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Influence of ejaculatory abstinence on seminal total antioxidant capacity and sperm membrane lipid peroxidation

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Objective: To determine whether the period of ejaculatory abstinence (EA) influences the total antioxidant capacity (TAC) of semen or lipid peroxidation (LPO) of sperm membranes.
Design: A prospective experimental trial.
Setting: Academic medical center for reproductive endocrinology and infertility.
Patient(s): Forty men from infertile couples planning intrauterine insemination.
Intervention(s): Men provided semen specimens after EA periods of 1 and 4 days.
Main Outcome Measure(s): Semen analysis, peroxidase staining, and assays for seminal TAC and sperm membrane LPO, with measures compared between days 1 and 4 within individuals (internal control) using paired *t* tests.
Result(s): The shorter period of EA (1 day vs. 4 days) resulted in statistically significant decreases in semen volume (−24%), sperm density (−28%), and total sperm count (−3.2%). There was a statistically significant increase in TAC with the shorter period of EA (1 day) compared with 4 days of EA. No difference was detected in sperm membrane LPO comparing 1 day of EA and 4 days of EA.
Conclusion(s): Higher seminal TAC obtained after a shorter period of EA could diminish oxidative stress–induced sperm damage by a mechanism independent of LPO. Shorter periods of EA may thus improve sperm quality by protecting from reactive oxygen species damage, even though lower numbers of motile sperm are produced after a shorter period of EA. This would be consistent with prior research indicating improved results after intrauterine insemination under these circumstances.
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Key Words: Abstinence, male infertility, seminal plasma oxidative stress, spermatozoa, total antioxidant capacity

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For selected couples with male factor infertility, ovulation induction with intrauterine insemination (IUI) produces significantly higher cycle fecundity compared to timed intercourse (1). We previously reported that shortening the period of ejaculatory abstinence (EA) resulted in a higher pregnancy rate after IUI despite the fact that fewer motile sperm cells were inseminated (2). These findings are consistent with the premise that improved sperm quality following a shortened EA period, rather than motile sperm number, is the principal factor that produced a higher IUI cycle fecundity.

Sperm cells in the cauda of the epididymis and vas deferens may be subject to a harmful seminal microenvironment before or after ejaculation that could impair sperm function in proportion to increasing time exposure. Maturing, epididymal spermatozoa require a level of reactive oxygen species (ROS) for the formation of disulfide-bridging for DNA compaction, but excessive ROS would induce oxidative damage to spermatozoa that could negatively affect fertilization potential. This balanced ROS

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microenvironment in the epididymis is regulated by the glutathione peroxidase system (3). Mature spermatozoa are particularly susceptible to ROS damage because they cannot respond to oxidative challenge with transcriptional activity secondary to loss of cytosolic organelles and because the external sperm membrane has a particular composition that is highly reactive to oxidative injury. Excessive ROS may be generated from leukocytes and dying spermatozoa in this seminal microenvironment. The ROS and/or deficient antioxidant defense systems in semen may cause sperm damage and have been implicated as an important cause of male factor infertility (4–6). Potential harmful effects of oxidative stress on human spermatozoa include damage to sperm membranes by lipid peroxidation (LPO), sperm DNA susceptibility to free radical attack, or sperm protein damage (7, 8). Theoretically, an increased antioxidant capacity in seminal fluid could neutralize superoxide free radicals and mitigate sperm damage.

Increasing the frequency of ejaculation may reduce spermatozoal exposure to harmful reactive oxygen species, thereby improving sperm viability and function. To test this hypothesis, we have determined whether the period of ejaculatory abstinence (EA) influences the total antioxidant capacity (TAC) of semen or impacts lipid peroxidation (LPO) of sperm membranes. A reduction of reactive oxygen stress by shortening the period of EA could be a mechanism to explain why a higher pregnancy rate after IUI results from semen specimens obtained after a short period of EA despite fewer motile sperm cells being inseminated (2).

MATERIALS AND METHODS

Study Participants

Forty men from couples with infertility being treated at the Reproductive Medicine and Infertility clinic at Carolinas Healthcare System and planning to undergo IUI were recruited to participate in this prospective experimental study after institutional review board approval. The average frequency of intercourse for the men before producing their first semen specimen for study was between once and twice weekly. Men were excluded from study entry for the following reasons: failure to follow the ejaculatory abstinence (EA) protocol, specimen less than 2 cc in volume, current smoking, presence of varicocele, history of vasectomy reversal, consumption of certain vitamins or supplements (vitamin E or C, coenzyme Q, taurine or glutathione), or genital infection within 6 months. The decision to exclude men with ejaculates of less than 2 cc in volume was secondary to technical problems with having sufficient volume and/or sperm for the TAC and LPO assays.

Study Protocol

All men provided two semen samples. The initial semen specimen was collected after 4 days of EA and the second specimen 1 day later. Semen analysis was performed after liquefaction at 37°C in accordance with World Health Organization guidelines (9). Quantification of seminal leukocytes was assessed by peroxidase staining. Raw semen (seminal plasma plus spermatozoa) was subjected to nondensity centrifugation at 300 $\times$ g for 7 minutes, and the supernatant (seminal plasma) was removed from the sperm pellet. Two aliquots of neat seminal plasma of 200 μL were transferred to cryovials and were cryopreserved as indicated herein for subsequent TAC assay. The sperm pellet was resuspended in the residual seminal plasma and was then diluted by careful addition of the TEST-yolk buffer freezing medium (Irvine Scientific) at a 1:1 (volume/volume) ratio. Gentle, uniform resuspension was performed for 5 minutes. The cryopreservation vials with the seminal plasma (for later TAC assay) and the resuspension of sperm with TEST-yolk buffer freezing medium (for later LPO assay) were placed in a -20°C freezer for 8 minutes and thereafter in liquid nitrogen vapor at -79°C for 2 hours. The vials were then transferred to liquid nitrogen at -196°C. For TAC and LPO testing, each reconstituted semen plus TEST-yolk buffer vial or seminal plasma vial was removed from liquid nitrogen and thawed at room temperature for 5 minutes, followed by incubation for 20 minutes at 37°C.

Assay for seminal total antioxidant capacity (TAC). Seminal plasma was assayed in triplicate for total antioxidant capacity (TAC) using a Randox Total Antioxidant Status kit (Crumlin) per the manufacturer's instructions. Briefly, samples were incubated with ABTS (2,2′-Azino-di-[3-ethylbenzthiazoline] sulphonate) radical cations, which have a relatively stable blue-green color in the absence of antioxidants. The degree of color suppression by antioxidants in the sample, which is proportional to the concentration, was measured at 600 nm using a DU 800 UV/Visible Spectrophotometer (Beckman Coulter). Unknown sample antioxidant concentrations were interpolated from a standard curve created from samples of known concentrations.

Assay for sperm membrane lipid peroxidation (LPO). Sperm membrane lipid peroxidation was measured using a Calbiochem Lipid Hydroperoxide (LPO) Assay Kit (Darmstadt) per the manufacturer's instructions. Thawed sperm resuspensions were diluted in Ham's F-10 (pH 7.4) with a 1:1 (volume/volume) ratio of resuspension to buffer medium, followed by centrifugation at 300 $\times$ g for 7 minutes. After discarding the supernatant, we resuspended the sperm pellet in fresh Ham's F-10 (pH 7.4) buffer to an adjusted final concentration of 20 $\times$ 10^6 spermatozoa/mL (10). Sample lipid hydroperoxides were first extracted in chloroform to eliminate interference by hydrogen peroxide or endogenous ferric ions. Lipid hydroperoxides present in this extract were measured directly using redox reactions with ferrous ions. The ferric ions produced were detected using thiocyanate ion as a chromogen. The absorbance of the reactions was measured at 500 nM using a Synergy HT Multi-Detection Microplate reader (BioTek). Unknown lipid hydroperoxide sample concentrations were calculated using a standard curve generated with samples of known concentrations.

Statistical Analysis

Measures for semen parameters, total antioxidant capacity (TAC), and lipid peroxidation (LPO) were compared statistically on semen specimens obtained after 4 days of ejaculatory

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