Pre-Incubation with Kisspeptin Improves the Adverse Effects of Freeze-Thawed Human Ejaculated Sperms

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Research article

Keywords: Kisspeptin, Motility, DNA Integrity, Cryopreservation, Lectin Histochemistry

DOI: https://doi.org/10.21203/rs.3.rs-29987/v1

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Abstract

Objectives: Sperm cryopreservation reduces sperm quality. Kisspeptin (KP), as an antioxidant, has beneficial effects on the sperm functions. Therefore, the present study was conducted to use kisspeptin in order to mitigate detrimental effects of the sperm freeze-thawing process and compare it with Glutathione (GSH), as positive control.

Materials and methods: 30 normal semen samples, prepared by swim-up procedure, were divided into 3 aliquots: negative control with no treatment; positive control receiving 1mM GSH; and experimental aliquot treated with 10 µM KP for 30 min. All aliquots were cryopreserved, and then thawed after 48 hr. Sperm motility was assessed according to WHO guidelines. Acrosomal reaction and capacitation were evaluated by Fluorescein Isothiocyanate-Conjugated Peanut Agglutinin (FITC-PNA), Wheat Germ Agglutinin (WGA), and Concanavalin A (ConA); percentage of positive cells was evaluated by flow cytometry. Sperm DNA quality was evaluated by Acridine Orange (AO), Aniline Blue (AB), Chromomycin A3 (CMA3), and TUNEL (Terminal deoxynucleotidyl transferase dUTP Nick End Labeling) staining methods. Statistical analyses were performed using ANOVA and LSD.

Results: Results of the study showed that, KP supplementation improved motility and led to an increase in the percentage of capacitated and acrosome-intact sperms compared to both controls. Freeze-thaw procedure damaged the DNA integrity severely, and KP pre-treatment significantly reduced frequency of apoptotic sperms as well as those with histone-protamine substitution impairment.

Conclusion: Pre-exposure of the sperms to KP can protect the sperm quality including motility and DNA integrity against detrimental influence of freeze–thaw cycle. Therefore, it can be considered as a good pre-additive substance to control the sperm quality during freezing and thawing procedure.

Background

Cryopreservation is referred to as a process in which the cells or tissues are protected by cooling at low temperatures to maintain their normal structure and function [1]. Human sperm cryopreservation can be considered as a common procedure in Assisted Reproduction Technologies (ARTs). This technique is performed to avoid iatrogenic effects of chemotherapy, radiotherapy and surgical treatments which may result in ejaculatory dysfunction or testicular failure and preserves the male fertility [2]. In addition, cryopreservation is suitable to keep fertility in some chronic diseases, such as autoimmune disorders and diabetes, which may result in testicular impairment [3].

In spite of extensive use of cryopreservation in ART programs, it has been widely reported that freezing and thawing cause serious detrimental changes in the structures (nucleus, membrane, and mitochondrion) and functions (i.e. motility) of ejaculated spermatozoa. Some of these adverse effects on spermatozoa include defective motility, reduced viability, alteration in the plasma and mitochondrial membrane compositions, abnormal acrosomal reaction, cell membrane glycoconjugate modification, and DNA fragmentation [4]. Changes in mitochondrial membrane fluidity as a result of change in the
mitochondrial membrane potential lead to release of Reactive Oxygen Species (ROS) [5]. Furthermore, cryopreservation has also been shown to diminish antioxidant enzyme activity in the spermatozoa, making them more vulnerable to oxidative stress. An increase in the ROS production and a decrease in the antioxidant capacity can induce cell apoptosis [6]. Release of apoptotic factors from the mitochondria leads to DNA fragmentation (DNAf). DNA quality is an important indicator of sperm fertilization potential, successful transmission of genetic information to the next generation, and normal embryonic development. Previous studies reported that oxidative stress causes DNA damage in the form of nucleotide modifications, DNA strand breaks, DNA base oxidation, chromatin cross linking, chromosomal deletions and rearrangements. Some studies have focused on the effect of using antioxidants on the sperm cryopreservation aimed at decreasing deleterious effects of cryopreservation [7,8].

Kisspeptin (KP) as an Arg-Phe (RF)-amide super family of overlapping neuropeptide is encoded by the KISS1 gene in human and binds to G-protein coupled receptor, GPR54. Kisspeptin receptor was found in mature sperm of the animal models such as mouse [9] and monkey [10]. For the first time, Pinto, Cejudo-Roman [11] showed that kisspeptin and GPR54 are present in human spermatozoa and modulated many sperm functions. In addition, previous studies revealed that KP could also have antioxidant function and protective effect against oxidative stress [12]. Kisspeptin has been shown to reduce in the sera of infertile men [13]. Amount of kisspeptin in human seminal fluid has been reported to be 60,000 times higher than that of the plasma [14]. KP treatment has also been found to improve human sperm motility, prevent acrosomal reaction, and induce capacitation [11]. Blood and semen kisspeptin concentrations may be considered as a potential marker of male reproductive health condition; and as an alternative strategy for treatment of infertility [14]. It contributes in regulation of sperm development and functions such as regulation of human sperm chemotaxis through G-protein signaling pathway [15]. Treating the sperm with kisspeptin has been shown to elevate the calcium content of the sperms, which in turn leads to sperm motility and hyper activation [11]. Also, kisspeptin plays a role in sperm-sertoli cell interaction in amphibians [16].

Although there is no experimental study on cryoprotective effect of KP on human spermatozoa; but, according to the evidence about beneficial effects of KP on antioxidant systems, we hypothesized that this protein might have a role in protecting impaired frozen/thawed ejaculated sperms. Therefore, the present study was designed to determine the effects of KP pre-incubation on motility, plasma membrane integrity, DNA quality, and apoptosis frequency in frozen-thawed human ejaculated spermatozoa.

Materials And Methods

Subjects

In this experimental study semen samples were collected from 30 healthy men, aged 18–35 years old, and referred to cytogenetic clinic in Shiraz, Iran. All the samples were considered normal based on World Health Organization (WHO) guidelines [17]. All the participants were asked to abstain from ejaculation 2-3
days before sample collection. The study design was approved by the Local Medical Ethics Committee of Shiraz University of Medical Sciences (IR.SUMS.REC.1396.S808). All the participants were informed about nature and purpose of the study and an informed consent form was obtained from all of them.

Swim-Up Method for Sperm Preparation

30 semen samples were allowed to liquefy at 37°C for 30 minutes. 1mL of each liquefied semen samples was transferred to a sterile conical centrifuge tube, and mixed with 2mL of Ham's F10 (Sigma) supplemented with 20% Human Serum Albumin (HSA) (Sigma). Then, sperm suspensions were centrifuged at 300g for 10 minutes. After centrifugation, the supernatant was decanted; then, 1 mL of the Ham's F10 was added to each pellet and the tubes were placed gently at 45° angle for 1hour at 37°C and 5% CO₂ to allow swimming -up of motile spermatozoa. Motile spermatozoa swam into the supernatant. This fluid was carefully aspirated and its volume was adjusted to 1 mL using Ham's F10. The samples were prepared individually and all experiments were performed for each individual sample separately.

Experimental Design

Each prepared sample was divided into three equal parts: Control group, 0.25 mL of the sample and 0.25 mL of Ham's F10 containing HSA; KP-treated group, 0.25 mL of the sample and 0.25 mL of 20 μM Kisspeptin-13 (Human KP, at final concentration of 10 μM, Sigma) [9,11]; and Reduced Glutathione (GSH)-treated group, 0.25 mL of the sample and 0.25mL of 2 mM GSH (at final concentration of 1mM,Sigma) [18]. All aliquots were incubated at 37°C for 30 minutes.

Each group was evaluated for motility according to the WHO guideline. Based on WHO criterion, the sperm motility can be classified into three categories: progressive in which the spermatozoa move forward linearly or in a large circle; non-progressive in which the spermatozoa move in site or slowly without any forward progress such as moving in small circles or beating flagellum; and immotile which no movement is seen among the spermatozoa [17]. The sperm motility was evaluated using light microscope through employing 10 randomly selected microscopic fields. In each field, number of progressive, non-progressive, and immotile sperms was counted and percentages of progressive, non-progressive, and immotile spermatozoa were calculated.

Sperm Cryopreservation and Thawing

A half mL of treated and control aliquots were diluted (1:1) with cryopreserved medium (Life Global, USA) dropwise in cryotubes at room temperature. The cryotubes containing the samples and freezing medium were exposed to liquid nitrogen vapor by locating them horizontally at 5 cm above the liquid nitrogen surface for 20 minutes; then, they were quickly plunged into liquid nitrogen for longer storage.

After 48 hours, the cryotubes were thawed at 37°C for 10 min; then, 1mL of fresh Ham's F10 was added to each sample. Finally, the tubes were centrifuged at 300g for 10 min. Spermatozoa were re-suspended in
Ham's F10 containing 20% Human Serum Albumin (HSA, Kedrion, Italy). After thawing, the sperm motility was re-examined.

**Assessment of Sperm Plasma Membrane Integrity**

**Lectins Histochemistry:** In the present study, Wheat Germ Agglutinin (WGA), Peanut Agglutinin (PNA) and Concanavalin A (ConA, All purchased from Sigma, USA) were used to detect non-capacitated, the acrosome intact, and acrosome-reacted spermatozoa.

The smears were prepared from all thawed samples, fixed with 2% paraformaldehyde for 20 minutes and subsequently washed with Phosphate Buffer Saline (PBS) for 30 minutes. The samples were incubated with Fluorescein isothiocyanate-conjugated WGA, ConA, and PNA at a dilution of 10 μg/mL for 2 hours at darkness. After washing with PBS, the samples were double stained with Hoechst (Sigma) for 5 minutes. The slides were evaluated using a fluorescent microscope (Nikon, Eclipse, E600).

**Lectins Flowcytometry:** Thawed samples were washed with 800 mL of PBS, were centrifuged at 170 g for 10 minutes and were fixed with 2% paraformaldehyde for 30 minutes at 4°C. Thereafter, the aliquots were centrifuged and the pellets were re-suspended in PBS. The aliquots were exposed to FITC-conjugated WGA, PNA, and ConA at a dilution of 10 μg/mL for 2 hours at 37°C in humidified environment and darkness. Frequency of the lectin-reacted spermatozoa was assessed, using FL1 channel of BD FACSCaliber™ flow cytometer (BD Biosciences, USA). The data were analyzed using FlowJo software.

**Assessment of Sperm DNA Quality**

The smears were prepared from each study group to record DNA and chromatin status, using Acridine Orange (AO), Aniline Blue (AB), Chromomycin A3 (CMA3), and TUNEL staining. A total of 200 sperms were evaluated per smear in randomly selected microscopic fields in all tests.

**Acridine Orange (AO) Test:** Thawed samples were spread on glass slides and allowed to air-dry. All the smears were fixed in methanol/acetic acid (3:1). Then, the slides were stained with 2–3 mL of 19% AO solution in citrate-phosphate buffer for 10 minutes. Stained spermatozoa were evaluated using fluorescence microscope (Nikon, Eclipse, E600). Three types of staining patterns were identified: green (double-stranded DNA), yellow (partially denatured DNA), and red sperms (completely single-stranded DNA) [8].

**Sperm DNA Structure Assay:** The sperm aliquots stained with AO (Sigma) were analyzed by flowcytometry as well. Briefly, 0.2 mL of thawed samples were diluted with TNE buffer (0.01 M Tris–HCl, 0.15 M NaCl, and 1 mM EDTA with a pH of 7.4) containing 10% glycerol at a final sperm concentration of 1-2×10^6 cell/mL. Then, the samples were immediately admixed with 0.4 mL of the detergent solution containing 0.1% Triton X-100, 0.15 M NaCl, and 0.08 N HCl with a pH of 1.2, at 4°C. After a short incubation of 30s, the cells were incubated in 1.2 mL of AO at the concentration of 6μg/mL in a solution containing 0.037 M citric acid, 0.126 M Na₂HPO₄, 0.001 M disodium EDTA, 0.15 M NaCl with a pH of 6.0
at 4°C. Then, the cells were analyzed by a FACSCaliber™ flow cytometer (BD Biosciences, USA). Strong green fluorescence (FL-1) and negative red fluorescence (FL-2) depicted regular sperm integrity. A sample of acid-treated sperms was used as positive control. DNA Fragmentation Index (DFI) was calculated using the following formula and obtained value was expressed as percentage:

\[ \text{DFI} = \frac{\text{Mean value of red fluorescence}}{\text{mean value of red fluorescence + green fluorescence}} \times 100\% \]

AO as a cationic metachromatic dye shows green fluorescence in monomeric state (when it bonds to DNA) and shows red fluorescence in polymeric state (when it bonds to RNA or denaturated single-stranded DNA). Cells with High DNA Stain ability (HDS) present in upper quarters of the dot blot chart represent immature cells indicating the cells with higher histone and lower protamine content [18]. The sperms with higher histone and lower protamine content have larger size.

**Aniline Blue (AB) Staining**

Air-dried smears were fixed in 3% buffered glutaraldehyde for 30 minutes. Then, the slides were stained with 5% aqueous Aniline Blue (AB) in 4% acetic acid (pH 3.5) for 7 minutes. Staining intensity of the sperm head was divided into three categories: unstained (gray/white), partially stained, and entire sperm head stained dark blue.

**Chromomycin A3 (CMA3) Staining**

For microscopic evaluation, the smears were fixed in methanol/glacial acetic acid (3:1) at 40°C for 5 minutes. Each slide was treated for 20 minutes with 100 µL of 0.25 mg/mL CMA3 in McIlvaine buffer containing 10 mM MgCl₂ (pH 7.0). The slides were rinsed in PBS and mounted using buffered glycerol. The samples were observed by fluorescent microscope (Nikon, Eclipse, E600). Two types of staining patterns were identified, namely bright green fluorescence of the sperm head (abnormal chromatin packaging), and dull green staining (normal chromatin packaging).

**TUNEL Staining**

To detect the DNA breaks, Dead End Fluorometric TUNEL System kit (Promega, United States) was used according to the manufacturer’s protocol. Briefly, the samples were fixed in 4% buffered formaldehyde for 25 minutes at 4°C, were washed twice in PBS and then were treated with 0.2% Triton X-100 in PBS for 5 minutes. Then, the samples were then incubated in equilibration buffer for 10 minutes. They were incubated in the reaction mix which was prepared according to the manufacturer’s instructions. Staining was performed at 37°C for 60 minutes in humidified chamber protected from light. The reaction was stopped by adding 2× Saline-Sodium Citrate (SSC) buffer after 15-min incubation and then was counterstained using Hoechst (0.1 mg/mL) in PBS for 10 minutes. The slides were evaluated using fluorescent microscope (Nikon, Eclipse, E600).

**Statistical Analysis**
Data normality was checked using Shapiro–Wilk test. Comparisons were performed using one-way Analysis of Variance (ANOVA) and least significant difference (LSD) or Tukey tests if the data were normally distributed, and Kruskal–Wallis and one-way ANOVA test were used if the data were not normally distributed. All data were expressed as mean values ± Standard Deviations (SD). The level of statistical significance was set at ≤0.05. SPSS software version 24 for windows was used to analyze the data. The graphs were illustrated by GraphPad Prism software (version 6.0).

**Results**

**Sperm Motility Assessment**

Percentages of motile spermatozoa were evaluated before freezing and after thawing. Comparison of results obtained before freezing showed that, both kisspeptin and GSH incubation led to a significant increase in the frequency of progressive sperms (both P= 0.003) and a significant decrease in immotile sperms compared to the controls (P= 0.004 and P=0.0001, respectively).

Comparison of each sample with its equivalent after freeze-thaw cycle showed a significant decrease in the total sperm motility. After thawing, frequency of the total motile spermatozoa was significantly higher in the KP and GSH-treated aliquots compared to the control group (P<0.0001) (Fig 1). Pre-incubation with both GSH and KP led to a significant increase in the frequency of progressive (P= 0.016; P= 0.06, respectively) and non-progressive (both P= 0.0001) sperms compared to the controls. In contrast, comparison of each sample with its equivalent after freeze-thaw cycle showed a significant increase in the frequency of immotile sperms. Frequency of immotile spermatozoa significantly decreased in the KP and GSH-treated aliquots compared to the controls after freeze-thaw cycle (P= 0.0001, Table 1).

| Groups    | Sperm motility (%) | Progressive (Mean±S.E.) | Non-progressive (Mean±S.E.) | Immotile (Mean±S.E.) |
|-----------|--------------------|-------------------------|----------------------------|----------------------|
| Control   | Before             | 71.9±2.37*              | 2.94±0.59                  | 25.16±1.93*          |
|           | After              | 8.76±1.07*              | 11.5±0.84*                 | 79.74±1.36*          |
| GSH       | Before             | 75.88±1.26              | 4.61±0.68                  | 19.51±1.08           |
|           | After              | 12.98±1.79              | 18.27±2.26                 | 69.13±2.11           |
| Kisspeptin| Before             | 78.71±1.49              | 4.45±0.52                  | 17.03±1.42           |
|           | After              | 14.18±1.2               | 19.82±1.59                 | 66.35±1.2            |

* Represent significant differences with GSH and KP-supplemented groups (P≤0.05).

**Effects of KP and GSH on the Sperm Plasma Membrane and Acrosome Integrity**
Frequency of intact spermatozoa was analyzed by flow cytometry of lectin-reacted sperms. Incubation of the sperm for 30 minutes before freezing led to a significant increase in the percentage of acrosomal intact spermatozoa in KP-treated aliquots compared to both GSH-treated aliquots and controls (both P<0.001). Since acrosome content can be stained with PNA, PNA-positive cells indicate acrosomal-intact sperms. Percentage of acrosomal intact spermatozoa in GSH-treated aliquots and control groups was statistically similar. Florescent microscopy showed acrosomal intact sperms versus acrosomal-reacted one. Acrosome in the anterior part of the head of intact spermatozoa was stained with PNA intensely (Fig 2).

Mannose residues in the posterior membrane of the acrosome were exposed in acrosomal-reacted spermatozoa and were stained using ConA. Treatment with KP and GSH for 30 minutes before cryopreservation showed no significant changes in the percentage of acrosomal-reacted spermatozoa compared to the controls. According to florescent microscopy, acrosomal-reacting sperms showed an intense staining with Con A, while non-reacting sperm heads showed a “weak” staining (Fig 2).

Flow cytometric assessment of the sperms reacting with FITC-conjugated WGA indicated that treatment for 30 minutes before freezing process led to a significant increase in the percentage of non-capacitated spermatozoa in KP group compared to both GSH-treated and control groups (P<0.0001). Percentage of non-capacitated spermatozoa in GSH-treated group was statistically similar to that of the control. Staining of the sperm smears showed that sperm populations reacted to WGA differentially according to their status (intact vs. capacitated). Non-capacitated sperm heads reacted with WGA uniformly (Fig 2).

**Effects of KP and GSH on the Sperm DNA Quality**

**Assessment of Acridine Orange (AO) Test**

Although both smears stained with AO and results of sperm chromatin structure assay showed that all samples contained some sperms with denatutrated nuclei, the pre-incubation with KP or GSH had no significant influence on percentage of sperms with red fluorescence reaction (DFI%, Fig 3). Results of smear analysis confirmed flowcytometric data (Fig 3).

**Assessment of Aniline Blue (AB) Staining**

There was a significant higher number of spermatozoa with normal histone content in KP-treated group compared to both GSH-treated and control groups (p=0.0001). However, no significant changes were observed in frequency of normal spermatozoa of GSH-treated group in comparison with the control group. KP-treated group provided a better condition compared to other groups (Fig 4, (a) and (b))

**Assessment of Chromomycin A3 (CMA 3) Staining**

Percentage of spermatozoa with normal protamine content was significantly higher in KP-treated group compared to the control group (p=0.002). GSH-supplementation could not provide a significant superior environment to improve histone-protamine substitution compared to the control condition. (Fig 4,c & d).
Assessment of TUNEL Staining:

Percentage of DNA-fragmented spermatozoa was significantly lower in KP-treated group compared to both GSH-treated and control groups (both P=0.002). Mean value of the percentage of apoptotic sperms in GSH- treated groups was the same as the control group, and GSH treatment was not good enough to reduce the frequency of apoptotic sperms. KP- treatment provided a better condition to protect the sperms from apoptosis compared to other groups (Fig 5).

Discussion

In spite of great improvement in cryopreservation techniques, poor sperm quality after thawing process has caused many problems, so that it dramatically reduces the chance of fertilization or pregnancy rate in ART programs [19]. Results of the current study indicated that KP provided a better condition to prevent the sperm quality loss during cryopreservation.

Considerable reduction in the sperm motility is the most commonly reported problem of cryopreservation on human spermatozoa [20]. Results of the current study showed that KP ameliorated the cell motility in both fresh and freeze-thawed sperms after incubation for 30 minute. Despite importance of the sperm motility in fertilization phenomenon, the mechanism by which it reduces has not been clarified. It has been demonstrated that antioxidant supplementation leads to an increase in the sperm motility. Sperm motility has been shown to improve by treating the methotrexate-treated rats with KP through its antioxidant property [21]. Results of a pervious study showed that sperm motility is partially dependent on the mitochondrial quality and function [22]. Mitochondria provide necessary energy to the sperm tail filaments, thus facilitating efficient progressive movement for penetration into the zona pellucida of the oocyte [4]. Pinto [11] suggested that KP modulates the sperm motility by activating the KP receptors on the sperms. Kisspeptin, can activate signal transmission pathways via GPR54, leading to its modulatory role in the sperm movement.

Our results showed that percentage of progressive motile spermatozoa significantly reduced after cryopreservation. Incubating the sperms with KP mitigates detrimental effects of freeze-thaw cycle on the sperm motility. Although the mechanism of the KP effects on sperm motility is out of the scope of this study, but in comparison with GSH, as an antioxidant, ameliorating effects of KP on the sperm motility may be exerted through its antioxidant-like properties shown in prior evidence on antioxidant activity of KP. Increased ROS production is one of the most critical problems during sperm freeze-thaw cycle [23] and it has adverse influence on the sperm motility[24].

The evidence shows that cryopreservation induces premature capacitation and spontaneous acrosomal reaction of the spermatozoa [25] and pre-treatment of the sperms with various anti-oxidants improves the plasma membrane integrity [26]. Cryo-induced capacitation is frequently mentioned as one of the factors associated with reduced performance of cryopreserved spermatozoa in the female reproductive tract accompanied by a loss of the acrosomal reaction potential and its contents. In the current study, a significantly higher number of spermatozoa in the KP- treated group maintained acrosomal content and escaped from early capacitation. In fact, KP signaling in the sperm could prevent unwanted acrosomal
reaction and prevented the sperm plasma membrane damage following freezing and thawing procedure. A previous study also showed that KP could not cause the human sperms to release acrosomal content and this is attributed to presence of KP receptor [11]. In contrast, it has been shown that administration of KP inhibitor reduced fertilization rate of non-capacitated sperms of the mouse [9]. Our results showed, number of both capacitated and acrosomal reacted sperms reduced as a result of KP administration. GSH, as a well known antioxidant, has no influence on the number of capacitated, and acrosome-reacted, and thawed sperms. Accordingly, it may be suggested that KP acts on capacitation and acrosomal reaction by another mechanism rather than its antioxidant activity.

Assessment of cryopreservation influence on the sperm chromatin as the basis of DNA integrity is essential in achieving fertilization and embryo development; as it is critical to guarantee success of ART [27]. Oxidative stress is considered as a possible mechanism for the cause of sperm DNA damage. It has been widely reported that ROS is attributed to poor semen quality and defective functional capability of human spermatozoa [28].

Results of a study showed that freeze-thaw procedure on the semen samples prepared from fertile and normal men induced sperm DNA fragmentation and defect in chromatin structure [29]. Live spermatozoa with DNA fragmentation after cryopreservation have not sufficient ability and quality for fertilization. Results concerning cell damage induced by cryopreservation are conflicting. Bell and Wang [30] found that, sperm cryopreservation of infertile men induced higher lipid peroxidation, which could be the result of loss of peroxidation protective enzyme activity [31] and higher ROS production. In contrast, some researchers did not find a significant enhancement in lipid peroxidation of the sperm after cryopreservation [32]. This disagreement between the results can be explained by different procedures of cryopreservation, eventual preparation of the semen before cryostorage, and various the tests evaluating DNA integrity.

In fact, sperm DNA damage has been attributed to high ROS levels in fresh and freeze/thawed semen [6]. Results of previous studies revealed that, supplementation of antioxidants reduced oxidative stress and sperm nuclear DNA damage (assessed by the comet assay), and improved the human sperm quality in the process of freezing/thawing [33]. In addition, Gadea and Gumba [34] reported that adding GSH to the thawing medium resulted in a reduction in ROS generation and DNA fragmentation, and improved fertilizing ability of frozen bull spermatozoa. Pre-treatment with KP, as an antioxidant, may also exert the same mechanism in reducing the number of DNA-damaged sperms.

Kisspeptin-10 treatment in the brain tissue has been shown to reduce the L-methionine-induced DNA fragmentation, associated with the increase in the GSH activity [35]. Oxidants can react with cellular and structural components and change their properties. Such interactions can change the “Ca^{2+} code” and modify essential pathways [36]. Calcium as a second messenger and signal molecule responds to oxidant stimuli [37]. Therefore, in the current study, pre-treatment of the sperms with KP, as an antioxidant resulted in modulation of DNA integrity, which may be due to calcium signaling regulation, as well as the other pathways, influenced by freeze-thaw induced shocks.
Under normal condition, DNA-protamine packaging protects the sperms from ROS attack; however, deficiency in protamination makes the sperm DNA vulnerable to ROS damage [38]. Oxidative stress is not the only mechanism explaining the origin of DNA damage. There are two other molecular mechanisms of sperm damage, namely suggested: defective chromatin packaging and apoptosis [6]. All of these mechanisms could simultaneously lead to DNA damage. Results of this study showed that KP pretreatment could improve protamination and it may explain preventive effect of KP on apoptosis; however, the DNA quality did not change.

The freeze-thaw procedure has been reported to cause chromatin defects, protamine packaging problems, and DNA fragmentation[28]. In this study, pre-incubation with KP led to a significant improvement in protamine-histone substitution as indicated by CMA3 and AB staining. As pre-treatment with GSH, as an antioxidant, had no effect on this criterion, it seems that KP cannot influence protamine-histone substitution through its antioxidant activity, which needs to be investigated in future studies.

Conclusion

Findings of the present study revealed that, pre-treatment of the sperms with KP can improve the sperm quality and lead to enhancement of freezing tolerance. KP has superior influence on the sperm freezing tolerance compared to GSH, as GSH just keeps the sperm motility while KP improves motility along with DNA quality after freeze-thaw cycle. Therefore, KP can be considered as a good candidate to improve quality of freeze-thawed sperm to be used in ART programs and increase fertilization and successful outcomes.

Abbreviations

AB: Aniline Blue
ANOVA: One-way Analysis of Variance
AO: Acridine Orange
ARTs: Assisted Reproduction Technologies
CMA3: Chromomycin A3
ConA: Concanavalin A
DFI: DNA Fragmentation Index
DNAf: DNA fragmentation
FITC: Fluorescein Isothiocyanate
Declaration

Acknowledgment

The authors wish to thank research vice chancellery of research deputy of Shiraz University of Medical Sciences by grant no 13379 and M Salmannjad and M Sani for technical supports. This study is a part of the requirements of PhD degree by S.F Hosseini. The authors would also like to thank Shiraz University of Medical Sciences, Shiraz, Iran and also Center for Development of Clinical Research of Nemazee Hospital and Dr. Nasrin Shokrpour for editorial assistance.

Availability of data and materials

All data are included in the published article.

Ethics approval and consent to participate
This study was approved by the Ethics Committee of Shiraz University of Medical Sciences, Feb, 2018(IR.SUMS.REC.1396.S808).

**Consent for publication**

Written informed consent for publication was obtained from all participants.

**Competing interests**

No conflicts of interest to declare.

**Funding**

This work was supported by research vice chancellery of research deputy of Shiraz University of Medical Sciences by grant no 13379.

**Authors’ contributions**

T.K.: technical support and revising the manuscript. S.F.H.: implementation of the study, data acquisition, analysis and interpretation, and drafting of the manuscript; T.T.K. technical support, data analysis and interpretation and major revising the manuscript. E.A.: funding acquisition, study design, technical support, data analysis and interpretation, major revising the manuscript, ethic approval. All authors provided final approval of the submitted manuscript.

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**Figures**

**Figure 1**

Percentage of the total motile (progressive and non-progressive) sperms in the samples pre-treated with KP and GSH before and after freezing and comparing them with the control aliquots. Results showed that both GSH and kisspeptin pre-treatment improved the total sperm motility.
Figure 2

The aliquots of representative semen samples stained with FITC-conjugated lectins and analyzed by flow cytometry; a) Controls; b) GSH, and c) KP-treated ones. The lectin histochemistry using Peanut Agglutinin (PNA) shows intact spermatozoa (arrowhead), and non-reacted one in the acrosomal region indicates spermatozoa releasing the acrosome content (arrow, e). The spermatozoa reacted with Con A are the spermatozoa releasing the acrosome content (arrowhead) and those which remained non-reacted indicate intact spermatozoa (arrow, e). The lectin histochemistry using WGA showed that, reacted spermatozoa indicate capacitated spermatozoa (arrowhead) and those with weak reaction represent non-capacitated spermatozoa (arrow, e).
*Significant difference with the other groups (P<0.0001)
Figure 3

. Acridine orange stains the sperm chromatin (a). The sperms with denaturated nuclei showed metachromasia (arrowhead), while normal double-stranded DNA showed green fluorescence (green arrows). Sperm chromatin structure assay using flow cytometry showed that, pre-treatment with KP or GSH had no significant influence on the sperm DNA Fragmentation Index (DFI %) compared to the controls (b). Acid-treated samples were used as technical control. Results showed that acid-treated samples contained a significantly higher number of the sperms with denaturated nuclei. Dot plots of representative sperm aliquots are also depicted (c). Gated area in acid-treated sample is depicted after excluding High DNA Stainability (HDS) cells and debris. The HDS population represents more rounded immature sperms.
Figure 4

Aniline blue chromatin staining of the spermatozoa pre-treated with KP before freezing procedure showed a significant higher number of sperms with normal histone content compared to other aliquots (a). The micrograph shows normal spermatozoa (white arrow) versus those with higher histone content (blue arrow) (b).
arrow) (b). CMA3 chromatin staining of spermatozoa pre-treated with KP and GSH before freezing procedure showed a significant higher number of sperms with normal protamine content compared to other aliquots (c). The histograph shows normal spermatozoa (green arrow) versus those with protamine deficiency (yellow arrow, d).

![Graph showing percentage of TUNEL-positive sperms](image)

**Figure 5**

TUNEL staining showed that, the aliquots pre-treated with KP before freezing procedure contain a significant lower number of TUNEL-positive sperms. The graph shows percentages of TUNEL-positive cells (a). Apoptotic spermatozoa (arrowhead) versus non-apoptotic spermatozoa (arrow, b).