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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;■:■–■. ©2016 by American Society for Reproductive Medicine.)
Key Words: Sperm cryopreservation, ROS, apoptosis, membrane integrity, AKT, BDNF

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

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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 $\pm$ 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 ($400 \times g$ 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