Glutathione content in sperm cells of infertile men

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Introduction

It is well known that the processes of lipid peroxidation are universal mechanisms of cell damage. However, the physiological levels of lipid peroxidation are necessary for normal functioning of almost all cells. Cell protection from the damaging effects of free radicals and lipid peroxidation products is generally determined by the degree of antioxidant protection. Under physiological norm a balance between the rate (intensity) of free radical processes and the activity of antioxidant systems is maintained.

Reduced glutathione (GSH) and associated enzymes that form the glutathione antioxidant system are of particular importance in maintaining cell viability in antradic and antiperoxide protection. The glutathione antioxidant system plays a leading role in antioxidant protection under pathological processes. Depletion of the glutathione system leads to cytotoxic and destructive lesions (Kalina et al., 2014). Reduced glutathione exists in cells in equilibrium with oxidized glutathione (GSSH) and the GSH/GSSH ratio characterizes the redox state of the system in different physiological states (Kurilova et al., 2008). GSH is present in the cell at high concentrations and is a major low molecular weight thiol under physiological conditions. It is known that GSH is the main component that quickly mobilizes at higher peroxide concentrations and restores them in the reaction leading to the formation of cytotoxic GSSG (Koval et al., 2008). The GSH content in cell depends on the balance of opposing processes such as de novo synthesis by gamma-glutamylcysteine synthetase, regeneration of GSSG by glutathionreductase and usage in neutralizing of H2O2 and free radicals (Lewis et al., 1997; Ochsendorf et al., 1998; Garrido et al., 2004; Ebisch et al., 2006; Aydemir et al., 2007).

Keywords: glutathione; glutathione peroxidase; spermatozoa; pathospermia; male infertility

Hyperproduction of reactive oxygen species can damage sperm cells and is considered to be one of the mechanisms of male infertility. Cell protection from the damaging effects of free radicals and lipid peroxidation products is generally determined by the degree of antioxidant protection. Glutathione is non-enzymatic antioxidant which plays an important protective role against oxidative damages and lipid peroxidation. The aim of the present work is to determine the content of reduced and oxidized glutathione in sperm cells of infertile men. Semen samples from 20 fertile men (normozoospermics) and 72 infertile patients (12 oligozoospermics, 17 asthenozoospermics, 10 oligoasthenozoospermics and 33 leucocytospermics) were used. The total, oxidized (GSSG) and reduced (GSH) glutathione levels were measured spectrophotometrically. The levels of total glutathione were significantly lower in the spermatozoa of patients with oligozoospermia, asthenozoospermia and oligoasthenozoospermia than in the control. Infertile groups showed significantly decreased values of reduced glutathione in sperm vs. fertile men, indicating an alteration of oxidative status. The oxidized glutathione levels in sperm cells of infertile men did not differ from those of normozoospermic men with proven fertility. The GSH/GSSG ratio was significantly decreased in the oligo-, astheno- and oligoasthenozoospermic groups compared to the normozoospermic group. In patients with leucocytospermia the GSH/GSSG ratio was lowered, but these changes were not significant. In addition, the glutathione peroxidase activity in sperm cells was decreased in patients with oligozoospermia, asthenozoospermia, oligoasthenozoospermia and with leukocytespermia. The most significant changes in glutathione peroxidase activity were observed in infertile men with leukocytespermia. Decreased GSH/GSSG ratio indicates a decline in redox-potential of the glutathione system in sperm cells of men with decreased fertilizing potential. Redistribution between oxidized and reduced forms of glutathione can be caused by depletion of intracellular stores of glutathione and intensification of lipid peroxidation processes. This leads to increased production of reactive oxygen species, further depletion of antioxidant pools and disturbances of structure and function of spermatozoa. Our results indicate that the evaluation of reduced glutathione level and GSH/GSSG ratio in sperm cells of infertile men can be helpful in fertility assessment.

Materials and methods

Patients. This study involved 72 infertile men with different forms of pathospermia. They were recruited between 2014 and 2016.
A detailed medical history was performed for all studied cases. Exclusion criteria: subjects currently on any medication or antioxidant supplementation were not included. In addition, subjects with infertility lasting over 10 years, azoospermia, testicular varicocele, genital infection, chronic illness and serious systemic diseases, smokers and alcoholic men were excluded from the study because of their well-known high seminal reactive oxygen species (ROS) levels and decreased antioxidant activity (Aitg et al., 2012).

Ejaculates from a total of 72 infertile and 20 fertile healthy individuals were obtained. The control group consisted of 20 healthy men with somatic fertility, normozoospermia and confirmed parenthood (married for 3–10 years and had healthy 1–3 children). Semen samples were obtained by masturbation and collected into sterile containers, following 3–5 days’ abstinence from sexual activity. After liquefaction at 37 °C with 5% CO₂ in air, semen samples were examined for volume, sperm concentration, pH, morphology and motility according to the World Health Organization guidelines. Before becoming involved in the study, all the men were familiarized with patient information leaflets and gave informed consent to participate in research. Terms of sample selection meet the requirements of the principles of the Helsinki Declaration on Protection of Human Rights, Convention of Europe Council on human rights and biomedicine and the provisions of laws of Ukraine. Approval for the study was taken from the ethics committee of Danylo Halytsky Lviv National Medical University.

According to semen analysis, oligozoospermia was found in 12 patients (16.7%), asthenozoospermia was detected in 17 patients (23.6%), oligoasthenozoospermia was observed in 10 patients (13.9%). Thirty-nine (54.2%) infertile men had leukocytes content in the semen lower than 1.0·10⁶ ml⁻¹, only in 33 patients (45.8%) was leukocytospermia noted (the leukocytes content ranged from 1.0·10⁶ ml⁻¹ to 3.0·10⁶ ml⁻¹) which indicates inflammation in this group of men.

Cell preparation. Sperm cells were washed from semen plasma by 3 times centrifugation at 3,000 g for 10 min in media which contained (mM): 120 NaCl, 30 KCl, 30 Hepes (pH 7.4). The content of total protein in the samples was determined by Lowry method using a kit to determine its concentration (“Simko Ltd”). The content of total glutathione was determined in saponin-permeabilized spermatozoa after complete reduction of glutathione through the use of glutathione reductase by means of Ellman’s reagent (Anderson, 1985). The level of 5-trinitrobenzoic acid was monitored with a spectrophotometer at 412 nm. To determine the content of oxidized glutathione (GSSG), 2-vinylpyridine was added to the incubation mixture to a final concentration of 2% 60 min before the determination (Griffith, 1980). The content of reduced glutathione (GSH) was calculated as the difference of contents between total glutathione and its oxidized form. Glutathione peroxidase activity was determined by the oxidation of glutathione.

Statistical analysis. Experimental data were processed by methods of variation statistics using software MS Office. The results are presented as the mean ± standard deviation of the mean. Analysis of variance (ANOVA) was used to compare the difference in the means between infertile and healthy men. Differences were considered statistically significant at P < 0.05 for all analyses.

Results

We found that in patients with normozoospermia the GSH content in sperm cells was 36.5±4.1 pmol of GSH/10⁶ cells (Fig. 1). Patients with oligozoospermia showed 1.9-fold lower GSH content (19.2 ± 2.1 pmol of GSH/10⁶ cells) than did control subjects, whereas patients with asthenozoospermia showed 1.8-fold lower GSH content (20.8 ± 1.9 pmol of GSH/10⁶ cells) than control subjects. In patients with oligoasthenozoospermia GSH content was 21.7 ± 2.7 pmol of GSH/10⁶ cells which was 1.7-fold lower than in normozoospermic men with proven fertility. The GSH content was 1.2-fold lower (29.8 ± 2.3 pmol GSH/10⁶ cells) in leukocytospermic patients vs men with normozoospermia, but these changes were not significant.

The content of GSH in sperm cells of fertile men was 34.0 ± 4.0 pmol/10⁶ cells (Fig. 2). We found the decreased GSH level in sperm cells of infertile men with different forms of pathospermia. However, the most expressed changes in the GSH content were observed in patients with oligozoospermia.

The GSH content decreased to 16.8 ± 1.9 pmol of GSH/10⁶ cells, which was twice lower than in normozoospermic men with proven fertility. In patients with asthenozoospermia and oligoasthenozoospermia the GSH level was 17.5 ± 1.8 and 18.3 ± 2.6 pmol of GSH/10⁶ cells that was 1.9-fold lower than control group. In leukocytospermic patients the GSH level was 27.1 ± 2.2 pmol of GSH/10⁶ cells which was 1.3-fold lower than in patients with normozoospermia, but these changes were not significant.

The GSSG content in sperm cells of normozoospermic and oligozoospermic men was 2.5 ± 0.3 pmol GSSG/10⁶ cells (Fig. 3). The content of oxidized forms of glutathione in spermatozoa in infertile men of other studied groups was increased, but these changes were not statistically significant. Patients with astheno-, oligoasthenozoospermia and leukocytospermia showed 1.3-, 1.4- and 1.1-fold higher GSSH content (3.3 ± 0.4, 3.4 ± 0.5 and 2.7 ± 0.3 pmol of GSSG/10⁶ cells correspondingly) than did control subjects.

In addition to determination of GSH, GSH and GSSG, the ratio of reduced to oxidized glutathione (GSH/GSSG ratio) was calculated from these measurements (Fig. 4). It is a more sensitive indicator of pro- and antioxidant status. The GSH/GSSG ratio in patients with normozoospermia was 15.2 ± 1.9. The ratio of the reduced to oxidized form of glutathione in the spermatozoa of men with oligo-, astheno- and oligoasthenozoospermia was much lower than in men with normozoospermia (7.2 ± 0.9, 6.1 ± 0.9 and 6.4 ± 0.9).
The GSH/GSSG ratio is somewhat lower in patients with leukocytospermia (12.1 ± 0.9). The ratio of the reduced to oxidized form of glutathione indicates a decrease in total capacity of the glutathione system in sperm cells of men with decreased fertilizing potential. The glutathione peroxidase activity was significantly decreased in patients with all forms of pathospermia compared with healthy men with proven fertility (Fig. 5). The enzyme activity in patients with oligozoospermia was 44% and in patients with astenozo- and oligoastenozoospermia 35% of that in normozoospermic men. The most significant changes in glutathione peroxidase activity were observed in infertile men with leucocytospermia (26% of activity in normozoospermic men).

Discussion

Other findings about GSH level in sperm cells are controversial. Overall, our results are consistent with several other studies. In particular, it was shown that GSH content in sperm cells of patients with oligozoospermia was significantly lower than in control group (Ochsendorf et al., 1998). Reduced intracellular GSH was observed in sperm of infertile men, but these differences were not statistically
significant (Garrido et al., 2004). However, the same study found the
presence of a moderate direct correlation between the GSH content and
disturbance of sperm morphology. In addition, it was shown that
intracellular GSH content in sperm cells of in smokers was lower
compared with nonsmokers (Viloria et al., 2010).

However, conflicting data have been obtained in other studies.
Ebisch et al. (2007) revealed high GSH content in spermatozoa of
subfertile men compared to fertile men. Lewis et al. (1997) showed
that the content of sulphydryl compounds was significantly higher
in spermatozoa from men with asthenozoospermia than in men
with normozoospermia. According to the researchers the increased
synthesis of thiol compounds is a compensatory mechanism to
protect cell from oxidative damage. Aydemir et al. (2007) found no
statistically significant differences in the values of intracellular
GSH between patients with idiopathic infertility and healthy men.

Conflicting data have also been obtained about GSH content in
semen plasma of men with infertility. A large number of studies
have found no statistically significant differences between GSH
content in seminal plasma of fertile and infertile men (Aydemir
et al., 2007; Wong et al., 2001; Ebisch et al., 2006). Hesham et al.
(2008) established a significant reduction of GSH in seminal
plasma of men with azoospermia and oligozoospermia. A decrease
in GSHt and GSH content was found in seminal plasma of men
with asthenozoospermia. However, the same study showed a slight
increase in GSH level in patients with combined form of
pathospermia (oligoasthenozoospermia) with simultaneous
decrease of oxidized form (GSSG) (Atig et al., 2012). In men with
violations of spermatogenesis by oligozoospermia, the GSH content
was decreased, but these differences were not statistically
significant (Atig et al., 2012). A reduction of GSH was found in
semen plasma in men with azoospermia and oligozoospermia
(Bhardwaj et al., 2000). The GSH/GSSG ratio was lower in seminal
plasma of men with idiopathic infertility and varicocele than in
healthy men (Micheli et al., 2016). On the contrary, other studies
found an increase in GSH content in seminal plasma of men with
azoospermia (Ochsendorf et al., 1998). Also, Rajmakers et al.
(2003) showed that GSH content was higher in seminal plasma of
fertile than in subfertile men.

These differences can be explained by the controversial
inclusion and exclusion criteria for patients in the studied groups,
different analytical approaches (techniques), patients’ lifestyle, their
habitats, health, diet, origin etc.

Taking into account that GSH not only protects cells from toxic
free radicals, but in general determines oxidation-reduction
characteristics of intracellular environment, reduction in GSH
content indicates a decrease in antioxidant capacity. This leads to
increased ROS production, further depletion of the pool of
bioantioxidants and increased lipid peroxidation, which causes
violations of the structure and functions of spermatozoa.

Reduced glutathione plays a leading role in the antioxidant
defense (Korzhov and Zhadan, 2007). Its antioxidative properties
are due to a direct interaction with ROS and functioning of
glutathione-dependent enzymes such as glutathione peroxidase
and glutathione transferase. Glutathione peroxidase catalyzes
the reduction of H₂O₂ or organic hydroperoxide, using reduced
 glutathione. As it is well known, glutathione peroxidase is a key
antioxidant enzyme which regulates ROS levels and protects sperm
cells from lipid peroxidation and oxidative stress. In fact, we
showed that glutathione peroxidase activity was significantly
decreased in patients with all forms of pathospermia compared with
healthy men with normozoospermia.

The intensity of free radical reactions and processes depends on
the concentration of oxygen and the functioning of the enzymatic
and nonenzymatic antioxidant systems. In our previous studies
(Onufrovych et al., 2016; Fafula et al., 2017) we found depletion of
antioxidant systems in particular inhibition of glutathione
peroxidase, glutathione reductase and glutathione transferase in
spermatozoa of infertile men. Despite the fact that the enzymatic
component of the antioxidant protection system is more sensitive to
changes in prooxidant-antioxidant balance than nonenzymatic, both
systems are closely related. On the one hand, nonenzymatic
antioxidants are substrates of reactions catalyzed by antioxidant
enzymes. On the other hand, as a result of enzymes work the
number nonenzymatic factors constantly increases and their level
depends on physiological needs of the cell. Therefore, it the
dynamic balance between content of nonenzyme components and
activity enzymatic systems is essential.

In addition, the reduction of GSH in sperm cells of infertile
men may be due to low activity of glutathione reductase, which
indicates the inactivation of GSH regeneration. It is well known that
 glutathione reductase uses NADPH as a restored equivalent, the
content of which is reduced under conditions of oxidative stress (De
Minicis and Brenner, 2008). It is also known that GSH/GSSG ratio
decreases rapidly under oxidative stress, leading to structural
defects and functional changes unless NADPH-dependent of GSH
regeneration occurs (Menendez et al., 2016).

It should be noted that GSH/GSSG ratio is significantly higher in
leucospermic patients than in other pathologies. According to
other researchers (Micheli et al., 2016) who found similar results in
infertile men with infectious factor, a slight decrease in GSH level and
maintaining GSH/GSSG balance is “sparring effect” caused by another
nonenzymatic antioxidant – ascorbic acid (vitamin C). However, this
effect is not a sufficiently powerful mechanism to neutralize ROS
hyperproduction and development of oxidative stress which is
evidenced by extremely high content of thiorubicuric acid reactive
substances in sperm cells of patients with leucospermia (Fafula
et al., 2017). The consequence of reduction of GSH can be H₂O₂
accumulation enhanced with subsequent production of hydroxyl radical
damage of macromolecular structures. In particular, it was shown
that prolonged intensification of lipid peroxidation is accompanied by
the accumulation of malondialdehyde and 4-hydroxynonenal that
can bind to DNA and proteins, modifying their structure and disrupting
the function (Hubsley, 2015; Spickett, 2013). GSSH reacts with thiol
groups of proteins with production of mixed disulfides that can alter the
catalytic properties of enzymes which is manifested by their ability to
maintain adaptive response to stress (Smirnov and Suhovskaya, 2014).

Thus, the changes in GSH content and activities of enzymes of
its metabolism found in sperm cells (Onufrovych et al., 2016;
Fafula et al., 2017) in patients with pathospermia may be
interpreted as a response to development of oxidative stress.
Decrease in intracellular GSH level and inhibition of glutathione
peroxidase leads to overproduction of ROS which results in
oxidative stress and injury in sperm cells.

Inhibition of antioxidant enzymes activities, a decrease in GSH
and enhanced lipid peroxidation indicate an irreversible destructive
changes (Mishchuk and Stolyar, 2008). Thus, the destabilization of
the glutathione system promotes even greater intensification of lipid
peroxidation processes and destruction of spermatozoa. Sperm
motility is associated with excessive oxidation of sulphydryl groups
that are part of GSH (Aig et al., 2012). GSH is localized mainly in
the middle part and tail of spermatozoon, which ensure its mobility
(Eskioeak et al., 2005).

Determination of GSH and the GSH/GSSG ratio can be used as
an additional biochemical indicator for the assessment of men’s
potential to father children. Disturbance in the glutathione system in
spermatozoa is one of the pathogenetic mechanisms leading to
infertility that must be considered when using antioxidants in the
treatment of male infertility.

Conclusions

Under conditions of pathospermia the reduced glutathione
content in spermatozoa decreased, which led to depletion of reduction
potential of the glutathione antioxidant system. Redistribution
between oxidized and reduced forms of glutathione can be caused by
depletion of intracellular GSH stores and intensification of lipid
peroxidation processes.
Support and acknowledgements

The publication contains the results of studies conducted under the President’s of Ukraine grant for competitive projects (project No О63/97-2016 from 10.08.2016 “Molecular biological regulatory mechanisms of disturbance of fertilizing ability spermatozoa and the development of new immuno-biochemical diagnostic methods of fertility in men” (scientific supervisor – Doc. Sci. D. Vorobets) of the State Fund for Fundamental Research (The President’s Order No 97/2016-pn dated: April 13, 2016).

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