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SEMINAL PLASMA LEVELS OF 15-F2α-ISOPROSTANE, MALONDIALDEHYDE AND TOTAL HOMOCYSTEINE IN NORMOZOOSPERMIC AND ASTHENOZOOSPERMIC MALES

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ABSTRACT

It has been proposed that oxidative stress plays an important role in male infertility. The aims of this study were to compare seminal plasma levels of 15-F2t-isoprostane (8-iso-PGF2α), malondialdehyde (MDA), and total (sum of free and bound) homocysteine (tHcy) from normozoospermic vs. asthenozoospermic men, and to examine the relationships between tHcy and lipid peroxidation products. The study was a case-control study with a simple random sampling. The case group was consisted of 15 asthenozoospermic males. This group was compared with 15 normozoospermic men. Seminal plasma levels of 15-F2α-isoprostane and tHcy were measured using commercially available enzyme immunoassay (EIA) kits. MDA levels were determined by the thiobarbituric acid (TBA) assay. The Mann-Whitney U test was used to compare two groups. Coefficients of correlation were calculated using Spearman's correlation analysis. All hypothesis tests were two-tailed with statistical significance assessed at the p value <0.05 level. MDA levels were higher in asthenozoospermic subjects than in control subjects (0.72±0.06 μM vs. 0.40±0.06 μM; $p<0.05$). No differences were seen in 15-F2α-isoprostane levels in asthenozoospermic subjects and controls (65.00±3.20 pg/ml vs. 58.17±4.12 pg/ml; $p>0.05$). Interestingly, tHcy levels were to be slightly higher in asthenozoospermic subjects than in controls (6.18±1.17 μM vs. 4.8±0.52 μM). Sperm motility was inversely correlated with seminal plasma 15-F2α-isoprostane and MDA levels, respectively ($p<0.05$). In summary, seminal plasma levels of 15-F2α-isoprostane and tHcy showed no significant difference between normozoospermic and asthenozoospermic men. Sperm motility was not correlated with seminal plasma levels of tHcy. No relationship was found between tHcy and lipid peroxidation.

KEY WORDS

15-F2α-isoprostane, Asthenozoospermia, Homocysteine, Lipid peroxidation, Malondialdehyde, Normozoospermia, Seminal plasma.

INTRODUCTION

One in six couples of reproductive age present with infertility (1, 2). Poor sperm forward motility (asthenozoospermia) is considered to contribute to the infertility of a significant number of males, and many cases of decreased sperm motility are not completely understood (3). One of factors that potentially can cause asthenozoospermia is oxidative stress (4).

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Oxidative stress arises as a consequence of excessive production of ROS and impaired antioxidant defense mechanisms (5). Reactive oxygen species (ROS) play a dual role in male infertility. On one hand, ROS play a key-role in processes such as capacitation, the acrosome reaction, and fertilization. On the other hand, excessive production of ROS can inflict severe damage to spermatozoa (6-9). Spermatozoa contain large quantities of polyunsaturated fatty acids (PUFA) and therefore they are susceptible to ROS-induced damage. It has been suggested that ROS induce membrane lipid peroxidation in sperm and that the toxicity of generated fatty acid peroxides are important causes of sperm malfunction (10). The most widely used assay for lipid peroxidation involves the measurement of malondialdehyde (MDA)-thiobarbituric acid (TBA) adducts due to its simplicity (11). Recently, it has been shown that 15-F2α-

isoprostane (8-iso-PGF2α) is a specific, chemically stable, and quantitative marker of oxidative stress in vivo (12, 13). 15-F2α-isoprostane is a prostaglandin isomer synthesised *in vivo*, independently of the activity of cyclo-oxygenase (14).

Homocysteine is a thiol-containing amino acid produced by the intracellular demethylation of methionine in the methylation processes. Total homocysteine level (reffered to as tHcy) is the sum of all homocysteine species. tHcy is prominently present in the oxidized form; mixed disulfides with proteins (15). Homocysteine is metabolized to either cysteine or to methionine. In any conditions which homocysteine production is high or its metabolism is impaired, homocysteine accumulates in the cell and is exported to the extracellular fluids (16). Homocysteine is receiving a lot of attention these days as a new risk factor for a variety of disease. One mechanism by which increased homocysteine has been imposed to influence its pathological effects is by promoting increased oxidative stress (15-19).

Available data on the impact of oxidative stress and sperm motility are based on the measurement of sperm and seminal plasma MDA (11, 20-24). To the best of our knowledge no information is available on the relationship between sperm quality parameters and seminal plasma 15-F2α-isoprostane or homocysteine levels. The objectives of this study were to compare seminal plasma MDA, 15-F2α-isoprostane, and tHcy levels in normozoospermic vs. asthenozoospermic males and their association with sperm motility and to investigate the relationship between seminal plasma tHcy levels and lipid peroxidation.

MATERIALS AND METHODS

We designed a case-control study with a simple random sampling. Following Institutional Review Board approval, semen samples were collected from males undergoing infertility screening. Semen samples were collected from males undergoing infertility screening. All specimens were collected into sterile plastic containers by masturbation at the clinical andrology laboratory at University Teaching Women Hospital after an abstinence period of 48-72 hrs and analyzed within 1h of collection. After allowing at least 30 min for liquefaction to occur, semen analysis was performed to measure sperm concentration, normal sperm morphology, percentage sperm motility in accordance with the recommendations of the World Health Organization (WHO) using Sperm Quality Analyzer IIC (SQA IIC, United Medical Systems Inc, Santa Ana, CA, USA) (25-27). The WHO criteria for sperm normality used were as follows: sperm concentration $\geq$ 20 Millions/ml of ejaculate, percentage sperm motility $\geq$ 50% and normal sperm morphology $\geq$ 30%. The case group was consisted of 15 asthenozoospermic males. This group was compared with 15 normozoospermic men. Samples with a leukocyte concentration >10^6 /ml of ejaculate were excluded from this study. Myeloperoxidase staining was performed to evaluate the leukocyte concentration in specimen (LeucoScreen; Fertipro). Liquefied semen samples were centrifuged at 3000 rpm for 5 minutes. The supernatant seminal plasma was then carefully removed and transferred to Eppendrof tubes. The seminal plasma was frozen at -80°C until examination.

15-F2α-isoprostane Measurement

15-F2α-isoprostane concentrations were measured using commercially available enzyme immunoassay (EIA) kit (Cayman Chemical, Ann Arbor, MI, USA). The procedure for the EIA was according to the instructions provided by the manufacturer. The sample volume that used was 50 µl. Absorbance was measured at a wavelength of 405 nm using enzyme-linked immunosorbent assay (ELISA) reader (STAT FAX 2100, USA). The concentration of 15-F2α-isoprostane was calculated from a semi-logarithmic standard curve of standard samples vs. $\%B/B_0$ [ratio of the absorbance of a particular sample or standard well to that of the maximum binding (B_0) well], and data was presented as pg/ml. The intra-assay coefficient of variation was <10%. The detection limit and specificity of 15-F2α-isoprostane assay were 5 pg/ml and 100%, respectively.

Total homocysteine Measurement

Total homocysteine (tHcy) levels were measured using Axis Homocysteine EIA kit (Axis-Shield AS. Germany). The procedure for the EIA was according to the instructions provided by the manufacturer. The sample volume that used was 50 µl. Absorbance was measured at a wavelength of 450 nm using ELISA reader (STAT FAX 2100, USA). The concentration of tHcy was calculated from a semi-logarithmic standard curve of standard samples vs. absorbance (450 nm). The intra-assay coefficient of variation was <10%. The detection limit of tHcy assay was 2.0 µM.

Malondialdehyde Measurement

The amount of Malondialdehyde (MDA) was determined by the TBA assay (23, 28). All reagents that were used in this assay were obtained from Merck (Darmstadt, Germany). In short, 0.50 ml of seminal plasma was added to 3.00 ml of 1.00% phosphoric acid, 1.00 ml of 0.60% TBA, and 0.15 ml of 0.20% butylated hydroxytoluene (BHT) in 95% methanol. The samples were heated in a boiling water bath for 45 minutes, cooled and 4.00 ml of 1-butanol was added. The butanol phase was separated by centrifugation at 3000 rpm for 10 minutes and absorbance was

measured at 532 nm. The concentration of MDA was expressed as μM.

Statistical Analysis

Based on a pilot study, using an α value of 0.05 and a β value of 0.2 (80% power), the minimum sample size required was 15 samples per group. The Mann-Whitney U test was used to compare two groups. Coefficients of correlation were calculated using Spearman's correlation analysis. All hypothesis tests were two-tailed with statistical significance assessed at the p value <0.05 level with 95% confidence intervals (CI). The data are expressed as the mean ± SEM. Statistical computations were calculated using SPSS 10 for windows software (SPSS Inc, Chicago, IL, USA).

RESULTS

Table 1 shows the profile of sperm quality parameters of the asthenozoospermic and normozoospermic males. The means percentages sperm motility and normal sperm morphology showed a significant difference between the groups ($p<0.05$). But, the sperm concentration difference between the two groups was not statistically significant ($p>0.05$). Seminal plasma MDA, 15-F2α-isoprostane, and tHcy levels of the samples are shown in (Table 2). The difference of mean MDA between the two groups was statistically significant ($p<0.05$), but means of 15-F_{2t}-isoprostane and tHcy levels did not show a significant difference between the two groups ($p>0.05$).

We also investigated the correlation between seminal plasma MDA, 15-F2α-isoprostane, and tHcy levels and sperm motility in overall and in each group. Seminal plasma 15-F2α-isoprostane levels showed an inverse significant correlation with sperm motility ($r= -0.41$, $p<0.05$) (Fig. 1) in overall, but this correlation was not in each group. No correlation was found between seminal plasma tHcy levels and sperm motility. Seminal plasma levels of malondialdehyde showed an indirect correlation with sperm motility ($r= -0.53$, $p<0.05$) (Fig. 2) in overall. Finally, the correlations of tHcys with MDA and 15-F2α-isoprostane were examined. Seminal plasma levels of tHcy were not correlated with MDA and 15-F2α-isoprostane levels.

DISCUSSION

The most relevant finding from this study was a significant inverse correlation between seminal plasma levels of 15-F2α-isoprostane and sperm motility. We observed that seminal plasma levels of 15-F2α-isoprostane and tHcy did not differ significantly between normozoospermic and asthenozoospermic males. Our study also showed that there was not any correlation between seminal plasma levels of tHcy and lipid peroxidation.

The results obtained in our study about MDA were in accordance with the studies of Frraczek M *et al.* and Keskes-Ammar L *et al.* (21, 24) and contradicted with the Suleiman SA *et al.* and Nakamura *et al.* studies (20, 22).

Because MDA is a by-product of oxidative stress, elevation of its level indirectly reflects high levels of ROS in seminal plasma. The link between ROS and reduced motility may be due to a cascade of events that result in a decrease in axonemal protein phosphorylation and sperm immobilization, both of which are associated with a reduction in membrane fluidity that is necessary for sperm-oocyte fusion. Another hypothesis is that hydrogen peroxide can diffuse across the membranes into the cells and inhibit the activity of some enzymes such as glucose-6-phosphate dehydrogenase (G6PD). This enzyme controls the rate of glucose flux through the hexose monophosphate shunt, which in turn controls the intracellular availability of nicotinamide-adenine dinucleotide phosphate (NADPH). This, in turn, is used as a source of electrons by spermatozoa to fuel the generation of ROS by an enzyme system known as NADPH oxidase. Inhibition of G6PD leads to a decrease in the availability of NADPH and a concomitant accumulation of oxidized glutathione. This can reduce the antioxidant defenses of the spermatozoa and peroxidation of membrane phospholipids (8, 9).

We also measured seminal plasma level of 15-F2α-isoprostane. Recent studies have focused on 15-F2α-isoprostane, as an index of lipid peroxidation. Quantification of 15-F2α-isoprostane has been suggested to be a reliable measure of oxidative injury *in vivo*. There are a number of favourable attributes that imply that measurement of 15-F2α-isoprostane may provide a reliable marker of lipid peroxidation *in vivo*. First, this is a stable compound. 15-F2α-isoprostane is also specific product of free radical-induced lipid peroxidation. 15-F2α-isoprostane has been found to be present in detectable quantities in all normal biological tissues and in free form in all normal biological fluids. This is important because it allows that definition of a normal range such that small increases in their formation can be detected in situations of mild oxidant stress. Finally, the levels of 15-F2α-isoprostane is unaffected by lipid content of the diet (13, 14). Our study did not show a significant difference in seminal plasma levels of 15-F2α-isoprostane between the two groups. 15-F2α-isoprostane, like MDA, showed an inverse significant correlation with sperm motility.

Another analyte that we measured was total homocysteine. One mechanism by which increased homocysteine has been proposed to influence its pathological effects is by promoting increased

Table 1. Sperm quality parameters in normozoospermic and asthenozoospermic males

Subjects	Concentration 10^6/mL mean±SEM	Motility % mean±SEM	Morphology % mean±SEM
Normozoospermic n= 15	99.87±8.59	52.00±1.07	42.66±2.06
Asthenozoospermic n= 15	78.47±7.54	34.67±2.46	33.33±1.26
p value	> 0.05	< 0.05	< 0.05

n= Number of subjects. SEM= Standard error of mean p value calculated by Mann-Whitney test.

Table 2. Seminal plasma malondialdehyde (MDA), 15-F2α-isoprostane, and total homocysteine (tHcy) levels between normozoospermic and asthenozoospermic males

Subjects	MDA µM mean±SEM	15-F2α- isoprostane pg/mL mean±SEM	tHcy µM mean±SEM
Normozoospermic n= 15	0.40±0.06	58.17±4.12	4.80±0.52
Asthenozoospermic n= 15	0.72±0.06	65.00±3.20	6.18±1.17
p value	< 0.05	> 0.05	> 0.05

n= Number of subjects. SEM= Standard error of mean, p value calculated by Mann-Whitney test.

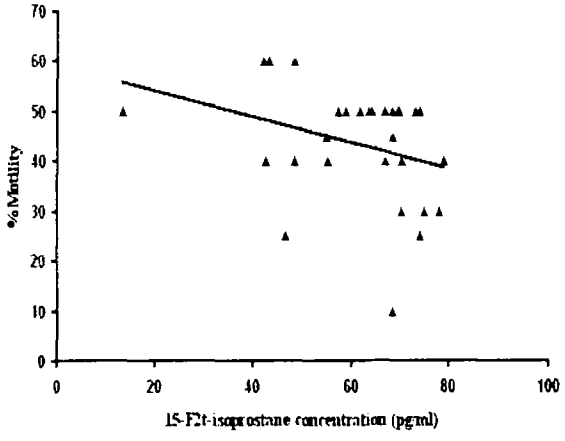

Fig. 1. Correlation between sperm motility and seminal plasma 15-F2α-isoprostane concentration (r= -0.41, p<0.05)

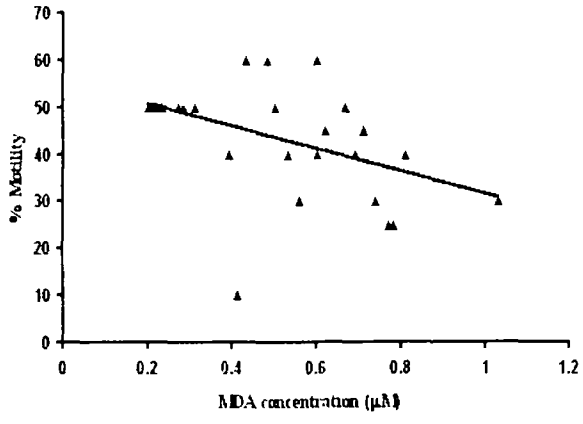

Fig. 2. Correlation between sperm motility and seminal plasma malondialdehyde (MDA) concentration (r= -0.53, p<0.05)

oxidative stress. One proposed mechanism is that because homocysteine is a thiol, it can undergo autooxidation and oxidation with other thiols. The resulting ROS-hydrogen peroxide and superoxide anion radical- generate oxidative stress (16-20). There is evidence of increased in vivo lipid peroxidation in certain animal models of hyperhomocysteinemia, as well as methionine-loaded human volunteers (30). However, the issue of whether homocysteine promotes increased oxidative stress *in vivo* is unresolved. Voutilainen et al study showed that there is a significant correlation between tHcy and F_2-isoprostane in hyperhomocysteinemic men (15). In our study seminal plasma levels of total homocysteine did not show a significant difference between normozoospermic and asthenozoospermic males. Our study did not show any correlation between seminal plasma tHcy levels and lipid peroxidation. This finding may be result from that seminal plasma mean tHcy level showed no significant difference between the two groups in our study.

In summary, seminal plasma15-F2α-isoprostane and tHcy levels between normozoospermic and asthenozoospermic men in current study were not significant, and no relationship was found between tHcy and lipid peroxidation. Our study also showed that sperm motility correlated significantly with seminal plasma levels of 15-F_{2t}-isoprostane and MDA.

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