NADPH oxidase 5 activation; a novel approach to human sperm cryoinjury

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Abstract Sperm cryopreservation leads to various structural and functional damages, some of which induce by oxidative stress. The reactive oxygen species (ROS) generates by mitochondria and membrane NADPH oxidases (NOXs). Among the NOXs, only NOX5 has been identified in the cell membrane of human sperm. This study was designed to clarify the possible role of NOX5 on sperm cryoinjury. Forty human semen samples were washed and randomly divided into fresh and cryopreserved groups. Each group was divided into 4 subgroups containing Ham’s F10 (control), 0.1% DMSO (vehicle), 100 nM of PMA (phorbol 12-myristate 13-acetate) and 1 µM of DPI (diphenyleneiodonium), as NOX5 activator and inhibitor. The samples of cryopreserved groups were preserved in liquid nitrogen for 1 month. The sperm kinematics, membrane integrity, ROS production, apoptosis rate, mitochondrial membrane potential (MMP), intracellular ATP and calcium concentration [Ca^{2+}]_i were evaluated. The percent of sperm with intact membrane and motile sperm reduced significantly after thawing (p < 0.01). The ROS production (p < 0.01) and the apoptotic rate increased, MMP dissipated, and the percentage of live cells with high [Ca^{2+}]_i decreased significantly in the cryopreserved control group relative to the fresh control group. DPI, in contrast to PMA, improved sperm progressive motility (p ≤ 0.01), membrane integrity in fresh and cryopreserved groups and reduced the ROS amount in cryopreserved group (p ≤ 0.01). Apoptotic rate, [Ca^{2+}]_i, ATP, and MMP did not change with DPI and PMA in cryopreserved groups. We conclude that NOX5 activity in fresh sperm is low, and it increases during cryopreservation. NOX5 inhibition improves the cryopreserved sperm quality.

Keywords Human sperm · Cryopreservation · NADPH oxidase 5 · Reactive oxygen species · Calcium
Introduction

Cryopreservation of human sperm is a therapeutic and valuable technique in assisted reproduction technology (ART). Despite successful achievements in developing sperm cryopreservation method, this procedure is accompanied with cell damages, which would impair the cell function (which is called cryoinjury). Cryoinjury is inevitable and has some detrimental effects on the sperm plasma membrane, chromatin integrity, viability, motility, velocity, and fertilization potential (Cankut et al. 2019; Saeednia et al. 2015; Zhang et al. 2016). In addition, it can dissipate mitochondrial membrane potential (MMP) and accelerates mitochondrial permeability transition pore (MPT) formation (Treulen et al. 2018). Pathological calcium overload (Treulen et al. 2018), intracellular ice crystal formation, osmotic shock, oxidative stress (OS), or their combinations are reported as postulated mechanisms for cryoinjury (John Morris et al. 2012; Sobhani et al. 2015).

Sperm is vulnerable to OS due to its restricted cytoplasmic content, inadequate antioxidant defense mechanisms (Ben Abdallah et al. 2009), and membrane polyunsaturated fatty acids (Ben Abdallah et al. 2009). Studies have shown that ROS production increases and the level of antioxidants diminish after freezing-thawing process, and these result in amplification of oxidative damages (Gadea et al. 2011; Taylor et al. 2009).

Mitochondria and membrane NADPH oxidases (NOXs) are involved in cellular ROS generation (Mussin et al. 2012; Sobhani et al. 2015). Elevation of intracellular calcium (Ca\(^{2+}\)) activates the mitochondrial oxidation-phosphorylation machinery and pathological Ca\(^{2+}\) overload can lead to excess ROS production and MPT formation (Brookes et al. 2004). Other sources for production of ROS in the cells are members of NOX family. NOX family has seven known members, NOXs 1-5 and DUOX1-2 (Bedard et al. 2012) and it was found that NOX5 is expressed in human spermatozoa (Ghani et al. 2013; Mussin et al. 2012). NOX5 has six transmembrane domains with a long NADPH-binding cytoplasmic C-terminal and EF hands in N-terminal (Bedard et al. 2012; Jagnandan et al. 2007). The EF hands bind Ca\(^{2+}\) and accelerate the activity of enzyme and causes ROS production (Banfi et al. 2004; Jagnandan et al. 2007). Therefore, Ca\(^{2+}\) is essential for NOX5 function.

Elevation of intracellular Ca\(^{2+}\) concentration induces a conformational change in the enzyme N-terminal that leads to NOX5 activation and ROS production (Banfi et al. 2004).

Another activator of NOX5 is protein kinase C (PKC). PKC phosphorylates the key residues of NOX5 and results in facilitation of the enzyme activation at lower levels of intracellular Ca\(^{2+}\) (Jagnandan et al. 2007). PMA (phorbol 12-myristate 13-acetate), as a PKC activator, increases NOX5 activity in the presence of Ca\(^{2+}\) (Chen et al. 2014). On the other hand, Diphenylene Iodonium (DPI) is used as a non-specific NOX inhibitor. For the first time, Cross and Jones (1986) observed that DPI could inhibit NADPH-dependent production of superoxide by a solubilized oxidase of pig neutrophils (Cross and Jones 1986). The inhibitory effect of DPI on sperm superoxide anion generation have been reported (Sabeur and Ball 2006).

The role of ROS on cryoinjury has already been shown, but the importance of NOX5 activity on ROS generation has not been reported. This study was designed to clarify the possible role of NOX5 activity on human sperm cryoinjury and furthermore, we tried to find a relationship between the cryopreservation induced changes of intracellular Ca\(^{2+}\), ATP concentration, MMP, and the rate of apoptosis with NOX5 activity.

Materials and methods

Sample collection and semen analysis

In this experimental study, forty semen samples from healthy adult men (20-40 years old) were used. Samples were collected by masturbation into sterile containers at least 3-5 days after their last sexual activity. Usual routine semen analysis was performed at Shiraz Fertility Center. Oligozoospermic, azoospermic, teratozoospermic and leukocytospermia samples, as well semen with abnormal appearance, pH and viscosity (based on World Health Organization (WHO) guidelines 2010) were excluded from the study. Other exclusion criteria were drug addiction, alcohol, and dietary supplements consumption. After the initial semen analysis, the samples were transferred to our laboratory in less than an hour.
Sperm cryopreservation and treatments

All the liquefied semen samples were washed with Ham’s F-10 medium (Sigma, N6633) and centrifuged at room temperature according to WHO 2010 guideline (WHO 2010). The pellets were re-suspended in 0.5 mL of the sperm medium and then incubated for 60 min at 37 °C under 5% CO2. The swim-upped sperm were collected and sperm concentration, motility, and viability of the samples were evaluated. The samples were divided into fresh (F) and cryopreserved-thawed (CT) groups randomly.

The sperm concentration were adjusted to $10^9$ sperm/ml and the samples of each group (both fresh and cryopreserved) were divided into four subgroups; control, solvent (0.1% DMSO, Sigma, D2650), DPI (diphényléniodonium) (Sigma, D2926) and PMA (phorbol 12-myristate 13-acetate) (Sigma, p8139). Final concentration of DPI and PMA in sperm incubation medium was 1 μM (Ghani et al. 2013) and 100 nM respectively (Ghanbari et al. 2018; Rotfeld et al. 2014). The solvent, DPI, and PMA were added to the cryopreserved medium before freezing. For sperm cryopreservation, the samples were mixed (1:1) with cryoprotective medium (ORIGIO11010010) within 30 min. Samples were treated as described before and loaded to a cryotube, tubes were placed on nitrogen vapor for 30 min, and plunged into liquid nitrogen, for at least 1 month. Thawing procedure was done at 37 °C in 5 min. The samples were washed and after 30 min of incubation, the following assessments were performed (Ebrahimi and Keshtgar 2019; Keshtgar et al. 2016).

Assessment of sperm motility

Kinematic characteristics of at least 200 sperm were recorded by Video Test Analyzer (VT) 3.1 using Olympus CX41 microscope. Percentage of progressive, non-progressive and immotile sperm were assessed and motility parameters such as straight-line velocity (VSL, μm/s), average path velocity (VAP, μm/s), and curvilinear velocity (VCL, μm/s) were evaluated (WHO 2010).

Assessment of ROS production

The amount of generated ROS was measured by chemiluminescent method using luminol (Sigma, A8511). Briefly, fresh prepared luminol (250 μm) and 12U/ml horseradish peroxidase (HRP) (Sigma, P6782) were added to 300 μl of sperm suspension in 96 black well plates. A microplate reader (synergy HT, Biotec) recorded light emissions every 10 s for 15 min at 37 °C and it was reported as relative light unit (RLU) (WHO 2010).

Assessment of apoptotic rate and sperm viability

The cells in early and late apoptosis state and viable sperm were identified by double staining with FITC-conjugated Annexin-V and propidium iodide (PI), using a phosphatidyl serine detection kit (IQ Products®, Netherlands) according to the manufacturer’s instruction. At first, the cells were washed and readjusted to $1.0 \times 10^6$ cell/ml by cold calcium buffer. The cell suspensions (100 μl) were incubated with 10 μl of FITC-conjugated Annexin-V on ice, in a dark place for 20 min. The cells were washed and incubated with 10 μl of PI for at least 10 min on ice. At least 50,000 sperm were analyzed using a BD FACSCaliberTM flow cytometer (BD Biosciences, USA) and FlowJo® (version 10.4.1) software (Gholami et al. 2019). The cells were classified into the following four categories: (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 (–)].

Assessment of membrane integrity

Hypo-osmotic swelling test (HOS test) was used to assess membrane integrity. Spermatozoa with intact membranes swollen within 5 min in a mixture of 1:1 hypo-osmotic medium containing 0.735 g of sodium citrate dihydrate (Sigma, W302600) and 1.351 g of D-fructose (Sigma, F0127) in 100 ml of purified water (final osmolality 100 mOsm/L). At least 100 sperm were evaluated after 5 and 30 min. Spermatozoa with intact membranes swell, and the shape of flagella stabilized after 30 min (Ramu and Jeyendran 2013).

Assessment of intracellular Ca2+

Briefly, sperm suspension containing $1 \times 10^6$ sperm were loaded with 4 μM fluo-3/AM (Sigma 73881) and 0.08% pluronic acid F-127 (Sigma P2443) at dark in
37 °C for 30 min. After incubation, samples were washed and 5 μm PI (Sigma P4170) was added to the medium. The data were analyzed by FlowJo software; the cells were placed into a quadrant according to their viability and intracellular Ca\(^{2+}\) content. First quadrant (Q1) represented the dead sperm with low intracellular Ca\(^{2+}\). Dead cells with high intracellular Ca\(^{2+}\), live cells with high intracellular Ca\(^{2+}\) and live cells with low intracellular Ca\(^{2+}\) sperm were allocated in quadrants of Q2, Q3, and Q4, respectively. Mean Fluorescence intensity (MFI) of flou-3/AM was calculated for each experimental subgroup (Ebrahimi and Keshtgar 2019).

**Assessment of MMP**

Mitochondrial membrane potential was assessed using MitoProbe\textsuperscript{TM} JC1 assay kit (M34152, Molecular probes, USA). Sperm were suspended in 1 ml warm phosphate-buffered saline (1 × 10\(^6\) sperm/mL), and JC-1 with the final concentration of 2 μM was added to the medium. The samples were incubated in 5% CO\(_2\) at 37 °C for 15–30 min. The assessment was performed by a flow cytometer using 488 nm excitation with 530 nm and 585 nm bands pass emission filters. Red to green ratio was calculated; change of the fluorescence intensity from red to green means the membrane potential decreases. Carbonyl cyanide 3-chlorophenylhydrazone (CCCP) was used as a MMP disrupter to confirm that the JC-1 response is sensitive to changes in membrane. For this purpose, the cells were treated with JC-1 and 50 μM CCCP simultaneously and green fluorescence intensity increment was observed considerably (Bravo et al. 2020; Contreras et al. 2017).

**Assessment of intracellular ATP**

The ATP contents of spermatozoa were determined using the bioluminescence assay kit CLS II (Roche Diagnostics GmbH, Germany). Briefly, 25μL of sample (1 × 10\(^6\) sperm per ml) was 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 min at 100 °C, samples were centrifuged at 1000 g for 60 s. Fifty μl of the supernatant was transferred to a fresh tube and frozen in −20 °C until further assessment. In the assessment day, a serial dilution of ATP standard (range between 10\(^{-5}\) and 10\(^{-10}\) M) was prepared and 50 μl of luciferase reagent added to the samples and standards. The luminescence was measured using a microplate reader (synergy HT, Biotek) after 1 s delay for 10 s. ATP standard curve was plotted and ATP concentration of all samples calculated. The data was expressed as nM of ATP per 10\(^6\) sperm (Aramli et al. 2013; Kordan et al. 2010).

**Statistical analysis**

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

**Results**

**Sperm motility**

After thawing, sperm progressive and non-progressive motility was significantly decreased and the percent of immotile sperm increased (Fig. 1a, b and c). DPI increased the percent of progressive motile sperm in F (from 72.8 ± 0.7 to 78.1 ± 0.9%) and in CT groups (from 36.9 ± 0.7 to 43.8 ± 1.1%) (p ≤ 0.01). PMA reduced progressive motile sperm in F and CT sperm to 61.7 ± 2.1 and 32.6 ± 1.0%, respectively (p ≤ 0.01) (Fig. 1a). Velocities of live motile sperm were recorded, and the analysis showed that freezing-thawing procedure reduced the VSL and VAP significantly (Fig. 1e, f). In F sperm, inhibition and activation of NOX5 by DPI and PMA had no effect on VCL and VSL but reduced the VAP (Fig. 1d, e and f).

Adding DPI to CT media prior to freezing did not protect sperm from the adverse effects of CT on sperm velocity, but PMA reduced VSL and VAP relative to F and CT control group (p ≤ 0.01).

**ROS production**

Chemiluminescence signals increased significantly in CT sperm. DPI reduced ROS production in CT group.
Meanwhile, PMA increased chemiluminescence signals significantly, in both F and CT sperm ($p \leq 0.01$) (Fig. 2a).

The chemiluminescence signals were calculated per 1000 live cell (Fig. 2b). RLU/10^3 live cells were reduced non-significantly by DPI in fresh sperm ($P = 0.054$), but the inhibitory effect of DPI on ROS production of CT sperm was significant. PMA had a stimulatory effect on ROS production of CT sperm (Fig. 2b).

Viability, frequency of apoptosis and membrane integrity

The percent of live cells were reduced dramatically, and high apoptosis rate was observed in cryopreserved-thawed sperm. DPI increased and PMA reduced significantly sperm viability in F group, meanwhile, similar non-significant changes in viability were observed in CT groups (Table 1).

Sperm membrane integrity was assessed by HOS test. The integrity of membrane was damaged severely in CT group. DPI showed a protective effect on membrane, but PMA decreased sperm with intact cell membrane.

Fig. 2  Fresh and cryopreserved-thawed sperm ROS production. The effects of DPI and PMA on a Relative light unit (RLU), b RLU per 1000 live cells ($n = 20$). Values are presented as mean ± SEM. * Significant difference with the same treated fresh sperm, † and ‡ significant difference with fresh and cryopreserved-thawed control ($p < 0.01$)
membrane in both F and CT sperm ($p \leq 0.05$) (Table 1).

Intracellular Ca$^{2+}$ and MMP

Intracellular Ca$^{2+}$ and cell viability were assessed using Fluo3/AM and PI double staining, by flowcytometric method. Figure 3a represent dot plots of intracellular calcium and viability of F and CT sperm. After thawing, the percentage of live cells with high intracellular Ca$^{2+}$ decreased significantly ($p \leq 0.05$), whereas, the percentage of dead cells with low intracellular Ca$^{2+}$ increased ($p \leq 0.05$). MFI of Fluo-3 AM in the CT group was lower than that of F sperm (Fig. 3b).

MMP was assessed using JC-1 staining, by flowcytometric method. Red and green fluorescent intensity indicated the mitochondria with high and low membrane potential, respectively (Fig. 4a and b). Red to green ratio decreased in thawed sperm significantly ($p \leq 0.05$) (Fig. 4b).

Sperm intracellular ATP

ATP concentration did not change with cryopreservation. Incubation of F sperm with DPI increased nonsignificantly ATP content, but this effect was not observed in thawed sperm (Fig. 5).

Discussion

The results of our study showed the significant impact of NOX5 activity on cryopreserved-thawed sperm. To

|        | Live (%) | Early Apoptosis (%) | Late Apoptosis (%) | Necrosis (%) | HOS positive (%) |
|--------|----------|---------------------|--------------------|--------------|-----------------|
| Control |          |                     |                    |              |                 |
| Fresh  | 81.74 ± 1.21 | 1.49 ± 0.39       | 9.53 ± 0.94        | 7.22 ± 0.77  | 88.9 ± 0.8      |
| Cryopreserved-thawed | 16.11 ± 5.1# | 2.9 ± 0.79       | 71 ± 6.7#          | 9.9 ± 6.9    | 39.8 ± 1.1#     |
| DPI    |          |                     |                    |              |                 |
| Fresh  | 84.72 ± 0.92# | 1.38 ± 0.54       | 7.2 ± 1.35         | 6.53 ± 1.27  | 92.1 ± 1.3#     |
| Cryopreserved-thawed | 18.4 ± 5.05# | 2.92 ± 0.8        | 68.3 ± 5.4#        | 10.3 ± 6.3   | 45.6 ± 1.5#+    |
| PMA    |          |                     |                    |              |                 |
| Fresh  | 79.34 ± 1.21# | 1.69 ± 0.49       | 11.1 ± 0.90#       | 7.81 ± 0.59  | 86.7 ± 1.5#     |
| Cryopreserved-thawed | 11.5 ± 2.3# | 2.9 ± 0.8         | 74.7 ± 5.6#        | 10.8 ± 7.1   | 36.5 ± 1.0#+    |

Values are presented as mean ± SEM, n = 10. # Significant difference with the same treated fresh sperm, * and + significant difference with fresh and cryopreserved-thawed control ($p < 0.05$). Thawed sperm

Fig. 3 Intracellular calcium of fresh and cryopreserved-thawed sperm. a Representative dot plots of intracellular calcium and viability of fresh and cryopreserved-thawed sperm, b The effects of DPI and PMA on MFI of Fluo-3 AM in fresh and cryopreserved-thawed sperm, (n = 5). Values are presented as mean ± SEM. # Significant difference with the same treated fresh sperm ($p < 0.05$)
In the present study, DPI (1 μM) reduced ROS production in cryopreserved-thawed human sperm. The ROS production of fresh sperm was reduced by DPI, which was not significant; hence, we conclude that NOX5 activity in fresh sperm is in its lower limits. We observed that PMA increased ROS production in F and CT sperm, but the effect of PMA on CT was more prominent than that of F sperm. PMA is a PKC activator (Banfi et al. 2004) and it was shown that PKCα directly bound to NOX5 and modified NOX5 phosphorylation and activity (Chen et al. 2014). Furthermore, amplitude and duration of calcium-dependent NOX5 activity was enhanced in the presence of PMA and the calcium sensitization was reversed by treating it with alkaline phosphatase (Jagnandan et al. 2007). These observations provide sufficient evidences that direct phosphorylation of NOX5 by PMA, mediates the stimulatory effect of PMA on NOX5 activity.

It has been shown that intracellular Ca\(^{2+}\) and phosphorylation of serine/threonine residues stimulate NOX5 activity (Banfi et al. 2004). Phosphorylation of NOX5 enhances its sensitivity to Ca\(^{2+}\) and stimulates the enzyme at lower levels of Ca\(^{2+}\) (Jagnandan et al. 2007). Some studies have shown that cryopreserved-thawed sperm contain higher intracellular Ca\(^{2+}\) in comparison to fresh sperm (Satorre et al. 2007). While, other studies revealed a different picture, in which intracellular Ca\(^{2+}\) was decreased significantly in cryopreserved-thawed sperm (Kumaresan et al. 2012; Wang et al. 2014). In the present study, we evaluated intracellular Ca\(^{2+}\) in F and CT sperm after 30 min, and unlike other studies (Singh et al. 2012), the best of our knowledge, the source of excess ROS generation during cryopreservation-thawing process is not defined. In this study, we observed that NOX5 did not have any considerable effect on fresh sperm, but its activity increased after thawing. The destructive effects of cryopreservation on sperm motility and viability are the well-known damages after cryopreserved–thawed procedure (Saeednia et al. 2015; Zhang et al. 2016) that were shown in our study, too. One possible cause of cryoinjury is OS (Sobhani et al. 2015), which leads to cell apoptosis and disruption of MMP (Li et al. 2010). Cryopreservation decreases antioxidants activity (Gadea et al. 2011; Sobhani et al. 2015), and increases ROS production (Asadmobini et al. 2017; John Morris et al. 2012). We showed similar results on ROS production and observed that luminal signals were elevated after thawing.
we found that the frequency of live-high Ca\(^{2+}\) sperm was reduced in CT sperm. Another study on intracellular Ca\(^{2+}\) of thawed boar spermatozoa showed that the proportion of sperm with high Ca\(^{2+}\) decreased during the initial 5 min of incubation (Kumaresan et al. 2014). However, it is possible that intracellular Ca\(^{2+}\) increases promptly after cryopreserved-thawing process, but this elevation cannot be sustained for long durations.

We showed a significant damage to membrane integrity and found that the frequency of late apoptosis was increased in CT sperm (Table 1). Simultaneously, a reduction in the MMP of CT sperm was observed. Studies showed that mitochondrial activity reduction (shift from high to medium potential) occurs during the cooling phase of cryopreservation (Treulen et al. 2018), because similar to the plasma membrane, mitochondrial membranes might endure injuries due to crystal ice formation (Morris et al. 2007). Regarding the results of HOS test and the decrement of MMP, we hypothesized that mitochondrial membrane was dis-integrated in CT sperm. In addition to the role of mitochondria in ATP synthesis, this organelle has a significant role in ion homeostasis (O’Rourke et al. 2005). Mitochondria can store Ca\(^{2+}\) and mitochondrial matrix Ca\(^{2+}\) is about ten micromolar (Ivannikov and Macleod 2013); The damaged mitochondrion could not store Ca\(^{2+}\), and the Ca\(^{2+}\) released into the cytoplasm. The cytoplasmic membranes of most cryopreserved-thawed sperm were not intact, and Ca\(^{2+}\) left these damaged cells and cause significantly reduction of Fluo3-AM fluorescent intensity in CT sperm. It is possible that during Ca\(^{2+}\) diffusion out of the mitochondria, Ca\(^{2+}\) bound to EF hand of NOX5, which activated this enzyme and produced more ROS. Inhibition of NOX5 activity by DPI improved motility and increased the percent of sperm with intact membrane, while PMA accelerated NOX5 activity and had a reverse effect relative to DPI.

On the other hand, a main source of ROS in cells is mitochondria (Brookes et al. 2004). However, we did not assess the mitochondrial ROS production specifically, but we showed that the basal ROS produced in fresh sperm was not affected by DPI and concluded that the basal ROS might be produced by mitochondria as the other studies which emphasized on the role of mitochondria in sperm ROS production and the correlation of mitochondrial integrity/functionality with loss of sperm function, particularly with the production of ROS (Koppers et al. 2008). Despite significant MMP dissipation in CT sperm, their ATP content was the same as F sperm. However, a considerable number of cryopreserved-thawed sperm were dead and they did not produce or consume ATP, so the ATP content did not show any change between F and CT sperm.

Rate of apoptosis was increased in CT sperm, and using DPI and PMA had no significant effect on apoptosis rate. Annexin V bind externalized phosphatidyl serine (PS), and translocation of PS to the outer membrane layer was reported previously in thawing human sperm (Schuffner et al. 2001). Mitochondria contains several pro-apoptotic molecules such as cytochrome C which recruits the procaspases, leading to the formation of mitochondrial permeability transition pore and triggered apoptosis (Crompton 1999; Gulbins et al. 2003). Therefore, we suggested that the mitochondrial membrane damage initiates during cryopreservation and induces the apoptotic process. However, we did not assess the apoptotic factors and this suggestion remained to elucidate.

ROS production in CT sperm was elevated significantly, however, PMA augmented and DPI diminished the ROS production in CT sperm. We conclude that NOX5 activity could increase during sperm cryopreservation-thawing process. On the other hand, the cell and mitochondrial membrane damaged severely with cryopreservation, and the damage would be worse by excessive ROS. The increment in ROS and damage to the membranes was accompanied to MMP dissipation, apoptosis, reduction of intracellular Ca\(^{2+}\), and sperm motility in CT sperm.

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Compliance with ethical standards

Conflict of interest The authors declare no potential conflicts of interest.

Ethical approval All participants signed a written informed consent, and the local Ethics Committee of Shiraz University of
Medical Sciences approved this research (IR.sums.REC.1391-6159).

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