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HAM'S F-10 MEDIUM AND HAM'S F-10 MEDIUM PLUS VITAMIN E HAVE PROTECTIVE EFFECT AGAINST OXIDATIVE STRESS IN HUMAN SEMEN

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ABSTRACT

Objectives. To investigate possible protective effects of vitamin E and Ham's F-10 medium (HF-10) on lipid peroxidation, apoptosis, motility, and vitality of spermatozoa.

Methods. Normozoospermic semen samples were obtained from 35 volunteers. Normal saline solution, HF-10 only, or HF-10 with vitamin E were added to split semen samples (control, group 1, and group 2, respectively). Sperm motility and vitality were evaluated at the end of 1, 2, and 24 hours. Superoxide dismutase, catalase, and malondialdehyde levels were assessed at the end of the first hour. Flow cytometric DNA analysis was performed at the end of 24 hours.

Results. Superoxide dismutase, sperm motility, and vitality were not different among the groups. The catalase values decreased in group 1, but not in group 2. Malondialdehyde values in supernatants decreased in group 2 and apoptosis of spermatozoa decreased in groups 1 and 2.

Conclusions. Our data suggest that vitamin E and HF-10 protect against the reactive oxygen species-mediated damage on spermatozoa. UROLOGY **67:** 384–387, 2006. © 2006 Elsevier Inc.

Elevated levels of reactive oxygen species (ROS) are related to male infertility as a consequence of oxidative stress on sperm function.[1] This stress originates from excessive generation of ROS by the spermatozoa and infiltrating leukocytes, and results in the peroxidation of unsaturated fatty acids in the sperm plasma membrane. Whatever the source of ROS, the lipid peroxides thereby generated exhibit powerful negative correlations with the movement characteristics of spermatozoa and their capacity for sperm-oocyte fusion.[2] Cells isolated from the ejaculates of a high proportion of patients exhibiting oligozoospermia are characterized by generation rates of ROS that considerably exceed those obtained for the normal fertile population.[3]

Spermatozoa are protected by various antioxidants and antioxidant enzymes in the seminal plasma or spermatozoa itself. When the gametes are cultured in vitro, they become more susceptible to oxidative damage. The addition of antioxidants brought beneficial effects in preventing loss of motility and inhibiting lipid peroxidation. Treating patients with antioxidants was shown to have a positive effect on improving fertilization in limited data.[4]

Apoptosis plays an important role in regulating spermatogenesis. However, the biologic significance of apoptosis in ejaculated sperm is not yet clear.[5]

The aim of this study was to assess the possible protective efficacy of vitamin E and Ham's F-10 medium (HF-10) as antioxidants[6,7] on lipid peroxidation, apoptosis, motility, and vitality of spermatozoa in vitro.

MATERIAL AND METHODS

The World Medical Association declaration of Helsinki Ethical Principles for medical research involving human subjects was accepted in the present study, and all subjects provided informed consent. This study was approved by the Commission of Scientific Research Projects of Karadeniz Technical University and by an ethical committee.

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Sperm Preparation, Motility, and Vitality Analysis

Semen samples of 35 normozoospermic idiopathic infertile men (aged between 29 and 43 years) attending the Urology Clinic were studied. Semen samples were produced by masturbation after 3 to 5 days of sexual abstinence, collected in clean plastic jars, and analyzed according to the World Health Organization andrologic criteria in the laboratory of the Department of Histology and Embryology.[8] The samples were allowed to liquefy for 30 minutes at 37°C in an incubator. Then, all samples were defined as possessing the following characteristics: volume 2.0 mL or greater, sperm concentration 50×10^6 spermatozoa/mL or greater, motility 50% or more, vitality 70% or more, normal morphology 30% or greater, and 1×10^6 leukocytes/mL or less. These parameters were studied at room temperature.[8]

Each human ejaculate that liquefied spontaneously was split into three fractions. The first, second, and third fraction was named the control group and groups 1 and 2, respectively (each group contained 0.5 mL of semen). The pH value of each media was evaluated and the pH of normal saline solution (0.9% NaCl) was corrected to 7.5 using NaOH solution. We added 0.5 mL of the saline solution to the control group; 0.5 mL of HF-10 (Irvine Scientific, Santa Ana, Calif) to group 1; and 0.5 mL of HF-10 and 40 μM vitamin E (alpha-tocopherol, Sigma, Germany) to group 2.[9] HF-10 (without vitamin E) served as the control for vitamin E. Sperm motility and vitality were evaluated at the end of 1 hour. Then the split seminal fractions were centrifuged at 1000*g* for 5 minutes immediately, and their seminal plasma was collected into plastic tubes; 0.5 mL of each seminal plasma in the tubes was kept at −20°C to be thawed and evaluated for malondialdehyde (MDA), catalase (CAT), and superoxide dismutase (SOD). Then, 0.5 mL of the saline solution (pH = 7.5) was added to the pellet of the control group; 0.5 mL of HF-10 was added to the pellet of group 1; and 0.5 mL HF-10 and 40 μM vitamin E were added to the pellet of group 2. Each pellet was mixed properly and kept at 37°C in the incubator for evaluation at 2 and 24 hours for sperm motility and vitality and flow cytometric DNA analysis at the end of 24 hours. Cell concentration and sperm motility and vitality were measured using a Makler Counter (Sefi-Medical Instrument, Haifa, Israel). The viability of spermatozoa was also evaluated using eosin Y.[10]

SOD and CAT-like Activities

SOD-like activity was measured by the reduction of nitroblue tetrazolium by the xanthine-xanthine oxidase system, which is a superoxide generator. Enzyme activity leading to 50% inhibition was accepted as 1 U. The results are expressed as units per milligram protein.[11]

CAT-like activity was measured as the removal of exogenous hydrogen peroxide using the method described by Yeung *et al.*[12] In brief, 10 μL 8.8 mM hydrogen peroxide was added to 195 μL seminal plasma diluted 41-fold in 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid-buffered medium (pH 7.4) and incubated for 60 minutes at 37°C. Then, 8 μL horseradish peroxidase (425 IU/mL), 8 μL Phenol Red (0.5), and, 5 minutes later, 20 μL 1 M NaOH were added, mixed well, and read at 630 nm against hydrogen peroxide standards.[13]

Determination of MDA

Seminal plasma samples were thawed for evaluation of MDA, CAT, and SOD. Lipid peroxidation in the samples was determined as the MDA concentration using the method of Yagi.[14] In brief, 0.3 mL seminal fluid was mixed with 2.4 mL of N/12 H_2SO_4 and 0.3 mL of 10% phosphotungstic acid. After allowing it to stand at room temperature for 5 minutes, the mixture was centrifuged at 1600*g* for 10 minutes. We discarded the supernatant, and the sediment was suspended in 4 mL distilled water. Next, 1 mL of 0.67% thiobarbituric acid was added, and the mixture was heated in boiling water for 60 minutes. The formed color was extracted into N-butanol. The mixture was centrifuged at 1600*g* for 10 minutes. The absorbance of the organic layer was read at 532 nm. Tetramethoxypropane was used as a standard, and MDA levels were calculated as nanomoles per milliliter.[14]

Flow Cytometric Analysis of Spermatozoa

Flow cytometric analysis was used to determine the percentage of apoptotic cells and DNA content of spermatozoa in semen. This analysis was done only at 24 hours in each group. Flow cytometry was performed on single nuclei of spermatozoa suspensions from the seminal pellet. Each pellet sample was processed using a modification of the method described by Hedley *et al.*[15] In brief, the samples were rehydrated, and the sperm suspension was digested with 0.5% pepsin in 0.9% NaCl (pH 1.5) for 30 minutes at 37°C to produce a suspension of bare nuclei. The suspension was filtered through an 80-μm nylon DNA mesh to remove aggregates. The nuclei were then washed and incubated for 10 minutes with Coulter DNA-Prep Reagents Kit (PN 6607055). The Coulter DNA-Prep Reagents Kit contains DNA-Prep LPR (less than 0.1% potassium cyanide, less than 0.1% NaN_3 nonionic detergents, saline, and stabilizers). DNA-Prep Stain contains 50 μg/mL propidium iodide (fluorescence excites at 488 nm and emits at 560 to 680 nm) and RNAse (type III-A, Bovine pancreas, less than 0.1% NaN3, saline, and stabilizers). Next, the sample was analyzed using a Coulter Epics Elite ESP (Coulter) flow cytometer after 1 hour of staining. The fractions of cells in semen were determined by the DNA distribution fraction of the cell, which was calculated using the MultiCycle DNA computer program (Phoenix Flow System, San Diego, Calif). These values were further analyzed by an apoptotic index. The occurrence of DNA fragmentation, as a feature of apoptosis, was determined by flow cytometric measurement of the percentage of nuclei with a hypodiploid DNA content, as previously described. Apoptotic cells were defined as cells in the sub-G_1 phase of the cycle. The coefficient of variation of the G_1 or diploid peak was used as a measure of the quality of the results. DNA histograms were only considered analyzable if the coefficient of variation was less than 10%. The peak with the least DNA content was taken as the diploid peak.[16]

Statistical Analysis

Data normality was assessed by the Kolmogorov-Smirnov test. In each group, comparisons among the measures (1, 2, and 24 hours) of motility and vitality were done with repeated measures analysis of variance (paired *t* test as post hoc) for normally distributed variables. Comparisons among groups were done with analysis of variance (Bonferroni as post hoc test) for motility and vitality, SOD-like activity, CAT-like activity, MDA values, and apoptotic index. The results are given as the mean ± standard deviation. $P < 0.05$ was accepted as significant.

RESULTS

The values for sperm motility and vitality are shown in Tables I and II. For the time points (1, 2, and 24 hours), the values for motility and vitality were not different among the three groups. Statistically, no significant difference was found for SOD values among the groups (Table III).

CAT-like activity values were different among the groups (Table III). Statistically, the difference was caused by the values of group 1. A significant

TABLE I. *Comparison of sperm motility between study groups at 1, 2, and 24 hours*

	1 hr	2 hr	24 hr	*P* Value
Control	70.5 ± 10.6	67.3 ± 11.3	1.2 ± 2.1	<0.0005
Group 1	71.0 ± 11.2	68.5 ± 11.4	1.5 ± 2.2	<0.0005
Group 2	70.1 ± 10.6	66.8 ± 12.7	1.5 ± 2.2	<0.0005
P value	0.938	0.821	0.948	

TABLE II. *Comparison of sperm vitality between study groups at 1, 2, and 24 hours*

	1 hr	2 hr	24 hr	*P* Value
Control	81.5 ± 6.0	79.3 ± 6.8	52.1 ± 4.6	<0.0005
Group 1	82.6 ± 5.8	80.4 ± 7.4	51.1 ± 5.1	<0.0005
Group 2	83.4 ± 5.6	82.1 ± 5.4	51.2 ± 6.2	<0.0005
P value	0.385	0.204	0.736	

TABLE III. *Seminal SOD- and CAT-like activity, MDA values, and apoptosis of study groups*

	Control	Group 1	Group 2	*P* Value
SOD-like activity (U/mL seminal plasma)	22.1 ± 2.6	19.8 ± 8.8	21.4 ± 7.2	0.358
CAT-like activity (mU/mL seminal plasma)	122.5 ± 4.1	111.1 ± 7.4	121.0 ± 12.7	<0.0005
MDA values (nmol/mL seminal plasma)	3.2 ± 0.7	3.1 ± 0.7	2.1 ± 0.5	<0.0005
Apoptosis (%)	21.4 ± 1.7	14.9 ± 1.0	7.9 ± 0.4	<0.0005

KEY: *SOD = superoxide dismutase; CAT = catalase; MDA = malondialdehyde.*

difference for MDA values was observed (Table III). Statistically, the difference was caused by the values of group 2.

According to flow cytometric DNA analysis, a significant difference was found for the apoptotic index (percentage) among the groups (Table III). Statistically, all groups were different from the control group and each other.

COMMENT

ROS produced by spermatozoa have been suggested, on the one hand, to be associated with defective sperm function,[17] and, on the other hand, to stimulate certain sperm functions, leading to fertilization.[12] Seminal plasma from infertile men has lower antioxidant levels than that of fertile men, particularly in patients whose semen have poor sperm motility. The presence of ROS in sperm of infertile groups is also associated with lower levels of antioxidants in seminal plasma.[18]

Vitamin E (alpha-tocopherol) and HF-10 are two potent antioxidants.[6,7] The production of ROS by sperm was reduced by supplementation in vitro with vitamin E.[19] Vitamin E occurs naturally in cell membranes[20] and protects spermatozoa against ROS-mediated damage.[21,22] It also minimally improved the postthaw motility.[23] In this study, we investigated the effects of vitamin E and HF-10 on spermatozoa. Our results have shown that vitamin E and HF-10 alone had no effect on motility and vitality, but that vitamin E with HF-10 decreased the MDA level in seminal plasma and decreased the ratio of apoptosis. Thus, these antioxidants may protect spermatozoa from oxidative stress with decreased apoptosis. Vitamin E and HF-10 led to an improvement in the markers of oxidative stress.

SOD and CAT are considered protective antioxidants for spermatozoa in semen.[13] They play a major role in protecting human spermatozoa against lipid peroxidation.[24,25] The mean SOD-like activity in the semen of infertile men was significantly greater than in the semen of fertile controls but the mean seminal CAT-like activity was not significantly different between these samples.[26] In this study, the difference for CAT values in group 1 was significant. The data showed that HF-10 decreased the baseline CAT value but the addition of vitamin E to HF-10 brought the value to that of the control group. This finding needs additional research.

MDA is an end product of lipid peroxidation[21] and is used for evaluation of lipid peroxidation occurring in seminal plasma and spermatozoa. The spermatozoal MDA concentration was shown to be greater in men with decreased sperm motility.[27] In this study, no relationship was found between the MDA concentration in the seminal plasma and

sperm motility and vitality in pellet suspensions. However, a significant positive correlation was found between the MDA concentration in seminal plasma and the apoptosis of spermatozoa in pellet suspensions.

Supplementation of sperm preparation medium (Percoll) with vitamin E did not influence baseline DNA integrity but provided sperm with dose-dependent protection against hydrogen peroxide-induced DNA damage.[19] No significant differences in the apoptotic index or the percentage of live and apoptotic sperm have been detected between subjects with normal and abnormal semen.[5] In our study, the apoptotic index was evaluated in three split semen with different diluents. The percentage of apoptosis in group 2 was the least among the groups and the percentage of apoptosis in group 1 was less than that of the control group.

CONCLUSIONS

Vitamin E with HF-10 decreased MDA levels in seminal fluid and decreased apoptosis of spermatozoa, suggesting a protective effect on spermatozoa by way of the inhibition of oxidative stress. However, future studies are needed to determine whether these antioxidants can produce a consistent improvement in the motility, vitality, and fertilization capacity of spermatozoa.

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