

ASSESSMENT OF THE IMPACT OF OXIDATIVE STRESS ON FROZEN SEMINAL PLASMA IN FERTILE AND INFERTILE MEN BY EXAMINING THE TOTAL ANTIOXIDANT CAPACITY

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Summary

The aim of the study was to explore the impact of oxidative stress on frozen seminal plasma in fertile and infertile men by examining the total antioxidant capacity. Patients: Infertile patients from male infertility clinic with various diagnoses and fertile men. Design: Seminal plasma from proven fertile men [n=50] and infertile patients [n=50] were examined for total antioxidant capacity (TAC) level, semen parameters such as morphology, motility and concentration, and DNA integrity test. Interventions: Seminal plasma TAC measurement by luminometric assay using the TAC assay kit, semen analysis parameters, DNA integrity test. Fertile men showed higher TAC values (median and SD): 1201 μ M (SD \pm 548), as compared with the infertile patients: 831 μ M (SD \pm 343). The result from sperm morphology of fertile patients showed a mean percentage of 4.8% (SD \pm 1.68) whereas the percentage in the infertile group was 2.68% (SD \pm 1.68). The same group of samples, analyzed for DNA damage showed a mean of DFI 10.38% (SD \pm 5.17%) in fertile men and a mean of DFI 17.22% (SD \pm 7.22%) in infertile men. Total antioxidant capacity of the seminal plasma as measured by the luminometric assay is a reliable and simple test for diagnosing and management of male infertility.

Key words: seminal plasma, male infertility, oxidative stress, total antioxidant capacity

Introduction

Infertility is a term referring to a failure of a couple to achieve a pregnancy within one year of regular unprotected intercourse. Subfertility is registered in 10-15% of couples of reproductive age. In 30-50% of these couples, infertility is caused by male reproductive dysfunction, and disturbances are found in 26% of infertile couples.

Causes of male infertility are identified at various levels. The testes themselves may be affected; infertility may be due to impairments of excurrent seminal ducts or in the accessory sex glands, or there may be a disturbance of semen deposition. Impairment of central structures such as the hypothalamus and the pituitary or the androgen target organs may also result in subfertility [1].

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Causes for male infertility include endocrine, urological, infectious genetic and other common problems with a complex etiology, and require a complete andrological diagnosis. The male reproductive tract is controlled by a well balanced hormonal system, with participation of hypothalamic (GnRH), pituitary (LH, FSH) and testicular hormones (androgens, inhibin B).

Semen analysis includes sperm concentration, sperm motility, sperm morphology, sperm cell vitality, pH and leucocyte count. The benefits from determining these parameters as predictors of fertility appear to be rather limited. Alternative tests such as sperm penetration, capacitation, acrosome reaction are available but they have not been approved as standard procedures. Previous studies, including our own studies, have demonstrated that the assessment of DNA integrity by flowcytometry (DNA integrity test [SDI]), has better predictive value for pregnancy outcome by natural or assisted reproduction.

The functional capacity of sperm cells decreases in patients with testicular pathology, endocrinological and genetic diseases, etc. Generation of free radicals that can not be neutralized by the antioxidant protection of cells is assumed to be a major factor [2-7].

Seminal plasma contains the entire enzyme complex, providing antioxidant protection of sperm cells [8]. This protection compensates for the loss of cytoplasmic sperm enzymes during maturation and transportation processes [5, 6, 9-11]. The total antioxidant capacity of seminal plasma is provided by the enzymatic (e.g. superoxide dismutase, catalase, and glutathione peroxidase) and nonenzymatic systems (e.g. ascorbate, urate, vitamin E)

When TAC levels are low, oxidative stress causes damage to the spermatozoa, and this correlates with reduction in function in fertile men. It is believed that free radicals cause damage to sperm DNA. Several reports have related low seminal plasma TAC levels to male infertility in general, as well as to failure in intrauterine insemination and in vitro fertilization (IVF) [6, 13-17].

Materials and Methods

Subject Selection

This study was approved by the Ethics Committee of Medical University – Pleven. Infertile patients (n= 50) as well as fertile healthy

men (n=50) were screened and selected on the basis of normal semen analysis according to the World Health Organization-2010 [18] guidelines. The mean age for the fertile group was 36.81 years (SD±4.59) and 35.9 years (SD±5.75) for the infertile group.

Semen Collection and Preparation

Semen sample were collected by masturbation after 3 to 7 days of sexual abstinence. After complete liquefaction at 36.6 C⁰ for 30 minutes, and 5 µL of each sample was loaded on an improved Neubauer counting chamber (Marienfeld superior, Germany) where conventional sperm analysis of concentration was carried out. Morphology was assessed, according to strict Kruger criteria, and motility was measured. All results were classified according the WHO, 2010 [18]. The infertile patient group (n=50) included men with a history of infertility of over one year of unprotected sexual intercourse. The fertile patients (n=50) had achieved pregnancy less than one year prior to the sperm analysis. All samples were centrifuged at 1000 g for 10 minutes. The seminal plasma was aliquoted and frozen in liquid nitrogen until the time of TAC assay. Before centrifugation, around 500µL were frozen directly in liquid nitrogen for further assessment of sperm DNA integrity.

The DNA integrity test

Sperm DNA damage was evaluated by flow cytometry after staining with acridine orange. On the day of analysis, the samples were defrosted and tested immediately. All buffers and samples were kept at 4 to 6 C⁰ and the test was run in a box with ice. After washing with TNE (0.15M NaCl, 0.01M TrisHCL, 1mM EDTA, pH 7.4) buffer, the sperm concentration was adjusted to approximately 1-2x10⁶ cells/ml and 200 µL of the sample was placed in 12x75 flowcytometer tubes. After that 400 µl of low-pH (pH 1.2) detergent solution containing 0.1% Triton X-100, 0.15M NaCl and 0.08M HCl was added for 30 sec., immediately followed by 1.2 ml staining buffer (phosphate-citrate buffer, pH 6.0) with 6 mg/l acridine orange (AO;chromatographically purified; cat. No.318337-1G, Sigma-Aldrich, USA). Cells were acquired were analysed using a FACScan flowcytometer (Becton Dickinson). A total of 5000 events were accumulated for each measurement at a low flow rate. A 488 nm light source was used. The study of the sperm

chromatin is based on the properties of metachromatic acridin orange (AO) staining. Acridin orange emits green light upon binding to double-stranded DNA (native) and red light when binding to a single-stranded DNA.

Data was analyzed by FlowJo software (TreeStar, Inc, Ashland, OR). Computer gates are used to determine the proportion of spermatozoa with increased levels of red fluorescence and green fluorescence. The results were presented as a percentage of sperm DNA fragmentation index (DFI).

The total antioxidant capacity assay of human seminal plasma

Semen samples were collected and all samples were centrifuged at 1000 g. Seminal plasma was stored in liquid nitrogen for analysis. Seminal plasma total antioxidant luminometric measurement was done using the luminescent NWLSSTM Total Antioxidant Capacity kit (TAC-Peroxy Assay)—AMS Biotechnology, UK. TAC kit is an artificial system where biologically relevant peroxy free radicals are generated by thermal decomposition of 2,2'-azobis(2 amidinopropane; ABAP) [2, 3]. These peroxy radicals react with an indicator molecule, luminol [LH₂], to generate a luminol radical (LH) that results in emission of blue light centered at ~425 nm. When nonenzymatic antioxidants are present, luminescence is inhibited until the antioxidants are exhausted. The time of inhibition or the induction time to light production is directly proportional to the total concentration of antioxidants. The antioxidant concentration is determined by comparing induction time to that of a water-soluble Vitamin E (tocopherol) analog Trolox.

Duplicate samples were run in 96-white microplates well provided with the test kit for 250 cycles, 17 secs each. Antioxidant capacity of original sample was determined using the following formula: (S original)=(S assay) * (Dilution Factor). The dilution factor for semen plasma was 40x. (Fig. 1) The results were analyzed with MARS data analysis software.

Statistical analysis

Statistical analysis was performed using Statgraphics plus 2.1 software for Windows. The data were presented as mean ± standard deviation (mean ±SD). Statistical significance was set at p ≤ 0.05.

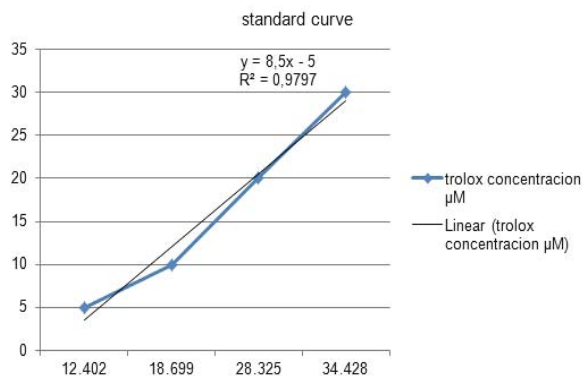


Figure 1. Standard curve for TAC measurement. Standard Trolox concentrations are represented on the y-axis and the absorbance at the x-axis

Results

Fertile patients were men, who had achieved pregnancy in a couple less than one year before analysis or the female partner was pregnant at the moment of analysis up to 12 w.g. (Table 1). The infertile patients were men of a sexually active nonconceiving couple that had not achieved pregnancy within one year (Table 2).

The results from sperm morphology of fertile patients showed a mean percentage of 4.8% (SD±2.0), while this percentage was 2.8% (SD±1.68) in the infertile group (p=0.001). The same group of samples, analyzed for DNA damage showed a mean of DFI 10.38% (SD±5.17%) for the fertile group, and a mean f DFI 17.22% (SD±7.22%) for the infertile one. The TAC level found for the fertile group was 1201.1 µM (SD±548) and in the infertile group the level was 831 µM (SD±343).

The study did not establish a statistically significant correlation between the percentage of sperm motility and sperm concentration in both two groups.

The percentage of sperm morphology (p=0.001) and TAC (p=0.0048) in the group of fertile patients were significantly higher than those in the infertile patients.

The same group of samples, analyzed for DNA damage showed a mean of DFI 10.38% (SD±5.17%) for the fertile men and a mean of DFI 17.22% (SD±7.22%) for the infertile men, i.e. there was a significant correlation (p=0.009; Fig. 2).

The TAC for the fertile group was 1201.1 µM (SD±548) and in the infertile group was 831 µM (SD±343) (p=0.004; Fig. 3).

Table 1. Summary statistics of basic semen parameters, DNA integrity test and TAC of fertile patients

Fertile parameter	N _o	Mean ± SD	Range
Age [y]	50	36.81 ± 4.59	30.00 - 46.00
Concentration [x 10 ⁶ /ml]	50	62.10 ⁶ ± 3.52	6.20 - 120.00
Motility [a+b] %	50	39.9 ± 5.35	32.00 - 54.00
Morphology %	50	4.8 ± 2.0	2.00 - 8.00
DFI %	50	10.38 ± 5.17	4.00 - 19.30
TAC [μ/m]	50	1201.1 ± 548	471.00 - 1196.00

Table 2. Summary statistics of basic semen parameters, DNA integrity test and TAC of infertile patients

Infertile parameter	N _o	mean ± SD	Range
Age [y]	50	35.90 ± 5,75	26.00 - 45.00
Concentration [x 10 ⁶ /ml]	50	50.60 ± 4.50	6.90 - 120.00
Motility [a+b] %	50	35.00 ± 12.20	01.00 - 53.00
Morphology %	50	2.8 ± 1.60	1.00 - 6.00
DFI %	50	17.22 ± 7.20	5.00 - 28.00
TAC [μ/m]	50	831 ± 343	250.00 - 1136.00

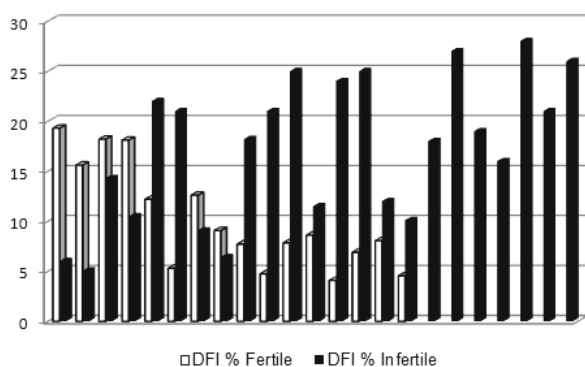


Figure 2. The sperm DNA integrity test values [DFI] in the infertile patient group versus the fertile group

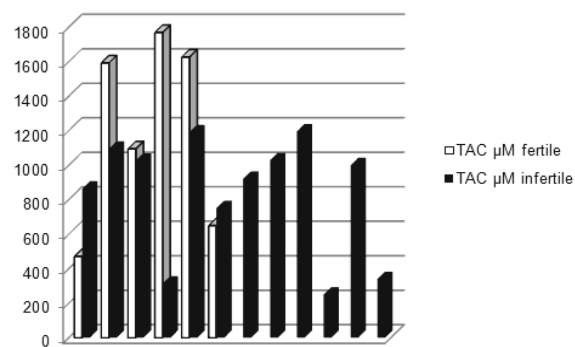


Figure 3. The seminal plasma TAC values in the infertile patient group versus the fertile patients group

Discussion

The pathogenetic relationship between ROS and the male infertility has been established [19-21]. Total antioxidant capacity of semen plasma provides protection from generating ROS. Therefore, when seminal total antioxidant

capacity decreases, male fertility also decreases. Other factors such as nutrition, vitamin supply, age, and infection have been reported to influence antioxidant capacity may be influenced by the factors such as [20, 17]. These factors were not considered in the process of patient selection in our study.

In our study, TAC levels established for the infertile patient group showed lower values, as compared to those in the fertile men group. (Fig. 2, Fig. 3)]. The males from the fertile group showed normal sperm morphology and lower DFI levels, while the infertile group showed significantly lower levels of TAC and high levels of DNA fragmentation. We also established a cutoff value of DFI (16%) in the fertile group. That is why we believe that DFI value should also be considered in patients with normal sperm parameters and failure to achieve natural pregnancy.

There are three main mechanisms related to damage of sperm chromatin: oxidative stress, defective chromatin packaging, and apoptosis [22]. Immature sperm, by producing high levels of ROS, can induce DNA damage in mature spermatozoa during their comigration from the seminiferous tubules to the cauda epididymis. This process activates sperm caspases and endonucleases by ROS [21, 23-26]. High levels of ROS decrease the total antioxidant capacity, thus causing seminal oxidative stress.

Some authors have reported high levels of ROS in semen samples of 25-40% of infertile men studied. High levels of ROS in seminal plasma is involved in altering plasma membrane through lipid peroxidation. Sperm cells are highly sensitive to damage induced by ROS because of the high content of polyunsaturated fatty acids in their plasma membranes and low concentration of ROS-neutralizing enzymes within the cytoplasm [19, 20]. On the other hand, DNA damage was found to result in impaired sperm morphology, lower motility, which eventually led to male infertility [18, 19].

Human semen contains many types of cells, such as mature and immature spermatozoa, round cells, leukocytes and epithelial cells. Leukocytes and immature spermatozoa are the main producers of ROS [20, 21]. Polymorphonuclear cells induce ROS in response to various stimuli such as chemicals and bacteria, and this process reduces the ability of reparative systems to compensate for the DNA damage. A pathological high ROS level is a concept that needs to be more accurately determined. In future studies, it would be useful to establish reference values for TAC, taking into consideration age and presence of factors such as infection, varicocele etc.

Conclusion

Measuring total antioxidant capacity of the seminal plasma by a luminometric assay is a reliable test in diagnosing and management of male infertility. This method, however, is hardly applicable in routine practice. Total antioxidant capacity provides more relevant information than that obtained by measuring the individual components of the antioxidant capacity because it considers the cumulative effect of all components of in the semen. Measurement of TAC can more accurately determine fertility and infertility. In addition, such a test can help select sub-groups of patients with infertility, who are found with high levels of oxidative stress and who might benefit from antioxidant supplementation.

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