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Original Article

Effect of Pre-Incubation of Cryopreserved Sperm with either Kisspeptin or Glutathione to Mitigate Freeze-Thaw Damage

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Abstract

Background: Sperm cryopreservation reduces sperm quality. Kisspeptin (KP) has beneficial effects on sperm functions. This study compares the effect of KP and Glutathione (GSH) on mitigating the detrimental effects of the freeze-thaw cycle on sperm.

Methods: An experimental study was conducted in Birjand (Iran) during 2018-2020. Thirty normal swim-up semen samples were treated with Ham's F10 medium (negative control), 1 mM GSH (positive control), or KP (10 µM) for 30 min before freezing. The motility, acrosome reaction, capacitation, and DNA quality of the frozen-thawed sperms were assessed according to the WHO guidelines. Statistical analysis was performed using paired *t* test, one-way analysis of variance, and least significant difference.

Results: Pre-incubation with KP significantly increased the percentage of sperm motility (34.00±6.7, P=0.003) compared to the control (20.44±7.4) and GSH-treated (31.25±12.2) aliquots. The frequency of non-capacitated spermatozoa was significantly higher in the KP-treated group (98.73%) than in the control (96.46%) and GSH-treated (96.49%) aliquots (P<0.001). The percentage of acrosome-intact spermatozoa in the KP-treated group (77.44%) was significantly higher than the control (74.3%) and GSH-treated (74.54%) groups (P<0.001). The sperm frequency with normal histone in the KP-treated group (51.86%) and with normal protamine (65.39%) was significantly higher than the controls (P=0.001 and P=0.002, respectively). The percentage of TUNEL-positive sperm was significantly lower in the KP-treated group (9.09±2.71) than both GSH-treated (11.22±2.73) and control (11.31±2.2) groups (both P=0.002).

Conclusion: Pre-incubation with KP protects sperm motility and DNA integrity from the detrimental effect of the freeze-thaw cycle. KP is suitable as a pre-treatment to control sperm quality during freezing-thawing.

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Keywords ● Kisspeptins ● Sperm motility ● DNA ● Lectins ● Cryopreservation

What's Known

- Kisspeptin has an antioxidant effect, and its receptors are present on the sperm surface.
- Kisspeptin plays a role in sperm quality and is a potential male reproduction health indicator.

What's New

- Pre-incubation of sperm with kisspeptin before cryopreservation preserves sperm motility and DNA integrity. It also inhibits spontaneous acrosome reaction and premature capacitation of sperm.

Introduction

Cryopreservation is the process of cooling sperm at low temperatures to protect and preserve the normal structure

and function of living cells. It is a common procedure used in assisted reproductive technology (ART) to prevent testicular failure and preserve male fertility due to the iatrogenic effects of chemotherapy, radiotherapy, and surgical treatments.[1] However, it was reported that the use of cryopreservation in ART has severe detrimental effects on the structure (nucleus, membrane, mitochondrion) and function (motility) of sperm. For instance, it was reported that sperm quality was impaired after 543 samples cryopreserved from 1995 to 2015 were thawed.[2] Other cryopreservation-induced damages include reduced sperm motility and viability, changes in the composition of sperm plasma and mitochondrial membranes, abnormal acrosome reaction, plasmalemma glycoconjugate modification, and DNA fragmentation (DNAf).[3] In addition, lower levels of cholesterol in the sperm plasma membrane make it susceptible to damage from the freeze-thaw cycle, which may lead to premature capacitation.[4] Physical damage caused by cryopreservation also affects cell surface glycoconjugates[5] or induces acrosome reaction by producing reactive oxygen species (ROS) due to changes in the fluidity and potential of the mitochondrial membrane.[6] Cryopreservation diminishes antioxidant enzyme activity in spermatozoa, making them more vulnerable to oxidative stress. Elevation of ROS and lower antioxidant capacity can induce apoptosis, leading to DNAf. A previous study assessed the effect of antioxidants on reducing the harmful effects of cryopreservation.[7] Superoxide dismutase and/or catalase in the semen play a role in the neutralization of ROS but are diluted by the cryoprotectant medium.[8] Glutathione (GSH) acts as an antioxidant by neutralizing ROS or activating exogenous antioxidants. Supplementing the freezing media with GSH, a popular antioxidant in infertility clinics, improves sperm quality.[7] Therefore, in the present study, we used samples pre-incubated with GSH as a positive control.

Kisspeptin (KP) is a neuropeptide that binds to G protein-coupled receptor 54 (GPR54). Studies showed that GPR54, a KP receptor, is found in mature sperm of mice[9] and monkeys.[10] For the first time, Pinto and colleagues showed that KP and GPR54 are present in human spermatozoa and modulate several sperm functions.[11] The potential of KP-based therapies was reported in several studies. It was shown that KP increases the activity of catalase and superoxide dismutase in male rats,[12] and antioxidant capacity in rat testis by KP injection.[13] It is also reported that serum KP levels are significantly low in infertile men[14] and treatment with KP increases sperm motility, prevents premature acrosome reaction,[11] and induces capacitation.[15] Therefore, KP may be considered a potential male reproductive health marker in the blood and semen, and an alternative strategy for treating infertility.[16]

Given the above, to the best of our knowledge, there are no experimental studies on the effect of KP on the freeze-thawing of human spermatozoa. We hypothesize that KP might play a role in preventing the detrimental effect of the freeze-thaw cycle on human sperm. The present study, therefore, aimed to assess the effect of pre-incubation of cryopreserved sperm with KP or GSH on motility, plasma membrane integrity, DNA quality, and apoptosis.

Materials and Methods

In an experimental study conducted during 2018-2020, semen was collected from 30 healthy men aged 18-35 years, who were referred to Emam Reza Hospital, Birjand University of Medical Sciences (Birjand, Iran). In accordance with the WHO guidelines, all samples were considered normal.[14] All participants were requested to abstain from ejaculation 2-3 days before sample collection. Semen with any abnormalities (sperm count and motility, morphology, pH, viscosity) were excluded from the experiment. The study was approved by the Ethics Committee of Shiraz University of Medical Sciences (IR.SUMS.REC.1396.S808).

Sperm Preparation

One milliliter of each sample of liquefied semen was mixed with 2 mL of Ham's F10 (Sigma, USA), supplemented with 20% human serum albumin (HSA) (Kedrion Biopharma, Italy), and centrifuged at 300 g for 10 min. The supernatant was decanted, and 1 mL of Ham's F10 was added. The tubes were gently positioned at a 45-degree angle for one hour at 37 °C and 5% CO_2 for swim-up separation of motile spermatozoa. This supernatant was carefully aspirated, and its volume was adjusted to 1 mL using Ham's F10.

Experimental Design

Each sample was divided into three equal parts and incubated at 37 °C for 30 min, namely control group: 0.25 mL of the sample and 0.25 mL of Ham's F10 containing HSA (N6908; Sigma, USA), KP-treated group: 0.25 mL of the sample and 0.25 mL of 20 µM human KP-13 at a final concentration of 10 µM (H5962.0001; BACHEM, Switzerland),[9, 11] and GSH-treated group: 0.25 mL of the sample and 0.25 mL of

2 mM GSH at a final concentration of 1 mM (G4251; Sigma, USA).[17]

The sperm motility was evaluated based on the WHO criterion and classified as progressive (moving forward linearly or in a large circle), non-progressive (moving in the site or slowly without any forward progression), and immotile (no movement).[14] The evaluation involved systematic random sampling of 10 microscopic fields. The number of progressive, non-progressive, and immotile sperms was counted in each field and expressed as percentages.

Freezing and Thawing of Sperms

A half milliliter of the aliquots was diluted (1:1) with a cryopreserved medium (LGSF-020; Life Global, USA), drop-wise in cryotubes at room temperature, and then positioned horizontally at 5 cm above liquid nitrogen vapor for 20 min. Then, they were quickly immersed in liquid nitrogen for extended storage. After 48 hours, the cryotubes were thawed at 37 °C for 10 min. One milliliter of fresh Ham's F10 was then added and centrifuged at 300 g for 10 min. Spermatozoa were resuspended in Ham's F10 containing 20% HSA. After thawing, sperm motility was re-examined.

Plasma Membrane Integrity Assessment

Lectins Histochemistry: Wheat germ agglutinin (WGA), peanut agglutinin (PNA), and concanavalin A (ConA) (all from Sigma, USA) were used to detect non-capacitated, acrosome intact, and acrosome-reacted spermatozoa, respectively. The smears of the thawed samples were fixed with 2% paraformaldehyde for 20 min. After washing with phosphate-buffered saline (PBS), the samples were incubated with fluorescein isothiocyanate (FITC)-conjugated lectins at 10 µg/mL dilution for two hours and double stained with Hoechst (Sigma, USA) for five min. The slides were evaluated using the Eclipse E600 fluorescent microscope (Nikon, Japan).

Flow Cytometry: Thawed samples were washed with 800 µL of PBS, centrifuged at 170 g for 10 min, and fixed with 2% paraformaldehyde for 30 min at 4 °C. Thereafter, the aliquots were centrifuged, and the pellets were resuspended in PBS. The aliquots containing 1×10^5 cells were exposed to FITC-conjugated lectins at a dilution of 10 µg/mL for two hours at 37 °C. The samples were assessed using the FL1 channel (wavelengths ≈495 nm) and FL3 channel (wavelength >575 nm) of the FACSCalibur™ flow cytometer (BD Biosciences, USA). The data were analyzed using FlowJo software (BD Biosciences, USA).

DNA Quality Assessment

The smears were prepared to measure DNA and chromatin status using acridine orange (AO) 65-61-2 (Sigma, USA), aniline blue 28631-66-5 (Sigma, USA), Chromomycin-A3 (CMA3) 7059-24-7 (Sigma, USA), and terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) staining. A total of 200 sperms were evaluated per smear in randomly selected microscopic fields. The microscopic slide was scanned from one side, and every three fields were selected. The counting continued until 200 sperms were evaluated.

Acridine Orange Test

The smears from thawed samples were fixed in methanol:acetic acid (3:1) and then stained with 2-3 mL of 19% AO solution in a citrate-phosphate buffer for 10 min. Stained spermatozoa were evaluated using a fluorescence microscope. Three types of staining patterns were identified, namely green (double-stranded DNA), yellow (partially denatured DNA), and red (completely single-stranded DNA).[5]

DNA Structure Assay

The sperm aliquots stained with AO were also analyzed using the flow cytometry technique. Briefly, 0.2 mL of thawed samples with 1 million events was diluted with TNE buffer (0.01 M Tris–HCl, 0.15 M NaCl, and 1 mM EDTA, pH: 7.4) containing 10% glycerol at a density of $1\text{-}2\times10^6$ sperm/mL. Then, they were immediately mixed with 0.4 mL of the solution containing 0.1% Triton X-100, 0.15 M NaCl, and 0.08 N HCl (pH 1.2) at 4 °C. After 30 seconds, they were incubated in 1.2 mL of AO at a 6 µg/mL concentration in a solution containing 0.037 M citric acid, 0.126 M Na_2HPO_4, 0.001 M disodium EDTA, and 0.15 M NaCl (pH 6.0) at 4 °C. These were analyzed using the FACSCalibur™ flow cytometer. Strong green (FL-1) and negative red fluorescence (FL-2) depicted normal sperm integrity (excitation wavelength at 488 nm and emission wavelengths at 520 nm for FL1 and 640 nm for FL3). A sample of acid-treated sperm was used as a positive control. DNAf index (DFI) was calculated using the formula below and expressed as a percentage.

DFI=Mean value of red fluorescence/(mean value of red+green fluorescence).

AO shows green fluorescence in the monomeric state (when it binds to DNA) and red fluorescence in the polymeric state (when it binds to RNA or denatured single-stranded DNA). Larger cells with higher histone and lower protamine content are found in the upper quartile of the dot blot chart. These cell populations

show high DNA stainability (HDS) and represent immature cells.[17]

Aniline Blue Staining

Air-dried smears were fixed in 3% buffered glutaraldehyde for 30 min and then stained with 5% aqueous aniline blue in 4% acetic acid (pH 3.5) for seven min. The staining intensity of the sperm head was divided into three categories, namely unstained (gray/white), partially stained, and entire sperm head stained dark blue.

Chromomycin A3 Staining

The smears were fixed in methanol:glacial acetic acid (3:1) at 40 °C for five min and treated with 100 µL of 0.25 mg/mL chromomycin A3 in a McIlvaine buffer containing 10 mM $MgCl_2$ (pH 7.0) for 20 min. The slides were mounted using buffered glycerol and observed with a fluorescent microscope. Two types of staining patterns of the sperm head were identified, namely bright green fluorescence and dull green staining (abnormal and normal chromatin packaging, respectively).

TUNEL Staining

The DeadEnd™ Fluorometric TUNEL System kit (Promega, USA) was used according to the manufacturer's protocol to detect DNA fragmentation. Briefly, the samples were fixed in buffered formaldehyde, washed twice in PBS, treated with 0.2% Triton X-100 in PBS, and incubated in an equilibration buffer. Then, they were incubated in the reaction mix prepared according to the manufacturer's instructions. The reaction was stopped by adding 2× saline-sodium citrate buffer and was counterstained with Hoechst (0.1 mg/mL). The slides were evaluated using a fluorescent microscope.

Statistical Analysis

Paired *t* test was used to compare sperm motility before and after the freeze-thaw cycle. In addition, one-way analysis of variance (ANOVA) and least significant difference (LSD) tests were used to analyze the data. All data were expressed as mean±SD. The level of statistical significance was set at 0.05. The data were analyzed using SPSS software for windows, version 24.0 (IBM, USA). GraphPad Prism software, version 6.0 (Graphpad, USA) was used to create the graphs.

Results

Sperm Motility Assessment

The percentage of motile spermatozoa was evaluated before freezing and after thawing. The results before freezing showed that both KP- and GSH-treated groups exhibited a significant increase in the frequency of progressive sperms (both P=0.003) and a significant decrease in immotile sperms compared to the control group (P=0.004 and P<0.001, respectively). The results of the paired *t* test revealed a significant decrease in the percentage of progressive and non-progressive motile sperms in each sample before freezing compared to those after thawing (all P<0.001). After thawing, the frequency of the total motile spermatozoa was significantly higher in the KP- and GSH-treated groups than the control group (P<0.001, figure 1). Pre-incubation with KP led to a significant increase in the frequency of progressive (P=0.016) and non-progressive (P=0.001) sperms compared to the controls. Before freezing, the frequency of progressive motile sperms in the GSH-treated group was the same as those in the control group (P=0.06). In contrast, after thawing, a significant increase in the frequency of immotile sperms (P<0.001) was observed. The frequency of immotile spermatozoa after thawing significantly decreased in the KP- and GSH-treated groups compared to the control group (both P<0.001). As indicated in table 1, the percentage of immotile sperms in the GSH-treated group was statistically similar to the KP-treated group (P=0.205).

Plasma Membrane and Acrosome Integrity

Incubation of the sperms with KP before freezing led to a significant increase in the percentage of acrosome-intact spermatozoa compared to both GSH-treated and control groups (both P<0.001). The percentage of acrosome-intact spermatozoa (PNA-positive) in the GSH-treated and control groups was

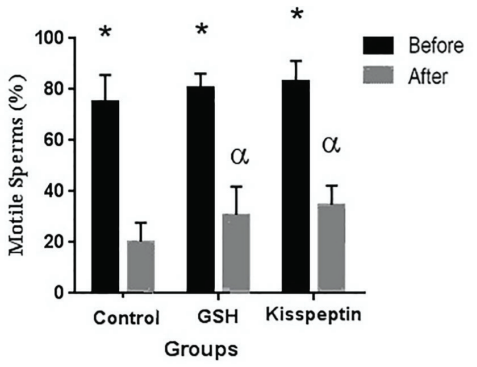

Figure 1: Data comparison revealed that the percentage of total motile (progressive and non-progressive) sperms in the samples pre-treated with KP and GSH was significantly higher than control aliquots both before and after freezing. Results showed that both GSH and KP pre-treatment prevented sperm motility loss. The total number of samples: 30. *Significant difference with matched groups after freezing (P<0.0001). αSignificant difference with control after freezing (P<0.05).

Table 1: Comparison of sperm motility in different groups before freezing and after thawing

Groups	Stage	Progressive (n=30) (Mean±SD)	Non-progressive (n=30) (Mean±SD)	Immotile (n=30) (Mean±SD)
Control	Before freezing	71.9±12.53*	2.94±3.12*	26.16±10.22*
	After thawing	8.76±5.73	11.5±4.52	79.74±7.34
GSH-treated	Before freezing	75.82±6.66*	4.61±3.6*	19.51±5.7*$^{\varphi}$
	After thawing	12.98±9.79	18.27±12.37	69.13±11.59$^{£}$
KP-treated	Before freezing	78.71±8.15*$^{\alpha}$	4.45±2.86	17.03±7.8 β*
	After thawing	14.18±6.59$^{€}$	19.82±8.72$^{£}$	66.35±6.6$^{\mu}$

*Significant difference with matched aliquots after thawing (all P<0.001), $^{\alpha}$Significant difference with control before freezing (P=0.003), $^{\varphi}$Significant difference with control before freezing (P=0.004), $^{\beta}$Significant difference with control before freezing (P<0.001), $^{€}$Significant difference with control after thawing (P=0.016), $^{£}$Significant difference with control after thawing (P<0.001), $^{\mu}$Significant difference with control after thawing (P<0.001), GHS: Glutathione, KP: Kisspeptin

statistically similar (P=0.343). Fluorescent microscopy showed intense PNA staining of the acrosome in the anterior part of the head of the intact spermatozoa (figure 2).

Mannose residues in the posterior membrane of the acrosome were exposed to acrosome-reacted spermatozoa and stained using ConA. Treatment with KP and GSH showed no significant change in the percentage of acrosome-reacted spermatozoa compared to the controls (P=0.114 and P=0.772, respectively). The percentage of acrosome-reacted spermatozoa in both KP- and GSH-treated groups was statistically the same (P=0.195). Based on the results of fluorescent microscopy, acrosome-reacted spermatozoa showed intense staining with Con A, while acrosome-non-reacted spermatozoa showed weak staining (figure 2).

Flow cytometric assessment of the sperm acrosome reaction with FITC-conjugated WGA

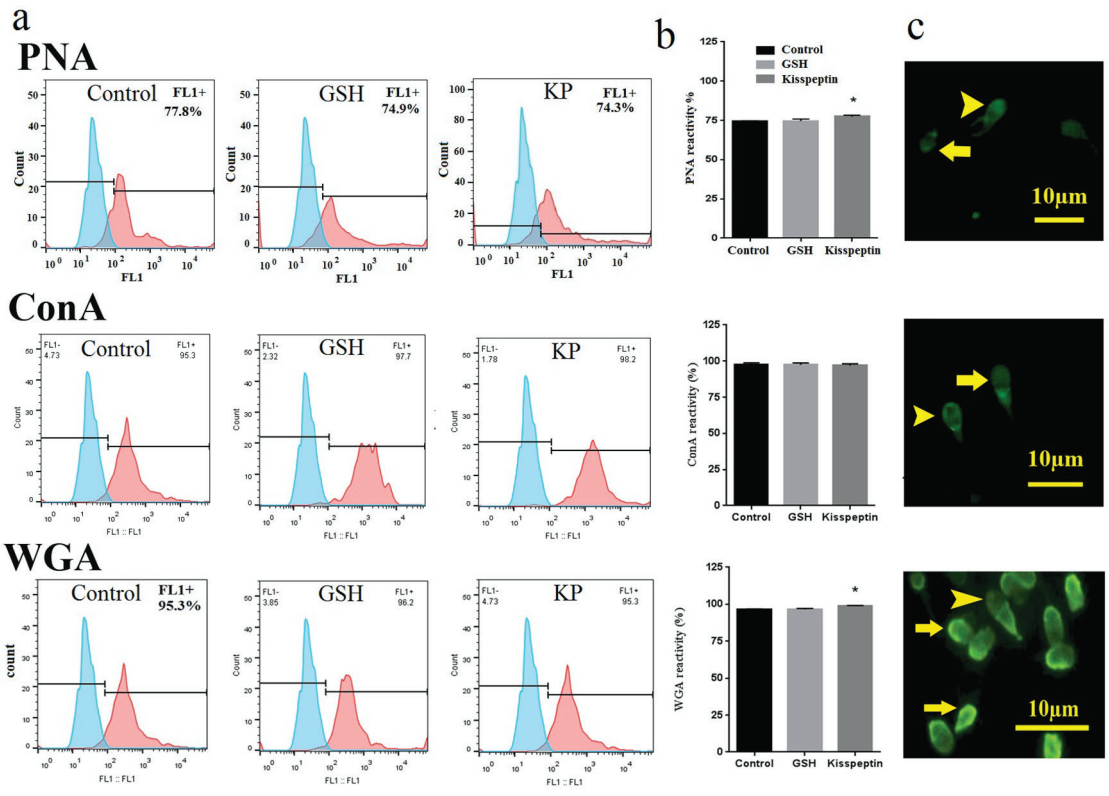

Figure 2: The semen samples were stained with FITC-conjugated lectins and analyzed using the flow cytometry technique. Each chart is a representative sample of the controls, GSH-, and KP-treated groups stained with either PNA, ConA, or WGA (panel a). Panel b shows a comparison between the percentage of lectin-positive cells in the control, GSH- and KP-treated groups. Panel c shows micrographs of sperms stained with PNA, ConA, and WGA. The top micrograph shows intact spermatozoon (arrowhead) and the release of the spermatozoon acrosome content (arrow). The middle micrograph shows the release of the spermatozoon acrosome content (arrowhead) stained with ConA and intact unstained sperm (arrow). The bottom micrograph shows sugar residues on the membrane of non-capacitated spermatozoa (arrow) stained with WGA. Sperms with weak reactions to WGA represent capacitated spermatozoa (arrowhead). The total number of samples: 30. *Significant difference with all other groups (P<0.001)

indicated that pre-freezing treatment with KP significantly elevated the percentage of non-capacitated spermatozoa compared to both GSH-treated and control groups (P<0.001). The percentage of non-capacitated spermatozoa in the GSH-treated and control groups was statistically the same (P=0.842). Histochemistry staining showed that sperm populations reacted differentially to WGA according to their status (intact vs. capacitated). Non-capacitated sperm heads responded to WGA uniformly (figure 2).

DNA Quality

***Acridine Orange Staining*:** Both AO-stained smears and the sperm chromatin structure assay showed that all samples contained some sperms with denatured nuclei. Pre-incubation with KP or GSH significantly decreased sperm frequency with red fluorescence (%DFI, figure 3) compared to the controls (P=0.001). The percentage of DFI was the same in the KP- and GSH-treated groups (P=0.889). The results of the smear analysis confirmed the flow cytometric assessment (figure 3).

***Aniline Blue Staining*:** There was a significantly higher number of sperms with normal chromatin in the KP-treated group than the other groups (both P<0.001). However, no significant changes were observed in the frequency of normal spermatozoa of the GSH-treated group compared with the control group (P=0.29, figures 4a and 4b)

***Chromomycin A3 Staining*:** The percentage of spermatozoa with normal protamine content was significantly higher in the KP-treated group than the control and GSH-treated groups (P<0.001, P=0.013, respectively). GSH supplementation did not create significantly better conditions for histone-protamine substitution compared to the control condition (P=0.175, figures 4c and 4d).

***TUNEL Staining*:** The percentage of DNA-fragmented spermatozoa was significantly lower in the KP-treated group than the other groups (both P=0.002). The percentage of apoptotic sperm in the GSH-treated group was equal to the control group (P=0.986), and GSH treatment was unable to reduce the frequency of apoptotic sperm. However, treatment with KP created better conditions for the protection of sperm from apoptosis than in other groups (figure 5).

Discussion

The results showed that treatment with KP improved sperm motility, diminished acrosome reaction, premature capacitation, and damage to plasma membrane and DNA. Significant

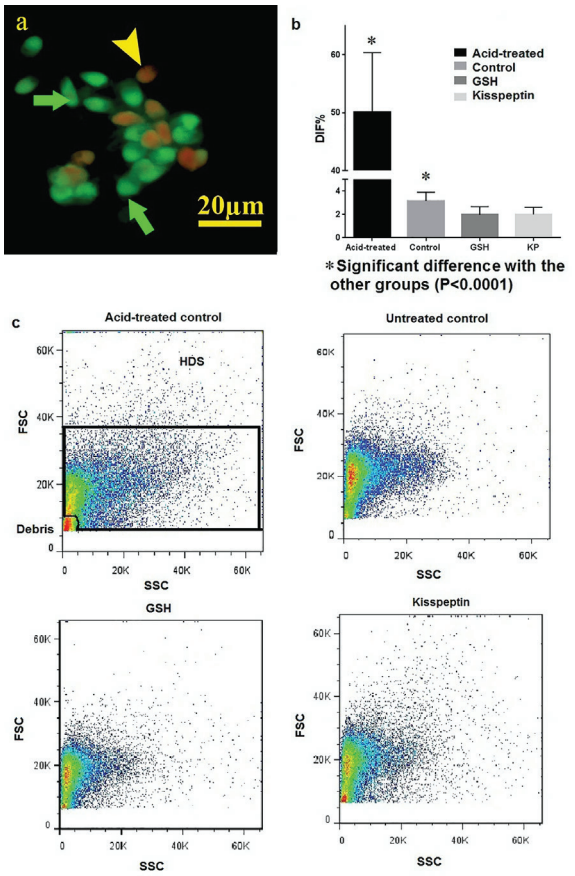

Figure 3: DNA integrity of the sperm chromatin was evaluated using acridine orange staining (a). Sperms with denatured nuclei showed metachromasia (arrowhead), while normal double-stranded DNA showed green fluorescence (green arrows). DNA fragmentation index was calculated by determining sperm frequency in the gated region (b). Acid-treated samples were used as technical controls. Dot plots of representative sperm aliquots are also depicted (c). The Gated area in the acid-treated sample is depicted after excluding high DNA stainability (HDS) cells and debris. The HDS population represents more rounded immature sperms. The total number of samples: 30. *Significant difference with the other groups (P<0.0001)

reduction in sperm motility is the most common problem in the cryopreservation of human spermatozoa.[18] KP receptors are found in the sperm head and neck. The administration of the KP inhibitor reduces sperm motility and activates signal transmission pathways via GPR54, leading to its modulatory role in sperm movement.[11] Oxidative stress induced by methotrexate has a negative impact on sperm motility. However, the antioxidant property of KP was shown to improve sperm motility in rats, and increase the activity of superoxide dismutase and catalase, GSH, Ca^{2+}, and total antioxidant capacity.[15, 19, 20] Increased ROS production is one of the most critical issues of the freeze-thaw cycle, as it adversely affects sperm motility.[21, 22]

Considering the effect of cryopreservation on enzymes capable of reducing oxidative

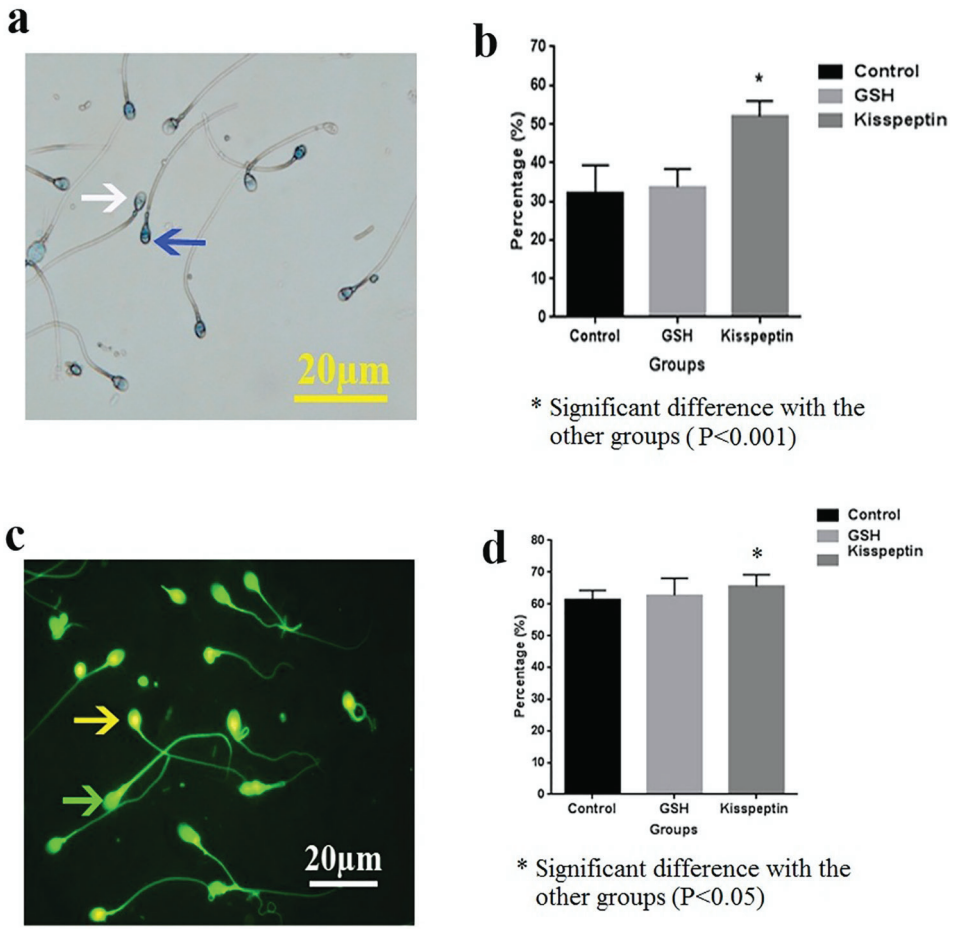

Figure 4: Normal histone content was evaluated using aniline blue staining. (a): The micrograph shows normal spermatozoa (white arrow) versus those with higher histone content (blue arrow). (b): The graph compares the percentage of sperms with normal histone in different groups. (c): Normal protamine content was evaluated by CMA3 staining. The micrograph shows normal spermatozoa (green arrow) versus sperms with protamine deficiency (yellow arrow). (d): The graph compares the percentage of sperms with normal protamine in different groups. The total number of samples: 30. *Significant difference with the other groups.

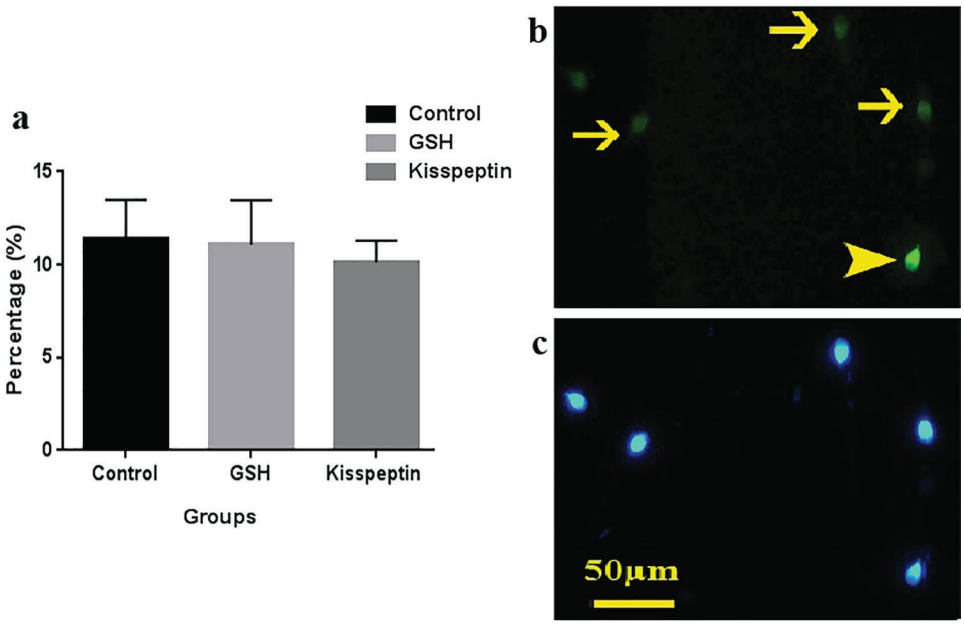

Figure 5: The graph shows a comparison between the percentage of TUNEL-positive cells in different groups (a). Apoptotic spermatozoa (arrowhead) and non-apoptotic spermatozoa (arrow) (b). Hoechst staining of the sperms in the same field (c). The total number of samples: 30.

stress,[3] many researchers add GSH in cryopreservation media to reduce oxidative stress-induced damage. As a positive control, we compared the effects of pre-incubation with GSH and KP on sperm quality following cryopreservation. Cryopreservation causes premature capacitation and spontaneous acrosome reaction. Pretreatment of sperm with various antioxidants improves the integrity of the plasma membrane.[23] Cryo-capacitation reduces the performance of spermatozoa in the female reproductive tract, accompanied by loss of acrosome reaction. Our results showed that a higher number of spermatozoa in the KP-treated group maintained acrosomal content, and escaped from early capacitation. KP prevented undesired acrosome reactions by interacting with KP receptors.[11] It is also reported that KP inhibitors reduce the fertilization rate of non-capacitated sperms in mice.[9] Since GSH, a well-known antioxidant, had no effect on the number of capacitated, acrosome-reacted, and thawed sperms, it is possible that the effect of KP on capacitation and acrosome reaction was due to a mechanism other than its antioxidant capacity.

Cryopreservation also compromises the integrity of sperm DNA, necessary for fertilization, embryo development, and the success of ART.[24] A possible mechanism of damage to sperm DNA is oxidative stress. It has been widely reported that ROS is associated with poor semen quality and impairment of human spermatozoa function.[20]

The freeze-thaw process on semen samples from fertile men causes sperm DNAf and abnormal chromatin structure.[22] After cryopreservation, live DNA-fragmented sperm has an insufficient quality for fertilization. Results concerning cell damage induced by cryopreservation are conflicting. It was found that sperm cryopreservation of infertile men induced higher lipid peroxidation,[25] which could be due to the loss of peroxidation protective enzyme activity[26] and higher ROS production. In contrast, another study reported no significant improvement in lipid peroxidation after sperm cryopreservation.[27] Such contradictory results could be due to different cryopreservation procedures, final preparation of semen before cryostorage, and difference in tests for evaluating DNA integrity.

Sperm DNA damage was attributed to high ROS levels in fresh and frozen-thawed semen.[28] It is reported that adding GSH to the thawing medium reduced ROS generation and DNAf, and improved fertilizing ability of bull spermatozoa.[29] The same mechanism may be exerted by pretreatment with KP.

Treatment of brain tissues with KP-10 reduces L-methionine-induced DNAf, associated with increased GSH activity.[30] Oxidants can react with cellular and structural components, change the Ca^{2+} code, and modify essential pathways.[31] Calcium is a signaling molecule that responds to oxidant stimuli.[32] In this study, pretreatment with KP, an antioxidant, resulted in the modulation of DNA integrity possibly by regulating calcium signaling.

DNA-protamine packaging protects sperm from ROS attack, and impaired protamination makes sperm DNA susceptible to ROS damage.[33] Therefore, oxidative stress is not the only mechanism that causes simultaneous DNA damage. Two other molecular mechanisms of sperm damage include defective chromatin packaging and apoptosis.[28] We showed that pretreatment with KP could improve protamination, and as a result it prevented apoptosis. However, it did not have any effect on DNA quality.

The freeze-thaw process causes defects in chromatin, problems with protamine packaging, DNAf,[34] and changes in protamine transcript in stallion sperm. A decrease in protamine-2 and an increase in protamine-3 mRNA were reported in frozen sperm compared to the controls. However, protamine-1 mRNA did not change.[35] This result shows the impact of cryopreservation on histone-to-protamine transition. In our study, pre-incubation with KP improved the protamine-to-histone transition. Since pretreatment with GSH did not affect this mechanism, it seems that it was not the antioxidant activity of KP that affected the protamine-to-histone transition. Further investigation on this topic is therefore necessary. Histone acetyltransferase activity is an essential step in protamine-to-histone transition[36] and KP induces histone acetylation/methylation.[37] Treating GT1-7 cells with KP up-regulates histone acetylation and chromatin conformation,[38] in which chromatin remodeling plays a critical role in the protamine-to-histone transition.[36] In addition, the presence of KP receptors in the dog epididymal fluid indicates a functional role of KP in sperm maturation and protamination.[39]

The main limitation of the study is the lack of measuring ROS and antioxidative enzyme activity, which is important for an in-depth understanding of the mechanism of the protective effect of KP.

Conclusion

Pretreatment with KP can improve sperm quality and tolerance to cryopreservation. KP provided a better effect on sperm freezing tolerance than GSH since KP improved both motility and DNA

quality during the freeze-thaw cycle, whereas GSH only improved sperm motility. Therefore, KP can be a suitable candidate for ART procedures as it improves the quality of frozen-thawed sperm.

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Authors' Contribution

T.K: Technical assistance and revision of the manuscript; S.F.H: Conducting of the study, data acquisition/analysis/interpretation, and drafting of the manuscript; T.T.K: Technical assistance, data analysis and interpretation, and critical revision of the manuscript; E.A: Facilitating research grant and ethical approval, study design, technical assistance, data analysis and interpretation, and critical revision of the manuscript. All authors have read and approved the final manuscript and agree to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

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References

1 Tournaye H, Dohle GR, Barratt CL. Fertility preservation in men with cancer. Lancet. 2014;384:1295-301. doi: 10.1016/S0140-6736(14)60495-5. PubMed PMID: 25283570.
2 MacKenna A, Crosby J, Huidobro C, Correa E, Duque G. Semen quality before cryopreservation and after thawing in 543 patients with testicular cancer. JBRA Assist Reprod. 2017;21:31-4. doi: 10.5935/1518-0557.20170009. PubMed PMID: 28333030; PubMed Central PMCID: PMCPMC5365198.
3 O'Connell M, McClure N, Lewis SE. The effects of cryopreservation on sperm morphology, motility and mitochondrial function. Hum Reprod. 2002;17:704-9. doi: 10.1093/humrep/17.3.704. PubMed PMID: 11870124.
4 Peris-Frau P, Soler AJ, Iniesta-Cuerda M, Martin-Maestro A, Sanchez-Ajofrin I, Medina-Chavez DA, et al. Sperm Cryodamage in Ruminants: Understanding the Molecular Changes Induced by the Cryopreservation Process to Optimize Sperm Quality. Int J Mol Sci. 2020;21. doi: 10.3390/ijms21082781. PubMed PMID: 32316334; PubMed Central PMCID: PMCPMC7215299.
5 Talaei T, Esmaeilpour T, Akiyash F, Bahmanpour S. Effects of cryopreservation on plasma membrane glycoconjugates of human spermatozoa. Iranian Journal of Reproductive Medicine. 2010;18:119-24.
6 Said TM, Gaglani A, Agarwal A. Implication of apoptosis in sperm cryoinjury. Reprod Biomed Online. 2010;21:456-62. doi: 10.1016/j.rbmo.2010.05.011. PubMed PMID: 20800544.
7 Amidi F, Pazhohan A, Shabani Nashtaei M, Khodarahmian M, Nekoonam S. The role of antioxidants in sperm freezing: a review. Cell Tissue Bank. 2016;17:745-56. doi: 10.1007/s10561-016-9566-5. PubMed PMID: 27342905.
8 Roca J, Carvajal G, Lucas X, Vazquez JM, Martinez EA. Fertility of weaned sows after deep intrauterine insemination with a reduced number of frozen-thawed spermatozoa. Theriogenology. 2003;60:77-87. doi: 10.1016/s0093-691x(02)01330-4. PubMed PMID: 12620582.
9 Hsu MC, Wang JY, Lee YJ, Jong DS, Tsui KH, Chiu CH. Kisspeptin modulates fertilization capacity of mouse spermatozoa. Reproduction. 2014;147:835-45. doi: 10.1530/REP-13-0368. PubMed PMID: 24567427.
10 Tariq A, Shahab M, Clarke I, Pereira A, Smith J, Sultan J, et al. Kiss1 and Kiss1 receptor expression in the rhesus monkey testis: a possible local regulator of testicular function. Open Life Sciences. 2013;8:968-74. doi: 10.2478/s11535-013-0219-4.
11 Pinto FM, Cejudo-Roman A, Ravina CG, Fernandez-Sanchez M, Martin-Lozano D, Illanes M, et al. Characterization of the kisspeptin system in human spermatozoa. Int J Androl. 2012;35:63-73. doi: 10.1111/j.1365-2605.2011.01177.x. PubMed PMID: 21651574.
12 Aydin M, Oktar S, Yonden Z, Ozturk OH, Yilmaz B. Direct and indirect effects of kisspeptin on liver oxidant and antioxidant systems in young male rats. Cell Biochem Funct. 2010;28:293-9. doi: 10.1002/cbf.1656. PubMed PMID: 20517893.
13 Abou Khalil NS, Mahmoud GB. Reproductive, antioxidant and metabolic responses of Ossimi rams to kisspeptin. Theriogenology. 2020;142:414-20. doi: 10.1016/j.

theriogenology.2019.10.039. PubMed PMID: 31711707.
14 Organization WH. WHO laboratory manual for the examination and processing of human semen. 2010.
15 Wahab F, Atika B, Shahab M, Behr R. Kisspeptin signalling in the physiology and pathophysiology of the urogenital system. Nat Rev Urol. 2016;13:21-32. doi: 10.1038/nrurol.2015.277. PubMed PMID: 26620614.
16 Zou P, Wang X, Chen Q, Yang H, Zhou N, Sun L, et al. Kisspeptin Protein in Seminal Plasma Is Positively Associated with Semen Quality: Results from the MARHCS Study in Chongqing, China. Biomed Res Int. 2019;2019:5129263. doi: 10.1155/2019/5129263. PubMed PMID: 30729125; PubMed Central PMCID: PMCPMC6343164.
17 Tejada RI, Mitchell JC, Norman A, Marik JJ, Friedman S. A test for the practical evaluation of male fertility by acridine orange (AO) fluorescence. Fertil Steril. 1984;42:87-91. doi: 10.1016/s0015-0282(16)47963-x. PubMed PMID: 6724015.
18 Kramer RY, Garner DL, Bruns ES, Ericsson SA, Prins GS. Comparison of motility and flow cytometric assessments of seminal quality in fresh, 24-hour extended and cryopreserved human spermatozoa. J Androl. 1993;14:374-84. PubMed PMID: 8288491.
19 Guvenc M, Aksakal M. Ameliorating effect of kisspeptin-10 on methotrexate-induced sperm damages and testicular oxidative stress in rats. Andrologia. 2018;50:e13057. doi: 10.1111/and.13057. PubMed PMID: 29862548.
20 Pasqualotto FF, Sharma RK, Nelson DR, Thomas AJ, Agarwal A. Relationship between oxidative stress, semen characteristics, and clinical diagnosis in men undergoing infertility investigation. Fertil Steril. 2000;73:459-64. doi: 10.1016/s0015-0282(99)00567-1. PubMed PMID: 10688996.
21 Tafuri S, Ciani F, Iorio EL, Esposito L, Cocchia N. Reactive oxygen species (ROS) and male fertility. Wu B, editors. New Discoveries in Embryology; London: Intechopen; 2015. p. 19-40. doi: 10.5772/60632.
22 Zribi N, Feki Chakroun N, El Euch H, Gargouri J, Bahloul A, Ammar Keskes L. Effects of cryopreservation on human sperm deoxyribonucleic acid integrity. Fertil Steril. 2010;93:159-66. doi: 10.1016/j.fertnstert.2008.09.038. PubMed PMID: 19027111.
23 Aliabadi E, Jahanshahi S, Talaei-Khozani T, Banaei M. Comparison and evaluation of capacitation and acrosomal reaction in freeze-thawed human ejaculated spermatozoa treated with L-carnitine and pentoxifylline. Andrologia. 2018;50. doi: 10.1111/and.12845. PubMed PMID: 28752591.
24 Meseguer M, Martinez-Conejero JA, O'Connor JE, Pellicer A, Remohi J, Garrido N. The significance of sperm DNA oxidation in embryo development and reproductive outcome in an oocyte donation program: a new model to study a male infertility prognostic factor. Fertil Steril. 2008;89:1191-9. doi: 10.1016/j.fertnstert.2007.05.005. PubMed PMID: 17681311.
25 Bell M, Wang R, Hellstrom WJ, Sikka SC. Effect of cryoprotective additives and cryopreservation protocol on sperm membrane lipid peroxidation and recovery of motile human sperm. J Androl. 1993;14:472-8. PubMed PMID: 8294232.
26 Alvarez JG, Storey BT. Evidence for increased lipid peroxidative damage and loss of superoxide dismutase activity as a mode of sublethal cryodamage to human sperm during cryopreservation. J Androl. 1992;13:232-41. PubMed PMID: 1601742.
27 Peris SI, Bilodeau JF, Dufour M, Bailey JL. Impact of cryopreservation and reactive oxygen species on DNA integrity, lipid peroxidation, and functional parameters in ram sperm. Mol Reprod Dev. 2007;74:878-92. doi: 10.1002/mrd.20686. PubMed PMID: 17186553.
28 Zini A, Libman J. Sperm DNA damage: clinical significance in the era of assisted reproduction. CMAJ. 2006;175:495-500. doi: 10.1503/cmaj.060218. PubMed PMID: 16940270; PubMed Central PMCID: PMCPMC1550758.
29 Gadea J, Gumbao D, Canovas S, Garcia-Vazquez FA, Grullon LA, Gardon JC. Supplementation of the dilution medium after thawing with reduced glutathione improves function and the in vitro fertilizing ability of frozen-thawed bull spermatozoa. Int J Androl. 2008;31:40-9. doi: 10.1111/j.1365-2605.2007.00756.x. PubMed PMID: 18190425.
30 Akkaya H, Kilic E, Dinc SE, Yilmaz B. Post-acute effects of kisspeptin-10 on neuronal injury induced by L-methionine in rats. J Biochem Mol Toxicol. 2014;28:373-7. doi: 10.1002/jbt.21573. PubMed PMID: 24863683.
31 Ermak G, Davies KJ. Calcium and oxidative stress: from cell signaling to cell death. Mol Immunol. 2002;38:713-21. doi: 10.1016/s0161-5890(01)00108-0. PubMed PMID: 11841831.
32 Ozcan M, Alcin E, Ayar A, Yilmaz B, Sandal

S, Kelestimur H. Kisspeptin-10 elicits triphasic cytosolic calcium responses in immortalized GT1-7 GnRH neurones. Neurosci Lett. 2011;492:55-8. doi: 10.1016/j.neulet.2011.01.054. PubMed PMID: 21276835.
33 Oliva R. Protamines and male infertility. Hum Reprod Update. 2006;12:417-35. doi: 10.1093/humupd/dml009. PubMed PMID: 16581810.
34 Pasqualotto FF, Sharma RK, Nelson DR, Thomas AJ, Agarwal A. Relationship between oxidative stress, semen characteristics, and clinical diagnosis in men undergoing infertility investigation. Fertil Steril. 2000;73:459-64. doi: 10.1016/s0015-0282(99)00567-1. PubMed PMID: 10688996.
35 Kadivar A, Shams Esfandabadi N, Dehghani Nazhvani E, Shirazi A, Ahmadi E. Effects of cryopreservation on stallion sperm protamine messenger RNAs. Reprod Domest Anim. 2020;55:274-82. doi: 10.1111/rda.13615. PubMed PMID: 31885108.
36 Wang T, Gao H, Li W, Liu C. Essential Role of Histone Replacement and Modifications in Male Fertility. Front Genet. 2019;10:962. doi: 10.3389/fgene.2019.00962. PubMed PMID: 31649732; PubMed Central PMCID: PMCPMC6792021.
37 Novaira HJ, Sonko ML, Radovick S. Kisspeptin Induces Dynamic Chromatin Modifications to Control GnRH Gene Expression. Mol Neurobiol. 2016;53:3315-25. doi: 10.1007/s12035-015-9269-0. PubMed PMID: 26081144.
38 Trevisan CM, Montagna E, de Oliveira R, Christofolini DM, Barbosa CP, Crandall KA, et al. Kisspeptin/GPR54 System: What Do We Know About Its Role in Human Reproduction? Cell Physiol Biochem. 2018;49:1259-76. doi: 10.1159/000493406. PubMed PMID: 30205368.
39 Gloria A, Contri A, Mele E, Fasano S, Pierantoni R, Meccariello R. Kisspeptin Receptor on the Sperm Surface Reflects Epididymal Maturation in the Dog. Int J Mol Sci. 2021;22. doi: 10.3390/ijms221810120. PubMed PMID: 34576283; PubMed Central PMCID: PMCPMC8466692.