Sperm DNA fragmentation induced by cryopreservation: new insights and effect of a natural extract from *Opuntia ficus-indica*

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Objective: To analyze the effect of cryopreservation on sperm DNA fragmentation (SDF) in two cytometric sperm populations, $PI^{brighter}$ and PI^{dimmer}, and to test the effects of *Opuntia ficus-indica* (OFI) extracts, which contain antioxidants and flavanoids, and of resveratrol on cryopreservation of human semen.
Design: In vitro prospective study.
Setting: Institutional study.
Patient(s): Twenty-one normozoospermic men undergoing semen analysis for couple infertility.
Intervention(s): Cryopreservation using the routine method in the presence of OFI extracts or resveratrol.
Main Outcome Measure(s): Measurment of SDF by TUNEL/PI flow cytometric method to evaluate sperm motility (by automated motion analysis, CASA system) and viability (by eosin/nigrosin staining) in the two populations of sperm PI^{br} and PI^{dim}.
Result(s): Cryopreservation induced an increase of SDF only in the PI^{br} sperm population. The increase was negatively dependent on the basal values of SDF in the same population. Addition of OFI extracts and resveratrol to the cryopreservation medium slightly but statistically significantly reduced SDF in the PI^{br} population without affecting the deleterious effect of cryopreservation on sperm motion parameters or viability.
Conclusion(s): The increase of SDF in the PI^{br} population, which is unrelated to semen quality, suggests that caution must be taken in using cryopreserved semen, as morphologically normal and motile sperm may be damaged. The addition of substances with multifunctional properties such as OFI extracts to cryopreservation medium is only slightly effective in preventing the dramatic effects on SDF. (Fertil Steril® 2012;98:326–33. ©2012 by American Society for Reproductive Medicine.)
Key Words: *Opuntia ficus-indica*, resveratrol, semen cryopreservation, sperm DNA fragmentation

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Sperm cyropreservation may represent, for some men, the only option to preserve their fertility potential. Indeed, sperm cryopreservation is now widely used not only in cases of malignancies or other conditions requiring therapies that may be harmful for testis function, but also in cases of azoospermia or severe oligozoospermia, as even a few sperm retrieved from the testis or present in semen can be cryopreserved for subsequent assisted reproduction techniques (ART). In particular, as men with (severe) oligozoospermia are considered at risk of a further decrease of semen quality, semen cryopreservation is often recommended.

The deleterious effects of cryopreservation on sperm function are well known, with dramatic effects on

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viability, motility, and chromatin integrity (1–3). It is believed that most of these deleterious effects are related to reactive oxygen species (ROS) generated during the procedure, and several studies have reported the generation of ROS during the cryopreservation of sperm (4–6). Spermatozoa are extremely sensitive to ROS-induced damage; the elevated content of polyunsaturated fatty acids in their membrane makes them susceptible to lipid peroxidation (7). Besides inducing alterations in the motility and viability of sperm, ROS may induce DNA damage, including sperm DNA fragmentation (SDF) and base oxidation (7). In particular, the increase of DNA damage after cryopreservation procedure represents a problem in cancer patients; these men may have increased SDF even before chemotherapy (8, 9), and the deleterious effects of the cryopreservation procedure may increase it.

The increase of SDF after cryopreservation and thawing is particularly relevant for the fertility status of these men, as there is good evidence that a sperm with fragmented DNA can be motile, apparently morphologically normal (10), and can fertilize an oocyte, at least in animal models (11), thus transmitting the genetic anomaly to the oocyte and the embryo. In particular, we have recently demonstrated the existence of two sperm populations characterized by different levels of SDF as well as by differing relationships with semen quality. These populations were named $PI^{brighter}$ (PI^{br}) and PI^{dimmer} (PI^{dim}), based on their avidity for the nuclear probe propidium iodide (PI). The PI^{dim} population, entirely formed of dead (12) and DNA-fragmented spermatozoa, negatively correlates with semen quality. The PI^{br} population is only partially fragmented, and the percentage of fragmented sperm is independent of semen quality (13). The fragmented PI^{br} sperm may be motile, may show an apparently normal morphology, may have a greater possibility than fragmented PI^{dim} sperm to participate in the process of fertilization, especially in ART. Thus, the evaluation of the impact of cryopreservation procedures on the PI^{br} population appears to be important for future ART applications.

Despite the wide use of sperm cryopreservation and the knowledge of its deleterious effects on sperm function, cryopreservation techniques have not improved in past few years. Because ROS are believed to be involved in the decrease of sperm quality during cryopreservation, attempts to reduce the effects of oxidative stress during cryopreservation have mainly focused on adding antioxidants to extension media (14–18) or using different cryoprotectants or techniques (19, 20). So far, few beneficial effects on sperm motility, viability, or DNA fragmentation have been reported. In fact, standard cryopreservation procedures as suggested in the latest edition of the World Health Organization (WHO) manual for examination and processing of human semen do not include the addition of antioxidants (21).

Prickly pear cactus, or *Opuntia ficus-indica* (OPI), has recently received a growing interest in the scientific community because of its multifunctional properties (22–24). Chemical analysis has revealed that OFI extracts contain ascorbic acid, polyphenols, carotenoids, taurine, and several types of flavanoids, in particular quercetin (22, 24). Furthermore, OFI extracts have high free-radical scavenging and antioxidant activity in vitro (22, 24). Recently, a study of an infertile couple who had had several previous unsuccessful attempts in ART reported an increase of sperm DNA condensation in the male partner after he had been treated with an OFI extract (25).

We used terminal deoxynucleotidyl transferase dUTP nick end labeling/propidium iodide (TUNEL/PI) to evaluate DNA fragmentation in the PI^{br} and PI^{dim} sperm populations after cryopreservation (13, 26). We then evaluated the SDF, motility parameters, and viability of cryopreserved human spermatozoa after supplementing the cryopreservation medium with OFI extracts.

MATERIALS AND METHODS

Chemicals

The OFI extract was a generous gift of Prof. Marc Cohen (Procrélys, Recherche et Traitement en Infertilité/AMP Natecia, Lyon, France). The OFI extracts were prepared by crushing the figs and drying the obtained grains at 40°C in the total absence of oxygen. The drying process of OFI extracts is a Nurilia patent. At the end, the product is crushed into powder. To control the final quality of the product, the aldehydes and quinone (oxidation products of alcohol and phenols) are evaluated by liquid chromatography, according European guidelines (CE no. 852/2004; CE no. 853/2004, and CE no. 2073/2005).

Test yolk cryopreservation medium was purchased from Irvine Scientific. Human tubal fluid (HTF) was purchased from Celbio. The in situ cell death detection kit, fluorescein, was purchased from Roche Molecular Biochemicals. Propidium iodide was obtained from Calbiochem. The Sperm Vitalstain was from Nidacon, and resveratrol and all the other reagents were obtained from Sigma Aldrich.

Sample Collection

Semen samples were collected according to WHO criteria (27) from a total of 21 patients, all normozoospermic by WHO criteria (27), who were undergoing routine semen analysis for couple infertility in the andrology laboratory of the University of Florence. Approval was obtained from the Hospital Committee for Investigations in Humans (Prot. N. 2010/0033789), and informed consent was obtained from the men to use their remaining semen after routine semen analysis.

Semen Analysis

Semen analysis was performed according to WHO guidelines (27). Sperm morphology and motility were assessed by optical microscopy, according to WHO criteria (27). Sperm viability was evaluated by using Sperm Vitalstain according to the manufacturer's instructions. The average sperm concentration was $96.3 \pm 87.0 \times 10^6$/mL, the forward motility was 52.7 ± 14.01, and the sperm normal morphology 6.42 ± 5.66 (all mean $\pm$ standard deviation [SD], $n = 21$).

Evaluation of Motion Parameters by CASA

We used computer-assisted semen analysis (CASA, Hamilton Thorn Research) in fresh samples and after thawing to