±9.78</td>
<td>42.18±7.97*</td>
</tr>
</tbody>
</table>

Dead cells with low Ca\(^{2+}\) (Fluo3-AM-PI+), dead cells with high Ca\(^{2+}\) (Fluo3-AM*-PI+), live cells with high Ca\(^{2+}\) (Fluo3-AM-PI), live cells with low Ca\(^{2+}\) (Fluo3-AM*-PI), mean fluorescent intensity (MFI), and ethylene glycol-bis (2-aminoethyl ether)-N, N', N'-tetraacetic acid (EGTA).  

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*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
and significant damage to membrane integrity was observed (figure 2). It is possible that Ca^{2+} 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+} 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 considerable number of the sperm.

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+} level; Furthermore, intact flagella membrane was required for normal motility. The inhibitory effect of EGTA on sperm motility has also been shown in other studies. Ethylene glycol-bis(2-aminoethyl ether)-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.

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

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

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.
Effect of EGTA on human sperm quality

In some studies, a calcium chelator was used for the preservation of sperm quality after cryopreservation thawing and freeze-drying procedures. 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. 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 in CT sperm. This observation elucidates the destructive effect of cryopreservation on mitochondria.

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.

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. 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 phosphatidylinerine, which is translocated to the outer layer of cell membrane. To come to a sound judgment about cell apoptosis, measuring apoptotic and anti-apoptotic factors appears to be necessary.

Conclusion

Cryopreservation reduced sperm motility and viability. It also increased ROS production, induced cell apoptosis, disrupted MMP, and reduced Ca++. 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.

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