Supplementation of freezing and thawing media with brain-derived neurotrophic factor protects human sperm from freeze-thaw-induced damage

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Objective: To investigate the effects of brain-derived neurotrophic factor (BDNF) supplementation to freezing and thawing media on frozen-thawed human sperm parameters.
Design: Laboratory study.
Setting: University hospital.
Patient(s): Semen samples from 21 healthy fertile men.
Intervention(s): We measured reactive oxygen species (ROS) by flow cytometry using the probes dichlorofluorescin diacetate for intracellular hydrogen peroxide (H_2O_2) and dihydroethidium for intracellular superoxide anion ($O_2^{-\bullet}$), sperm plasma membrane integrity by flow cytometry, caspase-3 activity using ELISA, and AKT phosphorylation status using Western blot in sperm that was cryopreserved and thawed in media either supplemented with BDNF or without BDNF supplementation (control).
Main Outcome Measure(s): Sperm motility, viability, ROS levels, caspase-3 activity and AKT phosphorylation.
Result(s): The percentage of motile and viable sperm cells was significantly higher in BDNF-supplemented groups as compared with the nonsupplemented (control) group. There was a significant difference in AKT phosphorylation status between BDNF-supplemented groups and the control group. Moreover, the levels of intracellular H_2O_2 and caspase-3 activity were significantly lower in the sperm cells that were frozen and thawed in media supplemented with BDNF compared with in the control group.
Conclusion(s): BDNF supplementation to sperm freezing or thawing media has protective effects against oxidative stress and apoptosis in frozen-thawed human spermatozoa and could improve sperm function, probably through the activation of AKT. (Fertil Steril® 2016;106:1658-65. ©2016 by American Society for Reproductive Medicine.)
Key Words: Sperm cryopreservation, ROS, apoptosis, membrane integrity, AKT, BDNF

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Human sperm cryopreservation, which is widely used in assisted reproduction techniques (ART) programs for both fertility preservation and research, was introduced in the 1960s (1, 2). In spite of the recent advances that have been made in the field of sperm cryopreservation, the process has the potential to compromise sperm function and quality through generation of reactive oxygen

species (ROS) and reduction in antioxidant activity (3). Negative impacts of cryopreservation on sperm functions may include impairment of sperm motility, viability, chromatin and plasma membrane integrity, fertilizing ability, early embryo development, implantation, and ultimately, a reduction in pregnancy rates (1, 4–6). Human spermatozoa are highly susceptible to oxidative stress (OS) damage due to low cytoplasmic antioxidant content and high polyunsaturated fatty acids (7, 8). Much evidence has shown that OS contributes to both direct and indirect cellular damage during the sperm cryopreservation process (6, 8, 9). It has been reported that generation of ROS occurs during the freezing and/or thawing process of spermatozoa (5, 10). It seems likely that the sudden increase in oxygen consumption by spermatozoa during thawing results in ROS-induced membrane damage (11).

Apoptosis is another mechanism that results in sperm damage during cryopreservation (12, 13). Previous studies have shown that cryopreservation and thawing of human spermatozoa can activate caspases-3, -8, and -9 (14). Moreover, several studies have confirmed that an imbalance between the antioxidant scavenging capacity and production of ROS inside the sperm can result in apoptotic-like changes within those spermatozoa (15–17). Currently, there is great interest in the use of antioxidants to prevent ROS generation and apoptosis during the sperm cryopreservation process (7, 18–20).

Brain-derived neurotrophic factor (BDNF), a polypeptide belonging to the neurotrophins family, is expressed in the central nervous system and plays a key role in the differentiation, maturation, survival, and regeneration of neuronal cells (21). The relationship between sex hormones and BDNF has revealed other functions of this neurotrophin outside the central nervous system (21).

It has been reported that BDNF could play a crucial role in both the female (22–26) and male (27, 28) reproductive systems. BDNF is expressed in Leydig and Sertoli cells of human testis, and its receptor (TrkB) has been found in spermatogonia (29, 30) and in the sperm (31–33), suggesting that BDNF may have a role in the paracrine regulation of spermatogenesis. In bovines, it has been reported that exogenous BDNF can raise the secretion of insulin and leptin in ejaculated sperm (31). Furthermore, it has been reported that BDNF expression was lower in oligoasthenozoospermic human semen compared with that of fertile men (28). Additionally, we have recently reported that BDNF supplementation to sperm preparation media could improve sperm function in human (33). The antioxidant properties and prosurvival activities of BDNF have been reported in many cell types (34–40).

Although positive correlations between BDNF and male fertility has been established in many studies (28, 30), its role in human sperm quality during cryopreservation is still undefined. The aim of the present study was to evaluate the effects of BDNF supplementation during freezing and thawing of human spermatozoa on different parameters that are known to be correlated with the fertilizing ability of the spermatozoa. Our results show that supplementation of freezing and thawing media with BDNF could ameliorate the cryodamage induced in sperm during cryopreservation; this occurs by modulating the oxidative damage, apoptosis, and regulation of AKT activation in human spermatozoa. We also investigated a possible correlation between these parameters.

MATERIALS AND METHODS

Population and Sample Collection

The semen samples were collected from 21 healthy fertile men (age range, 23–40 years) by masturbation into sterile containers after 72 hours of sexual abstinence. The men confirmed paternity for the last 2 years before the study. Semen collection was conducted in the andrology laboratory of Dr. Shariati Hospital (Tehran, Iran). Samples having normal classical parameters, according to the World Health Organisation criteria (WHO), were chosen (41). Normospermic semen samples had the following characteristics: volume ≥ 1.5 mL, cell concentrations $\geq 15 \times 10^6$ cells/mL, total motility $\geq 40\%$, and sperm cell morphology $\geq 4\%$. To omit parameters that may influence ROS generation, samples with leukocytospermia ($\geq 1 \times 10^6$ white blood cells/mL) and men with any history of prolonged illness, varicocele, or endocrine disorders such as diabetes; drug intake including vitamins such as carotene, ascorbate, and tocopherol or minerals such as selenium and zinc; and smoking or alcohol consumption were not included in the present study. Written informed consent was obtained from all participants, and the study was approved by the Ethics Committee (no. Ir.tums.rec.1395.2793) of Tehran University of Medical Sciences (Tehran, Iran). The mean ± SD of semen characteristics of the patients who participated in this study are shown in Supplemental Table 1.

Semen Analysis and Preparation

After the semen samples were liquefied, semen analysis was performed using computer-assisted sperm analyzer system (CASA motility module; Microptic). The sperm cells were isolated by washing the liquefied semen two times (400 × *g* for 5 minutes) in human tubal fluid (HTF) medium (Irvine Scientific) containing 10% human serum albumin (HSA; Life Global). Subsequently, the spermatozoa were purified using the swim-up technique in the same medium. Due to the limitation in the sperm concentrations, the supernatant containing motile sperm cells was collected and divided into three equal groups: [1] spermatozoa were frozen in media supplemented with BDNF (group 1), [2] spermatozoa were frozen in media without BDNF supplementation but the thawing media was supplemented with BDNF and the spermatozoa were incubated in the thawing media for 60 minutes (group 2), [3] spermatozoa were frozen and thawed in media without BDNF supplementation (control, group 3). The final concentration of BDNF used in either freezing or thawing media was 0.133 nM.

Sperm Freezing and Thawing

Sperm aliquots were gently mixed in equal proportions, drop by drop, with sperm freezing media at room temperature. Subsequently, sperm aliquots were placed in 0.5-mL straws. The samples were then subjected to static cooling at 4°C for 10 minutes. Straws containing the specimens were then placed in liquid nitrogen vapors at −80°C for 10 minutes. The straws were then transferred into liquid nitrogen

at −196°C for storage. After two weeks, the samples were thawed by incubating the straws at 37°C for 30 seconds (19). After thawing, the samples were resuspended in HTF medium supplemented with 10% HSA and centrifuged at 300 × *g* for 7 minutes. After that, the sperm pellets were resuspended in HTF medium and then processed to measure different parameters related to biological functions of the sperm. Unless otherwise stated, to keep a stable temperature during the experiment, all centrifugations were done at 25°C using a temperature-controlled centrifuge.

Assessment of Motility

After thawing, motility was evaluated using a CASA system in each group. A total of 200 spermatozoa in many different fields were evaluated and classified into the following grades: progressive motile, nonprogressive motile, and immotile cells, according to WHO criteria (WHO, 2010).

Measurement of Intracellular Hydrogen Peroxide (H_2O_2) and Superoxide Anion ($O_2^{-\bullet}$) Levels

To detect intracellular H_2O_2 and $O_2^{-\bullet}$, 2′, 7′ -dichlorofluorescin diacetate (DCFH-DA, Sigma) and dihydroethidium (DHE, Sigma) as fluorescent oxidative probes were used, respectively (42). The sperm cells were incubated with DCFH-DA (25 μM) for 40 minutes or with DHE (1.25 μM) for 20 minutes, at 25°C in the dark. After incubation, the green fluorescence (DCFH-DA) between the 500 and 530 nm wavelengths (in the FL-1 channel) and the red fluorescence (DHE) between the 590 and 700 nm wavelengths (in the FL-2 channel) were assessed by Becton Dickinson flow cytometer FACScan (Becton Dickinson) equipped with a 488-nm argon laser as a light source.

Evaluation of Viability and Sperm Membrane Integrity

Viability and plasma membrane integrity were detected according to the method described by Mahfouz et al. (43) using a double stain, YO-PRO-1 with a propidium iodide (PI) apoptosis assay kit (Invitrogen). Briefly, the cell suspensions were washed with cold phosphate-buffered saline and then treated with PI (50 μg/mL) and YO-PRO-1 (10 μM) for 20 minutes at 25°C in the dark. The samples were then evaluated for fluorescence emission: YO-PRO-1 green fluorescence was detected in the FL-1 channel (i.e., 530/30 band pass), and PI red fluorescence was detected in the FL-3 channel (i.e., 610/20 band pass). In this assay, three different populations of sperm samples were detected: [1] negative for both PI and YO-PRO-1, indicating viable cells (intact plasma lemma); [2] positive for YO-PRO-1 but negative for PI, indicating apoptotic cells (altered plasma lemma); and [3] positive for both PI and YO-PRO-1, indicating dead cells (damaged plasma lemma) (44). A nonstained aliquot from each sample was used as a negative control to measure the background autofluorescence.

The sperm population was gated on the basis of the log forward and side-scatter properties to exclude debris and nonsperm particles. Approximately 10,000 gated sperms were analyzed for each assay at a flow rate of <300 cells/second, and the obtained data were analyzed using FlowJo software (ver. 7.6.1) (43).

Measurement of Caspase-3 Activity

After thawing, caspase-3 activity was evaluated using a colorimetric assay kit (Abcam) according to the manufacturer's instructions. Briefly, sperm cells were resuspended in cold cell lysis buffer and incubated on ice for 10 minutes. The cell lysates were centrifuged at 10,000 ×*g* for 1 minute at 4°C, and the supernatants were frozen at −80°C until examination. The concentration of proteins was measured by Bradford assay. Fifty microliters of 2× reaction buffer containing 10 mM DTT and 5 μL of 4 mM caspase-3 substrate (DEVD-pNA) was added to 100 μg proteins from each sample and then incubated in a 96-well plate at 37°C for 4 hours. All measurements were taken in duplicate sets. The pNA light emission was quantified using an ELISA plate reader (GEN5, Bio Tek) at 405 nm. Comparison of absorbance (OD 405 nm) of pNA from the high-yield units with the control allowed the determination of the fold increase in caspase-3 activity.

Western Blot Evaluation of AKT Phosphorylation Status

Post-thaw sperm cell pellets were briefly resuspended in lysis buffer (50 mM Tris/HCl pH 7.4, 1% NP-40, 0.1% sodium dodecyl sulfate [SDS], 150 mM NaCl, 1 mM EDTA, 1 mM PMSF, 1 mM Na_3VO_4, 1 mM NaF) supplemented with a protease inhibitor cocktail (Sigma). Lysates were briefly sonicated (45). The suspensions were centrifuged at 12,000 × *g* for 20 minutes at 4°C. The total protein concentration of each sample was determined by the Bradford assay (Bio-Rad), according to manufacturer's instructions. Equivalent amounts of total cellular proteins were separated by 10% SDS polyacrylamide gel electrophoresis and subsequently transferred onto polyvinylidene difluoride membranes (Millipore) (46). The membranes were placed in the blocking buffer (5% nonfat dried milk) for 60 minutes at room temperature and then incubated with primary antibody against each target protein (anti-pan-AKT, Anti-pS473 AKT, and Anti-pThr308 AKT, cell signaling) overnight at 4°C. The membranes were then washed and incubated with a peroxidase-conjugated secondary antibody (cell signaling) for 60 minutes at room temperature. Antigen-antibody reactions were evident after incubating the membranes with a peroxidase substrate (Amersham ECL Advance Kit, GE Healthcare). Human leukemia K562 cell lysate was used as a positive control. Band intensity was calculated using Image J software.

Statistical Analysis

Data were analyzed using SPSS software (ver. 16). Data were tested for normality analysis of the parameters with the Kolmogorov-Smirnov test. One-way analysis of variance was used to compare mean values across groups, followed by Tukey's or Tamhane's tests. The results are given as mean ± SD. In this study, $P < .05$ was considered statistically significant. Correlation between the various sperm parameters was evaluated using the Pearson correlations coefficient ($P < .05$).

TABLE 1

Effects of BDNF supplementation to freezing or to thawing media on intracellular hydrogen peroxide (DCF positive spermatozoa) and superoxide anion levels (HE positive spermatozoa) in post-thaw samples.

Group	Superoxide anion (HE^{+ve} sperm)	Hydrogen peroxide (DCF^{+ve} sperm)
1	20.89 ± 12.17	48.62 ± 17.41[a]
2	19.54 ± 7.88	45.60 ± 13.39[b]
3	24.22 ± 8.81	62.05 ± 12.94

Note: Data presented as mean ± SD.
[a] $P<.05$ vs. control group.
[b] $P<.01$ vs. control group.

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RESULTS

Effects of BDNF on Sperm Motility

Supplementation of either sperm freezing or thawing media with 0.133 nM BDNF significantly increased the percentages of both progressive motility and total motility as compared with the control group (Supplemental Fig. 1). However, the differences between group 1 and group 2 were not significant. The percentage of nonmotile sperm cells was remarkably higher in the control group (group 3, 58.8 ± 4.84) compared with the BDNF-supplemented groups with freezing (group 1, 52.04 ± 8.15; $P<.01$) and thawing media (group 2, 48.52 ± 7.33; $P<.001$). No significant difference was observed between group 1 and group 2.

Effects of BDNF on Intracellular Levels of H_2O_2 and $O_2^{-\bullet}$

The percentage of sperm cells with DCF^{+ve} fluorescence (intracellular H_2O_2) was significantly lower in both group 1 and group 2 as compared with group 3. The percentage of sperm with DHE^{+ve} fluorescence (intracellular $O_2^{-\bullet}$) decreased in both group 1 and group 2 compared with group 3; however, the difference was not statistically significant (Table 1).

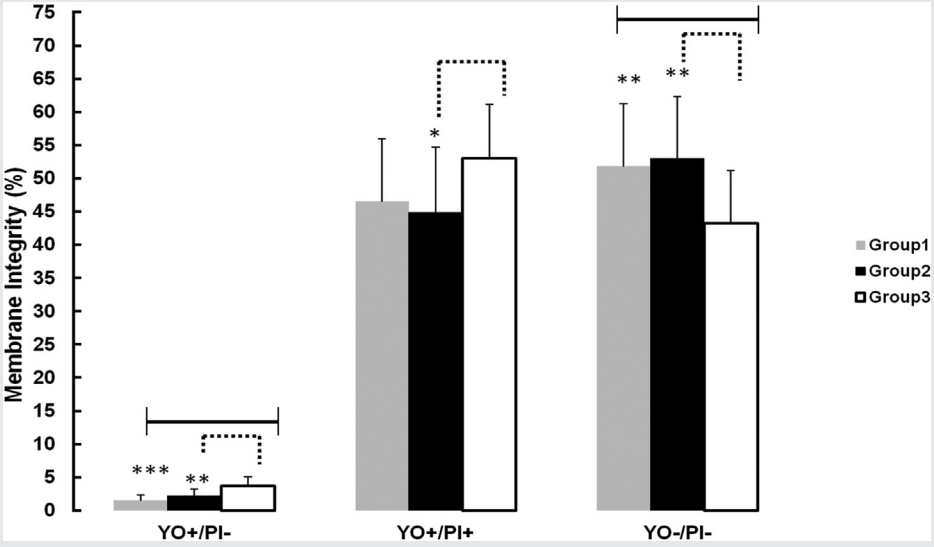
Effects of BDNF on Viability and Sperm Membrane Integrity

The percentage of viable sperm cells with intact plasma lemma (YO−/PI−) was significantly lower ($P<.05$) in group 3 as compared with groups 1 and 2. Furthermore, the percentage of apoptotic altered plasma lemma spermatozoa with (YO+/PI−) was significantly higher in group 3 (3.73 ± 1.36) as compared with group 1 (1.58 ± 0.76, $P<.001$) and group 2 (2.21 ± 0.97, $P=.001$). The percentage of dead sperm (YO+/PI+) was significantly lower ($P<.05$) in group 2 as compared with group 3 (44.85 ± 9.79 vs. 53.03 ± 8.14, respectively; Fig. 1). No significant difference was observed in sperm viability between group 1 and group 2.

Effects of BDNF on Caspase-3 Activity

The activity of caspase-3, was significantly lower in group 1 (0.05 ± 0.02; $P=.001$) and group 2 (0.04 ± 0.01; $P<.001$) as compared with group 3 (0.07 ± 0.01; Fig. 2). However, the difference between group 1 and group 2 was not significant.

FIGURE 1



Comparison of sperm viability and membrane integrity between BDNF-supplemented groups (group 1, BDNF supplemented to freezing medium; group 2, BDNF supplemented to thawing medium) and the control group (group 3, no BDNF supplementation). Three sperm populations can be identified: viable sperm (intact plasma lemma), negative for both YO-PRO-1 and PI; apoptotic sperm (altered plasma lemma), positive for YO-PRO-1; and dead sperm (damaged plasma lemma), positive for both YO-PRO-1 and PI. Each column represents mean ± SD. *$P<.05$ vs. control group; **$P<.01$ vs. control group; *** $P<.001$ vs. control group.

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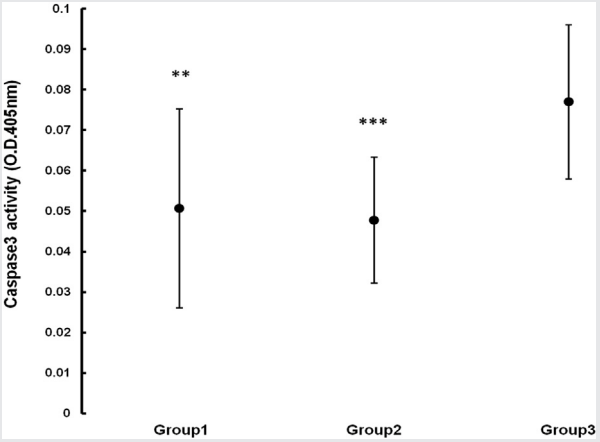
FIGURE 2



Comparison of the activity of caspase-3 between BDNF-supplemented groups (group 1, BDNF supplemented to freezing medium; group 2, BDNF supplemented to thawing medium) and group 3 (no BDNF supplementation). All data are mean ± SD. **P<.01 vs. control group; *** P<.001 vs. control group.

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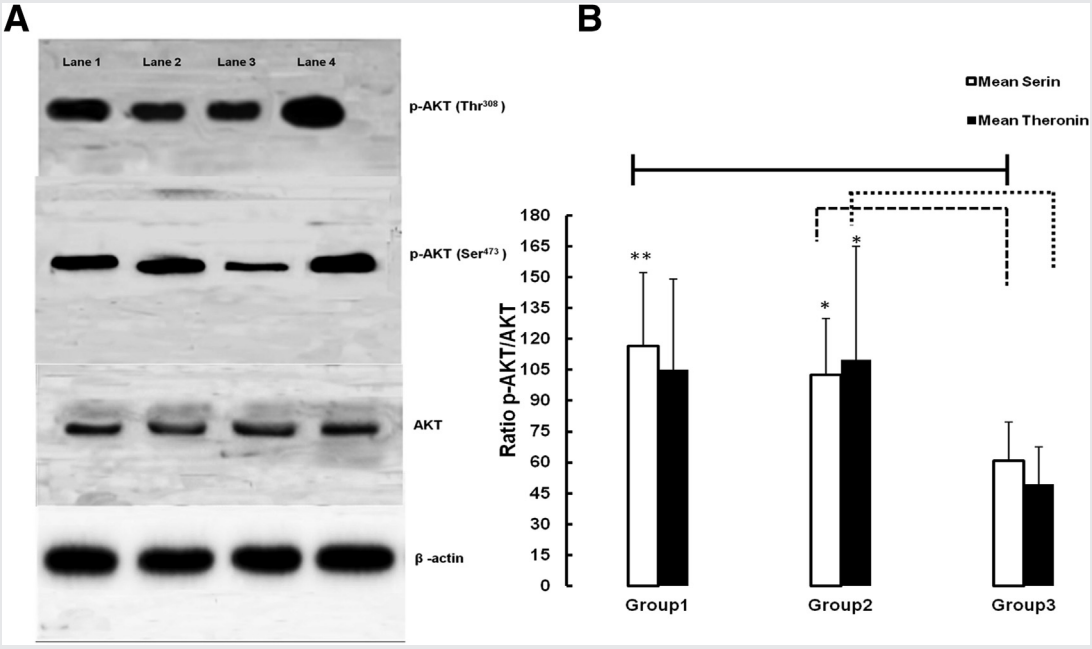
Effects of BDNF on AKT Phosphorylation Status

To determine the molecular mechanism involved in the protective effects of BDNF, we evaluated the effects of BDNF supplementation during sperm freezing or thawing on phosphorylation of the two AKT-specific phosphorylation residues (serine/Ser473 and threonine/Thr308; Fig. 3A). The AKT phosphorylation signal, mainly in the Ser473 residue, was significantly higher in group 1 and group 2 as compared with group 3. However, no significant difference was observed in the mean density of AKT phosphorylation in the Thr308 residue between group 1 and group 3 (Fig. 3B).

Correlation between the ROS Levels and Progressive Motility, Total Motility, and Caspase-3 Activity of Sperm

Our results showed that superoxide anion level was strongly and positively correlated to hydrogen peroxide level in the three groups. There was a moderate negative association between $O_2^{-\bullet}$ level and progressive motility in group 2. We also found a strong negative correlation between $O_2^{-\bullet}$ level and total motility in group 2. Furthermore, our results showed that caspase-3 activity was closely and directly correlated with $O_2^{-\bullet}$ level in group 3. Correlation analysis of H_2O_2 level showed a strong and negative relation between H_2O_2 level and total motility in groups 2 and 3 and H_2O_2 level and progressive motility in group 2. A moderate negative correlation was found between H_2O_2 level and progressive motility in group 1. The level of H_2O_2 in the spermatozoa also showed a strong and positive relation with caspase-3 activity in group 2 and group 3 (Supplemental Table 2).

FIGURE 3



Effects of BDNF treatment during freezing or thawing of human spermatozoa on AKT phosphorylation status. (**A**) The panel shows a representative Western blot analysis of AKT from sperm lysate in group 1 (lane 2), group 2 (lane 1), and the control group (lane 3). K562 cell lysate was used as a positive control (lane 4). β-actin served as loading control. (**B**) Densitometry analysis of normalized data with unphosphorylated AKT (for phospho-AKT [Ser473] and phospho-AKT [Thr308]). Each column represents mean ± SD. *P<.05 vs. control group; **P<.01 vs. control group.

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Correlation between the ROS Levels, Viability and Sperm Membrane Integrity

We found a moderate negative association between $O_2^{-\bullet}$ level and viability in group 2. Furthermore, a moderate positive correlation was observed between the percentage of cells with altered plasma lemma and $O_2^{-\bullet}$ level in group 3. Our findings showed that the percentage of cells with damaged plasma lemma was closely and directly correlated with $O_2^{-\bullet}$ level in group 2. There was also a strong and negative correlation between H_2O_2 level and viability in all groups. The level of H_2O_2 in the spermatozoa also showed a strong and positive relation with the percentage of cells with damaged plasma lemma in groups 1 and 2 and the percentage of cells with altered plasma lemma in group 3. A moderate direct correlation between H_2O_2 level and the percentage of cells with damaged plasma lemma was observed in group 3 (Supplemental Table 3).

DISCUSSION

The results of the present study demonstrate that supplementation of BDNF during freezing or thawing of human spermatozoa improves sperm viability and motility and reduces the level of intracellular H_2O_2 and caspase-3 activity. This indicates that supplementation of both sperm freezing and thawing media with BDNF can protect spermatozoa against OS and apoptosis induced as a consequence of the cryopreservation procedure. This protective effect may be mediated through a transient activation of the phosphatidylinositol-3-kinase (PI3-K)/AKT pathway.

Although sperm cryopreservation provides a valuable method in infertility management for many men (14), it is well established that this procedure increases membrane permeability, phosphatidylserine externalization, caspase activation, apoptotic (5, 14) and necrotic cell death (14, 47), ROS generation, and decrease in antioxidant capacity and mitochondrial membrane potential (3, 5, 12), which eventually impair sperm functions and reduce the fertilizing ability of frozen-thawed spermatozoa (48).

Many studies have indicated that sperm damage can also occur during the thawing process (5). For example, Gosálvez et al. reported that sperm DNA damage has been increased sequentially throughout 4-hour period after thawing (49).

The prosurvival and antioxidant capacity of BDNF has been demonstrated in several other cell types including neuronal cells (50) and cardiac cells (51). On the other hand, several lines of evidence have demonstrated that the antioxidant activity of BDNF may be due to its ability to scavenge free radical ions, modulate the activity of antioxidant enzymes, enhance expression of sestrin2 (a stress-responsive gene involved in the cellular defense against oxidative damage), and induce the phosphorylation of CREB, which subquently suppresses the release of cytochrome C into cytosol (35, 52).

Heaton et al. also showed that BDNF and vitamin E supplementation provides protective effects through a variety of mechanisms such as the enhancement of mitochondrial membrane potential, reduction of ROS generation, and Bax translocation in cerebellar granule cells (38). It has also been shown that BDNF can protect neuronal cells against ouabain-induced oxidative damage by modulating the activity of antioxidant enzymes, especially by decreasing SOD and increasing CAT activity (35). Our findings show that BDNF reduced cryodamage by scavenging H_2O_2, without altering the baseline of intracellular $O_2^{-\bullet}$ (Table 1). Similarly, a previous study reported that BDNF has a protective effect on PC12 cells against ROS-induced cell death via modulation of ROS as it decreased the conversion of $O_2^{-\bullet}$ to H_2O_2 (36). Studies showed that H_2O_2 is a primary ROS responsible for oxidative damage in spermatozoa in vitro and that it has high oxidative activity and the ability to cross membranes and can be converted to OH•, a highly reactive and toxic radical to the cell (53–56). Moreover, it seems that the superoxide radical ion, a short-lived free radical, does not react directly with polypeptides, sugars, or nucleic acids, and its ability to peroxidize lipids is controversial (57).

Many studies have shown that supplementation of sperm freezing and/or thawing media with antioxidants could protect sperm cells against ROS-mediated damage that occurs as a consequence of the cryopreservation process (4, 5, 58). The results in the present study demonstrate that the addition of BDNF to freezing or thawing media can enhance post-thaw sperm motility. Moreover, our data show that intracellular ROS levels were negatively associated with total and progressive motility in BDNF-supplemented and control groups. Previous studies have shown that the cryopreservation of human spermatozoa is associated with a significant decrease in sperm motility (5). Furthermore, a negative correlation between ROS levels and motility has been reported in frozen-thawed human spermatozoa (59).

Previous studies showed that apoptotic mechanisms are involved in cryodamage in mammalian spermatozoa (12, 14, 60). Assessment of caspase activity has been suggested as an assay for the determination of sperm apoptosis (61). Our results show that supplementation of freezing or thawing media with BDNF could protect spermatozoa against apoptosis in agreement with previous studies (39, 62). These findings are in agreement with the research conducted by Almeida et al. who showed that BDNF inhibits glutamate-induced caspase-3-like activity in neuronal cells (37). In addition, a close association has been established between caspase activation and membrane damage (63, 64). Many previous studies suggested that BDNF could decrease apoptosis in different types of cells through various mechanisms including activation in the PI3-K/AKT pathway, increase in Bcl-2 anti-apoptotic protein expression, and decrease in activation of caspases-2 and -3 (24, 37, 50, 51, 65).

Membrane integrity is known to be one of the most important factors that enhance sperm motility and viability; however, sperm membrane integrity is greatly affected by the freezing and thawing processes (14, 59). Our results show that sperm viability significantly increased in BDNF-supplemented groups compared with in the control group. The results indicate that viability was negatively correlated with the levels of intracellular ROS in frozen-thawed sperm cells. Han et al. reported that treatment of neuronal cells with 10 μg BDNF improved cell viability (50). Numerous studies have shown the importance of free radicals in the cryopreservation process (5, 8, 14). Furthermore, some researchers have reported that high

levels of ROS and low levels of antioxidants can stimulate apoptosis (15, 59). In agreement with our findings, many studies have shown a direct correlation between levels of intracellular ROS and the increase in apoptotic-like changes in frozen/thawed spermatozoa (15, 59, 66).

Some studies claim that AKT can maintain sperm motility, viability, and mitochondrial membrane potential by inhibiting activation of caspases-3 and -7 and the apoptosis-like mechanism (60, 67). It has also been proposed that dephosphorylation of AKT occurs when there is imbalanced ROS and/or a lack of prosurvival factors (68). It has been established that ROS can inhibit the activity of the PI3-K/AKT signaling pathway (69). In view of these studies, it seems likely that the ROS generation that occurs during sperm cryopreservation might be able to suppress the PI3-K/AKT pathway.

It has been shown that BDNF can stimulate the cascade that activates AKT in many types of cells, which in turn could inhibit apoptosis (37, 65). In view of these findings, we investigated changes in the phosphorylation status of AKT to evaluate the potential ability of BDNF in regulating the survival of human spermatozoa after freezing and thawing procedures. Our results show that the PI3-K/AKT pathway was activated by BDNF in human spermatozoa and contributed to the survival of sperm after cryopreservation. This result was consistent with the study conducted by Xia et al. who showed that the protective effect of BDNF in neuronal cells against induced apoptosis is mediated by the PI3-K signaling pathways (65).

Conclusion

Our results suggest that BDNF supplementation to both sperm freezing and thawing media could improve sperm motility, viability and the fertilizing ability of spermatozoa after cryopreservation, indicating that BDNF supplementation to sperm freezing or thawing media has protective effects against ROS production and apoptosis. Based on our data, we speculate that the effects of BDNF on the quality of frozen-thawed sperm might be modulated by the PI3-K/AKT signaling pathway, which plays an important role in reducing ROS levels and apoptosis in spermatozoa. It remains to be tested whether the supplementation of BDNF during freezing and thawing of spermatozoa could affect the fertilizing ability and embryonic development after ART treatment in actual infertile patients. These findings are of great importance for improving the efficiency of sperm cryopreservation and to help infertile/subfertile men.

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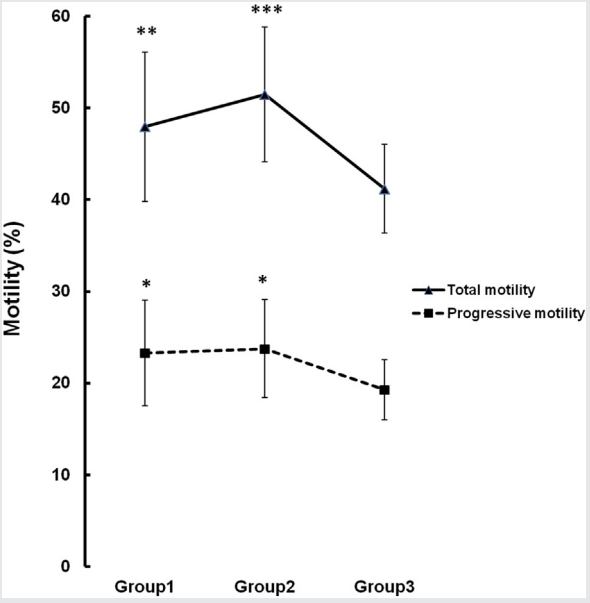
SUPPLEMENTAL FIGURE 1



Effects of BDNF supplementation to freezing or to thawing media on total and progressive motility of cryopreserved human spermatozoa. Each column represents mean ± SD. *$P<.05$ vs. control group; **$P<.01$ vs. control group; *** $P<.001$ vs. control group.

Najafi. BDNF protects frozen-thawed human sperm. Fertil Steril 2016.

SUPPLEMENTAL TABLE 1

Semen characteristics of participants.

Characteristic	Mean ± SD
Age, y	32.52 ± 4.74
Volume, mL	3.98 ± 1.20
Sperm concentration, $\times 10^6$	70.19 ± 17.84
Total motility, %	65.42 ± 10.27
Normal morphology, %	14.28 ± 6.31

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SUPPLEMENTAL TABLE 2

The relationship between ROS levels (intracellular superoxide anion and hydrogen peroxide) and hydrogen peroxide, superoxide anion, progressive motility, total motility, and caspase-3 activity of frozen-thawed human spermatozoa in BDNF-supplemented and control groups.

Group	H_2O_2	$O_2^{-\bullet}$	Progressive motility	Total motility	Caspase-3 activity
Superoxide anion					
1	r = 0.580[b]	–	r = −0.212	r = −0.160	r = 0.290
	P= .006	–	*P*= .356	*P*= .489	*P*= .202
2	r = 0.889[b]	–	r = −0.505[a]	r = −0.716[b]	r = 0.464[a]
	P= .000	–	*P*= .019	*P*= .00	*P*= .034
3	r = 0.763[b]	–	r = −0.311	r = −0.511[a]	r = 0.578[b]
	P= .000	–	*P*= .170	*P*= .018	*P*= .006
Hydrogen peroxide					
1	–	*r* = 0.580[b]	*r* = −0.476[a]	r = −0.273	r = 0.108
	–	*P*= .006	*P*= .029	*P*= .232	*P*= .642
2	–	*r* = 0.889[b]	*r* = −0.691[b]	*r* = −0.769[b]	*r* = 0.595[b]
	–	*P*= .00	*P*= .001	*P*= .00	*P*= .004
3	–	r = 0.763[b]	r = −0.167	r = –0.812[b]	r = 0.695[b]
		P= .000	*P*= .469	*P*= .00	*P*= .00

[a] *P*< .05 vs. control group.
[b] *P*< .01 vs. control group.

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SUPPLEMENTAL TABLE 3

Correlation between ROS levels (intracellular superoxide anion and hydrogen peroxide) and membrane integrity of frozen-thawed human spermatozoa in BDNF supplemented and control groups.

Group	Intact membrane	Altered membrane	Damaged membrane
Superoxide anion			
1	r = −0.213	r = 0.039	r = 0.208
	P= .353	*P*= .867	*P*= .365
2	r = −0.536[a]	r = 0.382	r = 0.554[b]
	P= .012	*P*= .087	*P*= .009
3	r = −0.374	r = 0.516[a]	r = 0.247
	P= .095	*P*= .017	*P*= .280
Hydrogen peroxide			
1	*r* = −0.645[b]	*r* = 0.319	*r* = 0.610[b]
	P= .002	*P*= .159	*P*= .003
2	r = −0.584[b]	r = 0.273	r = 0.589[b]
	P= .005	*P*= .231	*P*= .005
3	r = −0.601[b]	r = 0.840[b]	r = 0.490[a]
	P= .004	*P*= .00	*P*= .024

[a] *P*< .05 vs. control group.
[b] *P*< .01 vs. control group.

Najafi. BDNF protects frozen-thawed human sperm. Fertil Steril 2016.