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Original article

Nitric oxide, malondialdheyde and non-enzymatic antioxidants assessed in viable spermatozoa from selected infertile men

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ABSTRACT

There are growing evidences that the semen of infertile male population shows higher reactive oxygen species (ROS) levels concomitant with lower antioxidant capacity compared to those detected in semen of fertile population. The plasma membrane of the sperm cell, which has high levels of polyunsaturated fatty acids, renders it particularly sensitive to ROS. The aim of this study was to compare the sperm parameters (concentration, motility, morphology and vitality) and the levels of malondialdehyde (MDA), as marker of lipid peroxidation (LPO), nitric oxide (NO), ascorbic acid (AA), total (GSHt) and oxidized glutathione (GSSG) in viable sperm in a group of 38 infertile patients and in a group of 55 control subjects with unknown reproductive potential. The comparison between variables in infertile patients and controls revealed that the sperm quality was reduced in the infertile group, whereas the levels of NO, AA and GSH were significantly increased in viable spermatozoa from infertile men; however, the endogenous levels of MDA were similar in infertile and control groups. Based on our results, we could speculate that the rise of GSHt and AA levels in viable sperm of infertile group help partially to counteract the damaging effect of ROS and partly prevent a substantial LPO. The observation of the concomitant increase of NO and antioxidant indices in viable spermatozoa of infertile subjects is a novel finding and we think that these results can be useful since the viable sperm population is conceivably used in assisted reproductive technology.

1. Introduction

The study of the unbalance between the production of reactive oxygen and nitrogen species (ROS and RNS) and the ability of the antioxidant defenses to scavenge them is becoming a cornerstone in the field of male infertility [1,2].

ROS play a central role in the physiological processes of sperm cells such as maturation, capacitation, acrosome reaction and fertilization [3].

Under pathological conditions, the production of ROS often increases, resulting in oxidative stress that can exert negative effects against spermatozoa and male reproductive system [2,4,5]. The sperm membranes, rich in polyunsaturated fatty acid, are particularly exposed to oxidative stress that causes lipid peroxidation (LPO) and consequently leads to a loss of plasma membrane integrity. In addition to this phenomenon, the excess of ROS induces chromatin damages which, in turn, leads to DNA fragmentation, all conditions contributing to sperm alteration and infertility [6,7]. One of used indices, able to monitor LPO is represented by malondialdehyde (MDA) which probably indicates a widespread diagnostic criterion for the oxidative damage to spermatozoa even though other markers were recently proposed [8]. MDA levels assessed both in seminal plasma and directly in spermatozoa are negatively correlated with sperm parameters [9–11]. Recently, our group suggested that seminal plasma MDA may represent a marker for understanding pathologies causing sperm motility decrease with particular regard to genitourinary infections and varicocele [12].

Nitric oxide (NO) represents a cells signaling molecule supporting a wide array of biological processes in cardiovascular system, immunity, reproductive system and many others. NO is also a potent free radical and one of the component of RNS. It is synthesized from L-arginine oxidation by NO synthases family (NOS) in almost all mammalian cells [5].

The wide presence of three isoforms of NOS in the testis and in spermatozoa is indicative of the important role of NO in

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spermatogenetic process [5,13]. NO shows both positive and negative effects. At physiological concentrations it plays a crucial role in sperm hyperactivation, capacitation and zona pellucida binding, as well as sperm motility, viability and morphology [4]. On the contrary, overproduction of NO plays a negative effect on sperm parameters and reduces the sperm motility [4]. In fact, increased NO levels were observed in the semen of infertile asthenozoospermic men [14].

NO, synthesized by human spermatozoa, was observed to support sperm motility [13] and to induce acrosomal reaction in the presence of follicular fluid [15]. Saeednia et al. [16] reported that endogenous NO was increased in frozen-thawed sperm of asthenozoospermic men, compared with that of basal sperm, indicating that endogenous NO concentration was risen in case of cryopreservation, procedure known to produce ROS and partially damage sperm.

To quench the injuries caused by oxidative stress, the cells are equipped with intracellular enzymatic and non-enzymatic antioxidants: some enzymatic antioxidants include superoxide dismutase, glutathione peroxidase and glutathione reductase, while non-enzymatic antioxidants include ascorbic acid (AA), reduced glutathione (GSH) and oxidized glutathione (GSSG), vitamins E and B, carotenoids, taurine, carnitines and many others [2].

AA is a water-soluble ROS scavenger with high potency that protects human spermatozoa against endogenous oxidative DNA damage [17]. Increased ROS in the seminal plasma of infertile men may decrease the effective concentration of AA, increasing the harmful effects of ROS to sperm cells that are associated with abnormal sperm parameters [18]. Several studies supported the hypothesis that AA treatment had a positive effect on sperm quality and supplementation of biologic AA was an effective method for the treatment of infertile males with poor sperm quality [19].

Glutathione (L-c-glutamyl-L-cysteinylglycine) is a tripeptide ubiquitously distributed in living cells. It plays an important role in the intracellular defence mechanism against oxidative stress. It directly reacts with free radicals and catalyses the reduction of toxic H_2O_2 in to water and hydro peroxides [20]. Different studies have also shown that supplementation of GSH in the extenders prior to freezing minimized the oxidative damage caused due to free radical generation [21,22].

Seminal plasma represents the major source of antioxidants that undoubtedly protects the sperm cells from oxidative attack [1,23]. The vast majority of studies measured these compounds in the liquid part of the semen [2,24]. At this purpose, our group have recently demonstrated that different pathologies such as varicocele and genitourinary infections are characterized by the presence in seminal plasma of specific markers of oxidative stress, including MDA and enzymatic and non-enzymatic antioxidants [25].

Although sperm cells need the help of a consistent antioxidant buffering ability of seminal plasma to counteract ROS, the spermatozoa themselves contain a small amount of antioxidants. In fact, unlike other types of cells, they have a small amount of cytoplasm that contains intracellular enzymatic and non-enzymatic antioxidants [23,26].

The aim of this study was to determine the concentrations of NO, MDA and the levels of AA, total GSH (GSHt) and GSSG in viable sperm cells. These indices of oxidative stress measured in cells and seminal parameters were compared between a group of infertile patients and a group of control subjects with unknown reproductive potential.

2. Materials and methods

2.1. Patients

From June 2015 through October 2016, we analysed 117 consecutive semen samples from subjects (aged 24–47 years) attending our Centre for semen analysis.

We enrolled 93 cases following the inclusion criteria: non azoospermic men with a normal karyotype evaluated by conventional cytogenetic analysis, BMI $< 25\ kg/m^2$ and no history of diabetes, radiotherapy, chemotherapy, chronic illness or medication, neither of the use of drugs, alcohol and dietary supplements. The patients declared that they were not professionally exposed to pesticides or heavy metal. Patients with leukocytospermia [27] were excluded. The subjects showed normal concentrations of follicle stimulating hormone (FSH), luteinizing hormone (LH) and testosterone (T) evaluated in serum by chemiluminescence using commercial kits (Beckman Coulter Access for FSH, LH and T, Beckman Coulter S.p.A., Milano, Italy).

The studied cases included subjects with mild smoking habits (< 10 cigarette/day), the presence of varicocele and genitourinary infections.

These subjects were grouped as followed:

- infertile patients (group 1, n = 38), individuals that did not obtain pregnancy after two years of unprotected sexual intercourse; the female factors were excluded.
- men that did not seek pregnancy, they attended our centre to control their semen parameters (group 2, n = 55).

In the group 1, 8 patients were mild smokers (21%), 8 had a varicocele (21%, six patients had a left- side grade 2 varicocele, a patient a left-side grade 3 varicocele and the last one had a bilateral grade 1 varicocele) and 2 had genitourinary infections (5%, both patients had a semen infection by *Enterococcus faecalis*).

In the group 2, 12 men had smoking habit (22%), 7 had varicocele (13%, five patients had a left- side grade 2 varicocele, a patient a left-side grade 3 varicocele and the last one had a right-side grade 2 varicocele), 3 had genitourinary infections (5%, a patient had a semen infection by *Enterococcus faecalis*, another by *Escherichia coli* and the last one by *Streptococcus agalactiae*).

All patients and subjects were informed with respect to this study and they provided an informed written consent before the inclusion on this research. The study was approved by the Ethics Committee of Azienda Ospedaliera Universitaria Senese, CEAOUS.

2.2. Semen analysis

Subjects enrolled in this study were asked to abstain from intercourse and masturbation for a period of 3–4 days before the semen collection. Samples were analysed after liquefaction for 30 min at 37 °C and volume, pH, sperm concentration, motility and morphology were assessed according to WHO guidelines 2010 [27]. The possible presence of leukocytes was explored by peroxidase stain; a concentration $> 1 \times 10^6$ cell/ml was considered out of range and identified as leukocytospermia.

Sperm vitality was determined by staining 10 μL of semen sample with 10 μL of 0.5% eosin Y (CI 45380) in 0.9% aqueous sodium chloride solution [27]. A few minutes after staining, the samples were observed by light microscope and stained (dead) cells and unstained (living) cells were scored.

After analysis the samples were centrifuged at 2000 rpm for 15 min, the seminal plasma was removed and the sperm cells stored at −80 °C until use.

2.3. Determination of NO, MDA and antioxidant indices

The indices such as NO, MDA, GSHt, GSSG and AA were calculated in the viable population of spermatozoa. The indices were evaluated directly in the spermatozoa and the final results were calculated considering the percentage of viable sperm assessed by eosin Y test. This procedure enabled us to exclude from the calculation dead sperm with broken plasma membrane that had release their content, including the evaluated indices, in the seminal plasma.

2.3.1. Lysis procedure

Sperm cells were suspended in 1 mL of phosphate buffer saline (PBS). The sperm specimens were lysed through the rapid freeze-