Evaluation of Lipid Peroxidation as an Indirect Measure of Oxidative Stress in Seminal Plasma

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Background: 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 $15\text{-}F_{2t}$-isoprostane ($8\text{-iso-}PGF_{2\alpha}$), malondialdehyde (MDA), and total (sum of free and bound) homocysteine (tHcy) in normozoospermic vs. asthenozoospermic men, and to examine the relationships between tHcy and lipid peroxidation products.

Materials and Methods: The study was a case-control study with a simple random sampling. The case group consisted of 15 asthenozoospermic males. This group was compared with 15 normozoospermic men. Seminal plasma levels of $15\text{-}F_{2t}$-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.

Results: MDA levels were lower 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 $15\text{-}F_{2t}$-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 slightly higher in asthenozoospermic subjects than in controls (6.18 ± 1.17 μM vs. 4.8 ± 0.52μM). Sperm motility was inversely correlated with seminal plasma $15\text{-}F_{2t}$-isoprostane and MDA levels, respectively ($p<0.05$).

Conclusion: Seminal plasma levels of $15\text{-}F_{2t}$-isoprostane and tHcy showed no significant differences between normozoospermic and asthenozoospermic men. Sperm motility correlated inversely with seminal plasma levels of $15\text{-}F_{2t}$-isoprostane and MDA. No relationship was found between tHcy and lipid peroxidation. However, higher sample size is required to confirm these findings.

***Key Words:** Seminal Plasma, $15\text{-}F_{2t}$-Isoprostane, Malondialdehyde, Homocysteine, Lipid Peroxidation, Asthenozoospermia, Normozoospermia*

Introduction

One in six couples of reproductive age presents with infertility (Hull *et al.*, 1995). Poor sperm motility is the major underlying cause of male infertility, the etiology of which is not completely elucidated. One of factors that potentially can cause asthenozoospermia is oxidative stress (Gagnon and de Lamirande 1999). Oxidative stress arises as a consequence of excessive production of Reactive oxygen species (ROS) and impaired antioxidant defense mechanisms.

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ROS play a dual role in male infertility (Sanocka and Kurpisz, 2004). On one hand, ROS plays 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 (Ichikawa *et al.*, 1999; Pasqualotto *et al.*, 2000; Agarwal *et al.*, 2003). Spermatozoa contain large quantities of polyunsaturated fatty acids (PUFA); therefore, they are susceptible to ROS-induced damage (Sanocka and Kurpisz 2004). It has been suggested that ROS induce membrane lipid peroxidation in sperm. The toxicity of generated fatty acid peroxides are important causes of sperm malfunction (Fraczek *et al.*, 2001; Agarwal and Saleh 2002). The most widely used assay for lipid peroxidation involves the

Table I. Sperm quality parameters in normozoospermic and asthenozoospermic males.

Variables	Normozoospermic (n=15)	Asthenozoospermic (n=15)	p
Concentration (10^6/ml)	99.87±8.59	78.47±7.54	>0.05
% Progressively Motile	52.00±1.07	34.67±2.46	<0.05
% Rapid Motile	15.67±1.45	3.67±1.14	<0.05
% Normal Morphology	42.66±2.06	33.33±1.26	<0.05

Variables are reported as mean±SEM.

Table II. Seminal plasma malondialdehyde (MDA), 15-F_{2t}-isoprostane, and total homocysteine (tHcy) levels between normozoospermic and asthenozoospermic males.

Variables	Normozoospermic (n=15)	Asthenozoospermic (n=15)	p
MDA (μM)	0.40±0.06	0.72±0.06	<0.05
15-F_{2t}-isoprostane (pg/ml)	58.17±4.12	65.00±3.20	>0.05
tHcy (μM)	4.80±0.52	6.18±1.17	>0.05

Variables are reported as mean±SEM.

measurement of MDA-TBA adducts due to its simplicity (Gomez *et al*., 1998; Antoine *et al*., 2002).

Recently, it has been shown that 15-F_{2t}-isoprostane (8-iso-PGF$_{2α}$) is a specific, chemically stable, and quantitative marker of oxidative stress in vivo (Lawson *et al*., 1999; Roberts and Morrow 2000; Roberts and Morrow 2002). 15-F_{2t}-isoprostane is a prostaglandin isomer synthesised in vivo, independently of the activity of cyclo-oxygenase (Meagher and FitzGerald 2000; Pratico *et al*., 2001).

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 (Voutilainen *et al*., 1999). Homocysteine is metabolized to either cysteine or methionine. In any condition which homocysteine production is high or its metabolism is impaired, homocysteine accumulates in the cell and is exported to the extracellular fluids (Jacobsen 2000). 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 (Voutilainen *et al*., 1999; Jacobsen, 2000; Zappacosta *et al*., 2001; Powers *et al*., 2002; Perna *et al*., 2003).

Available data on the impact of oxidative stress on sperm motility are based on the measurement of seminal plasma and sperm MDA (Suleiman *et al*., 1996; Gomez *et al*., 1998; Fraczek *et al*., 2001; Rhemrev *et al*., 2001; Dandekar *et al*., 2002; Nakamura *et al*., 2002; Keskes-Ammar *et al*., 2003). The results of these findings are controversial. Suleiman *et al*., (1996) did not observe any correlation between seminal plasma levels of MDA and sperm motility. Study of Frazcek *et al*. (2001) showed that seminal plasma level of MDA is higher

in asthenozoospermic males than this level in normozoospermic men. Nakamura *et al*. (2002) investigation showed that there is not significant difference in seminal plasma level of MDA between asthenozoospermic and normozoospermic males. Keskes-Ammar *et al*., (2003) observed that there is an indirect correlation between seminal plasma levels of MDA and sperm motility. To the best of our knowledge no information is available on the relationship between sperm parameters and seminal plasma 15-F_{2t}-isoprostane or homocysteine levels and also the findings on the relation of seminal plasma levels of MDA in normozoospermic vs. asthenozoospermic males are controversial. Therefore, we designed a case-control study with simple random sampling to (i) compare seminal plasma MDA, 15-F_{2t}-isoprostane, and tHcy levels in normozoospermic vs. asthenozoospermic males and to examine their association with sperm motility and also to (ii) investigate the relationship between seminal plasma tHcy levels and lipid peroxidation, as measured by MDA and 15-F_{2t}-isoprostane.

Materials and Methods

Semen Samples

The 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 Tabriz Medical School after an abstinence period of 48-72 hrs, and were analyzed within 1h of collection. After allowing at least 30 min for liquefaction, semen analysis was performed to measure sperm concentration, normal sperm morphology, percentage sperm motility, and semen volume in accordance with the recommendations of the World Health Organization (WHO 1999). Samples with a leukocyte concentration >10^6/mlof ejaculate were excluded from this study. Myeloperoxidase staining was
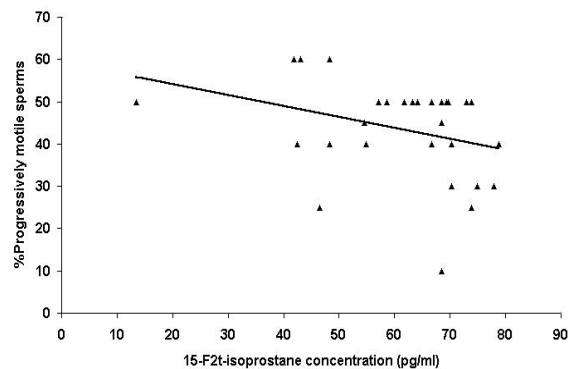
Figure 1. Correlation between percentage of progressively motile sperm and seminal plasma 15-F2t-isoprostane concentration (r= -0.41, p<0.05).
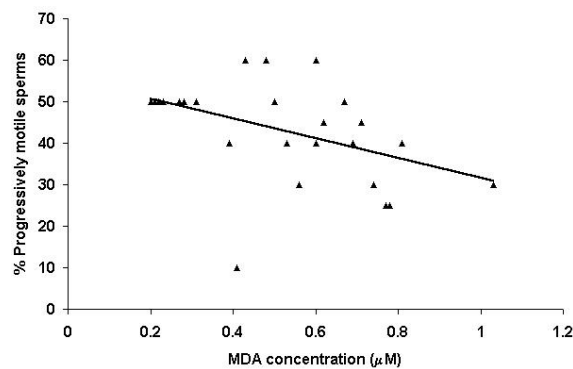
Figure 2. Correlation between percentage of progressively motile sperm and seminal plasma malondialdehyde (MDA) concentration (r= -0.53, p<0.05).

performed to evaluate the leukocyte concentration in specimen (LeucoScreen; Fertipro). After semen analysis, subjects were determined to be either normozoospermic, as the control group, (n=15) or asthenozoospermic, as the case group, (n=15). Liquefied semen samples were centrifuged at 700g for 10 minutes. The supernatant seminal plasma was then carefully removed and transferred to Eppendrof tubes. The seminal plasma was frozen at -80ºC until examination.

Assays

a) 15-F_{2t}-isoprostane. The concentrations of 5-F_{2t}-isoprostane 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-F_{2t}-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 sensitivity and specificity of the 15-F_{2t}-isoprostane assay were 5 pg/ml and 100%, respectively. (Cayman Chemical, Ann Arbor, MI, USA).

b) Ttotal homocysteine (tHcy). 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 sensitivity of the tHcy assay was 2.0 μM.

c) Malodialdehyde (MDA). The amount of MDA was determined by the TBA assay (Dandekar *et al*., 2002). All reagents that were used in this assay were obtained from Merck (Darmstadt Germany). Briefly, 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

A total of 30 samples were tested. Of these, 15 were normozoospermic and 15 were asthenozoospermic. The profile of sperm quality parameters of these samples is shown in Table I. The means percentages of progressively motile sperms, rapid motile sperms (WHO 1999), and normal sperm morphology showed a significant difference and
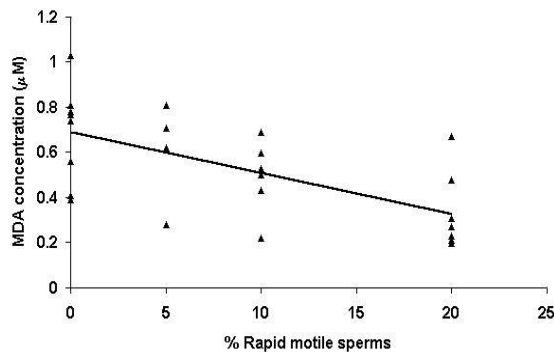
Figure 3. Correlation between percentage of rapid motile sperms and seminal plasma malodialdehyde (MDA) concentration (r = -0.58, p<0.05).

tHcy levels did not show a significant difference between two groups. Seminal plasma MDA, $15\text{-}F_{2t}$-isoprostane, and tHcy levels of the samples are shown in Table II. The means of $15\text{-}F_{2t}$- isoprostane between the two groups (p>0.05). While, the mean values for MDA between the two groups were significantly different (p<0.05).

Seminal plasma $15\text{-}F_{2t}$-isoprostane levels showed an inverse significant correlation with progressively motile sperms (r= -0.41, p<0.05) (Fig. 1) and also with normal sperm morphology (r= -0.42, p<0.05). The correlation between seminal plasma $15\text{-}F_{2t}$-isoprostane levels and rapid motile sperm was significant only in asthenozoospermic group (r=-0.60, p<0.05). Seminal plasma levels of malondialdehyde showed an indirect correlation with progressively motile sperms (r= -0.53, p<0.05) (Fig. 2), but this correlation was not observed in each group. We also observed an indirect correlation between the malondialdehyde levels and the percentages of rapid motile sperms (r= -0.58, p<0.05) (Fig. 3). However, this relationship was not found with $15\text{-}F_{2t}$-isoprostane and tHcy levels. No relationship was found between malondialdehyde and normal sperm morphology.

Finally, the correlation between tHys and lipid peroxidation, as measured by MDA and $15\text{-}F_{2t}$-isoprostane, was examined. Seminal plasma levels of tHcy showed no correlation with lipid peroxidation.

Discussion

The most relevant finding from this study was a significant inverse correlation between seminal plasma levels of $15\text{-}F_{2t}$-isoprostane and sperm motility. We observed that seminal plasma levels of $15\text{-}F_{2t}$-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.

Suleiman SA *et al.*, (1996) study showed that there is no relationship between seminal plasma MDA concentration and sperm motility. Investigation of Keskes-Ammar *et al.*, (2003) showed that sperm motility inversely correlates with semen MDA levels. This correlation was also observed in our study. Frraczek M *et al.*, (2001) observed that seminal plasma level of MDA is higher in asthenozoospermic males than in normozoospermic. This finding was also observed in our study. Nakamura *et al.*, (2002) investigation showed that there is not a significant difference in seminal plasma MDA levels between normozoospermic and asthenozoospermic men. But in our study this difference was significant.

We also measured seminal plasma level of $15\text{-}F_{2t}$-isoprostane. Recent studies have focused on $15\text{-}F_{2t}$-isoprostane, as an index of lipid peroxidation. Quantification of $15\text{-}F_{2t}$-isoprostane has been suggested to be a reliable measure of oxidative injury in vivo. Measurement of $15\text{-}F_{2t}$-isoprostane may provide a reliable marker of lipid peroxidation in vivo, because, it is a stable compound. In addition, $15\text{-}F_{2t}$-isoprostane is specific product of free radical-induced lipid peroxidation. $15\text{-}F_{2t}$-isoprostane has also 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 the definition of a normal range such that small increases in its formation can be detected in situations of mild oxidant stress. Finally, the levels of $15\text{-}F_{2t}$-isoprostane is unaffected by lipid content of the diet (Pratico *et al.*, 2001; Roberts & Morrow, 2002). Our study did not show a significant difference in seminal plasma levels of $15\text{-}F_{2t}$-isoprostane between the two groups. $15\text{-}F_{2t}$-isoprostane, like MDA, showed an inverse significant correlation with percentages of progressively motile sperm. It also correlated with percentages of normal sperm morphology. But, MDA showed no correlation with percentages of normal sperm morphology.

We also measured total homocysteine. Homocysteine is receiving a lot of attention these days as a new risk factor for a variety of abnormality. One mechanism by which increased homocysteine has been proposed to influence its pathological effects is by promoting increased 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 (Jacobsen 2000; Powers *et al.*, 2002; Perna *et al.*, 2003). Voutilainen *et al.*, (1999) study showed that there is a significant correlation between tHcy and F2-isoprostane in hyperhomocysteinemic men. Our study did not show any correlation between seminal plasma tHcy levels and lipid peroxidation. This finding is related to our results that seminal plasma mean tHcy level showed no significant difference between the two groups in our study. Studies that found this correlation were carried out in hyperhomocysteinemic subjects.

In summary, seminal plasma $15\text{-}F_{2t}$-isoprostane and tHcy levels between norozoospermic 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. We also concluded that homocysteine metabolism may not impair in asthenozoospermic males.

A significant limitation of our study was a small sample size. Higher sample size is required to investigate the metabolism of 15-F_{2t}-isoprostane and tHcy in asthenizoospermic males and their relationship with sperm motility.

Acknowledgements

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