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ACTIVITY OF SUPEROXIDE DISMUTASE AND CATALASE AND CONTENT OF MALONDIALDEHYDE IN SEMINAL PLASMA OF INFERTILE PATIENTS

AKTIVNOST SUPEROKSID DIZMUTAZE I KATALAZE I SADRŽAJ MALONDIALDEHIDA U SEMINALNOJ PLAZMI INFERTILNIH PACIJENATA

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Summary – The reactive oxygen species, the highly reactive metabolites of oxygen, play a crucial role in both the normal function and the metabolism of sperm cells. Oxygen radicals achieve their physiological effects in the cells only if there is a proper balance between their production and degradation. In case of radicals' production exceeding the antioxidant capacity of the semen, there is an oxidative damage of the membrane lipids and proteins as well as the DNA damage followed by the fragmentation and decondensation of DNA. The ejaculates were obtained from seventy-seven infertile and fertile healthy individuals. The semen samples were collected and classified according to the WHO criteria. The activities of superoxide dismutase and catalase as well as the concentration of malondialdehyde were measured spectrophotometrically. The fertile, healthy donors showed the significantly higher activities of both superoxide dismutase and catalase, as well as the lower concentration of malondialdehyde compared to the infertile donors. The activities of superoxide dismutase and catalase, as well as the HOS test, correlated positively with the sperm cell number, but negatively with the concentration of malondialdehyde. The activity of superoxide dismutase and the concentration of malondialdehyde were highest in the group of patients with the lowest success of the HOS test. The assessment of the antioxidant enzymes and malondialdehyde in addition to the semen analysis and the HOS test may be greatly useful in diagnosing infertility in men having oxidative stress in their etiology.

Key words: *Superoxide Dismutase; Catalase; Oxidative Stress; Infertility; Malondialdehyde; Spermatozoa + metabolism; Male; Semen Analysis + methods; Sperm Count; Membrane Lipids; Lipid Peroxidation*

Introduction

The reactive oxygen species (ROS), the highly reactive metabolites of oxygen, play a crucial role in both the normal function and the metabolism of sperm cells. ROS exert their physiological effects as the signaling molecules in the cells depending on their nature and concentration in the processes of hyperactivation, capacitation and sperm acrozomal reactions [1]. Moreover, they play a key role in the sperm-egg attachment [2,3]. However, the physiological roles of radicals in the cells are achieved only if there is a balance between the production and degradation of ROS [4]. When the overproduction of free radicals exceeds the antioxidative capacity of both the sperm and the seminal plasma, this subsequently leads to the oxidative damage of membrane lipids and proteins [5], as well as to the DNA damage followed by the fragmentation and decondensation [6,7]. Various studies have shown that these molecules are capable of inducing the peroxidative cell damage, particularly of the lipid membranes, due to their high reactivity [8]. The sperm cells, owing to their specific structure, appear to be very sensitive to the oxidative insult. The sperm plasma membrane is very rich in polyunsaturated fatty acids which undergo the peroxidation easily. Antioxidative enzymes and other antioxidants are concentrated at the central parts of the sperm, so a great deal of sperm membrane remains unprotected. In the situation when ROS are produced profusely in the semen, whether they originate from the defective sperm or white blood cells, oxygen free radicals play an important role in determining the etiology of a poor sperm function due to the peroxidative damage of lipid membranes [9,10] and this makes them a possible cause of male infertility. However, both the sperm and the seminal plasma possess the enzymatic as well as non-enzymatic antioxidative defense mechanisms. The capacity of these systems should be investigated since the cause of a poor sperm function may not only be due to the increased production of the oxygen free radicals, but also to a reduced activity of antioxidants in the seminal plasma.

Materials and methods

The investigation included 77 patients between aged from 24 to 54 years, who asked for a consultation with a gynecologist at the Clinic for Gynecology and Obstetrics, Clinical Center in Kragujevac regarding their marital infertility. The first information about the fertile abilities of men are provided by the spermogram test. In order to obtain a sperm sample for analysis a period of sexual abstinence of at least 48 hours, but no longer than seven days is necessary before sampling. The basic parameters of spermogram - number, motility, vitality and sperm morphology were determined according to the WHO procedures [11]. Based on these parameters, the subjects were divided into four groups: normozoosper-

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Abbrevations:
ROS – reactive oxygen species
HOS – hypoosmotic sweelling test
WHO – World Health Organization

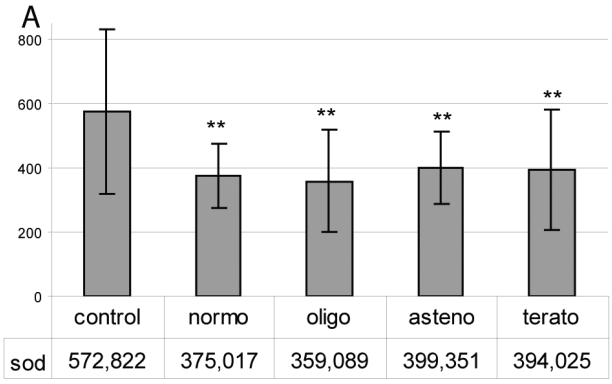
mic group - 18 men, oligozoospermic group (sperm count less than 20 x 10^9/L) - 23 men, astenozoospermic group (less than 50% of progressively moving sperm cells) - 20 men and teratozoospermic group (less than 30% of sperm with normal morphology) - 16 men. The control group consisted of nineteen fertile men who made a woman conceive in the previous year according to the standards of WHO, and who were of similar ages as the group of the infertile patients. The azoospermic patients (with no sperm in the ejaculates) and the patients with the urological infection (with leukocytospermia more than 1 x 109/L) were excluded from the study. The physical integrity of the sperm membrane was examined by the hypo-osmotic swelling test – HOS test. The HOS test is based on the semi-permeability of intact cell membrane that allows sperm swelling in hypo-osmotic conditions. The sperm exposure to hypo-osmotic conditions makes water get into the cell, with resulting swelling of the cytoplasmic space and twisting of the sperm tail [12,13]. After the sperm incubation for 30 minutes in the hypo-osmotic conditions at 37 °C, the number of sperm cells with a twisting tail was examined microscopically. The light microscope with the magnification of 400 x was used to count 100 sperm cells in one or more microscopic fields. The sperm cells with functional membrane twist their tails, while those whose membrane integrity is compromised do not show the phenomenon of swelling, specifically do not twist their tails. For the greater accuracy, at least two samples were counted and the result was expressed as the mean counts. The reference values for the HOS test were > 60%. Based on the results of HOS test, the groups were categorized as following: the control group was the group of subjects with more than 65% of the sperm cells with the functional membranes (with the twisted sperm tail) (15 patients); the experimental group with the lower HOS test score: 45-65% of sperm cells with the twisted sperm tail (25 patients); the experimental group with the HOS test performance of 26-44% (25 patients) and the experimental group of the HOS test performance of <25% (12 patients).

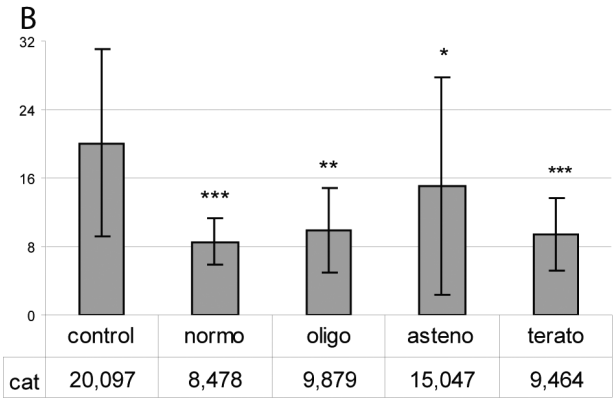
In order to investigate the antioxidative capacity of seminal plasma, the activities of superoxide dismutase and catalase were determined, while the concentration of malondialdehyde, the marker of the lipid peroxidation, was measured as an indirect indicator of the activity of free radicals in the seminal plasma. The activity of superoxide dismutase (E.C. 1.15.1.1.) in the seminal plasma was determined spectrophotometrically by the commercial RANDOX kit [14]. The method of Goth [15] was used for the spectrophotometrical determination of catalase activity (E.C. 1.11.1.6.) in the seminal plasma. The method of Stocks and Dormandy [16] was applied to determine the concentration of malondialdehyde spectrophotometrically.

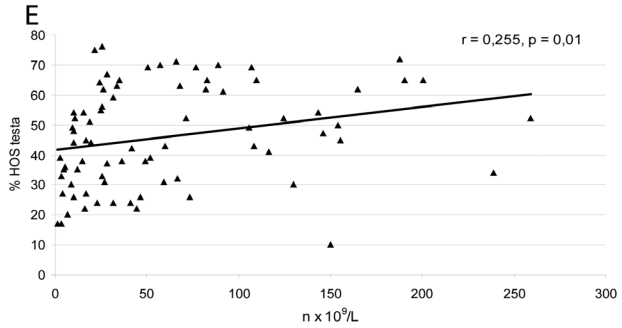
The results were expressed as the mean ± standard deviation. The statistical processing of results was performed using a commercial software package SPSS (version 11.0, SPSS Inc., Chicago, IL). The following tests were used for statistical processing: the analysis of variance (ANOVA), the correlation analysis and the Kruscal-Wallis test. The statistical significance was assessed at a value $P<0.05$.

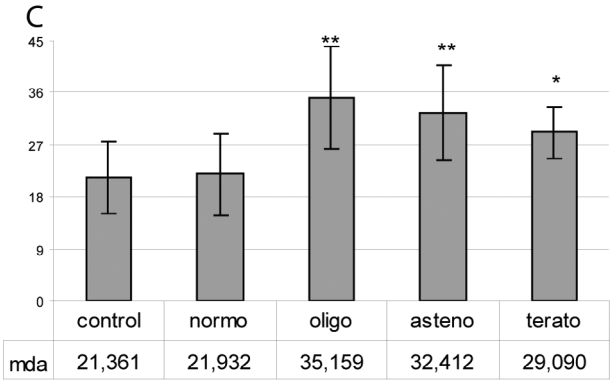
Results

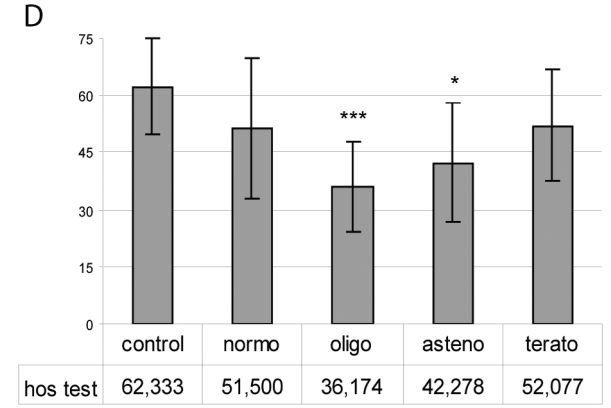
The measured activity of superoxide dismutase was significantly lower in all groups of infertile patients compared to the control group, $p<0.01$ **(Graph 1A).** The lowest measured value of superoxide dismutase was in the group of olygospermic patients. In addition, there was a positive correlation between the activity of superoxide dismutase and the number of sperm cells ($r=0.389$, $p=0.0005$). By monitoring the activity of superoxide dismutase in the groups formed according to the outcome of the HOS test, the results demonstrated an increase in the enzyme activity with the decreased success of the HOS test. Thus, the highest measured activity of superoxide dismutase was recorded in the group with the lowest success of HOS tests, less than 25%, $p<0.05$ **(Graph 2A)**. Furthermore, the determination of catalase activity in the seminal plasma of the infertile patients showed the highest activity of the measured catalase in the group of astenozoospermic patients ($p<0.05$) and the lowest enzyme activity in the group of normozoospermic men ($p<0.001$) compared to the fertile controls **(Graph 1B)**. In addition, a positive correlation was observed by comparing the values of sperm cell number and the catalase activity ($r=0.268$, $p=0.02$). The results also demonstrated no significant differences in the activities of catalase in the seminal plasma of the groups formed according to the success of the HOS test (the values of the catalase activity (kU/L) in the HOS groups were: >65% - 14,134; 45-65% - 11,093; 26-44% - 11,876; <25% - 11,203, $p>0.005$). The increased values of malondialdehyde, a specific marker of lipid peroxidation were found in all groups of patients with a disorder of spermogram parameters. The highest concentration of malondialdehyde was measured in the group of olygozoospermic patients ($p<0.01$), while these values were slightly lower in the group of astenozoospermic patients ($p<0.01$) and in the group of teratozoospermic patients, $p<0.05$ **(Graph 1C)**. Moreover, the results demonstrated a negative correlation ($r=-0.294$, $p=0.04$) between the values of the sperm number and the concentration of the plasma malondialdehyde in the seminal plasma of infertile patients. In addition, the concentration of malondialdehyde increased with the decrease in the success of the HOS test **(Graph 2B)**; the highest concentration value of the malondialdehyde was measured in the group with the lowest success of the HOS test, less than 25% ($p<0.001$). Furthermore, the sub-











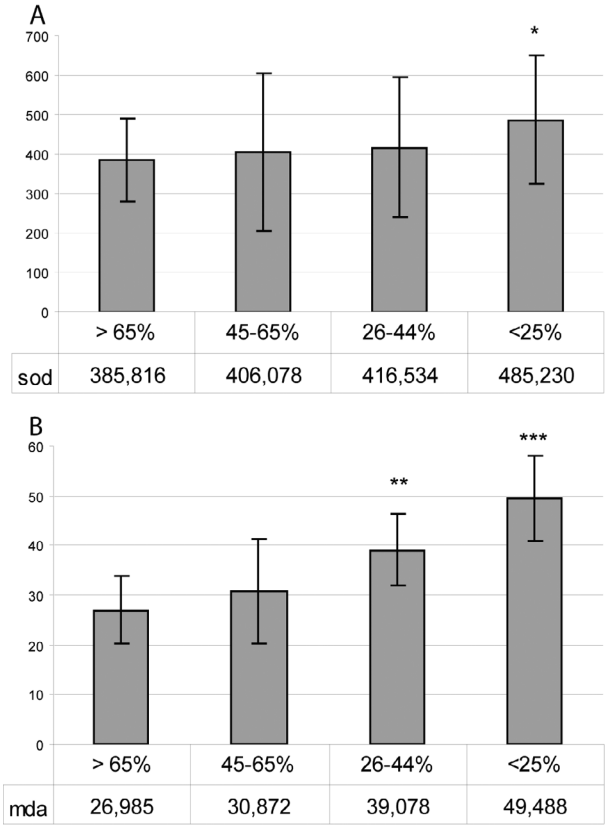
Graph 1. Values of determined parameters in seminal plasma (distribution of patients according to the results of semen analysis)
A – activity of superoxide dismutase (sod) [U/mL]; B – activity of catalase (cat) [kU/L]; C – concentration of malondialdehyde (mda) [nmol/mL]; D – score of HOS test (hos test; %); E – correlation between the sperm cell number and HOS test score (r = 0.255, p = 0.01)
Legend: $^{*}p<0.05$; $^{**}p<0.01$; $^{***}p<0.001$

Grafikon 1. *Vrednosti određivanih parametara u seminalnoj plazmi (raspodela pacijenata prema rezultatima spermograma)*
A – aktivnost superoksid dizmutaze (sod) [U/mL]; B – aktivnost katalaze (cat) [kU/L]; C – koncentracija malondialdehida (mda) [nmol/mL]; D – uspešnost HOS testa (hos test) (%); E – korelacija broja spermatozoida u uzorku i uspešnosti HOS testa (r = 0,255, p = 0,01)
Legenda: $^{*}p<0,05$; $^{**}p<0,01$; $^{***}p<0,001$

jects from the normozoospermic and control group demonstrated the highest percentage of the success of the HOS test, whereas the group of oligozoospermic patients showed the lowest level of performance of the HOS test, p<0.001 **(Graph 1D)**. Finally, there was a positive correlation between the number of sperm cells and performance of the HOS test (r = 0.255, p = 0.01) **(Graph 1E)**.

Discussion

During the process of fertilization, the fusion of the membrane of sperm cells and oocytes should occur. To make the fertilization occur, it is necessary to have the sperm membrane integrity preserved both physically and biochemically, and this process can be checked by the HOS test, i.e. by exposing the cell to the hypo-osmotic conditions when water gets into the interior of the sperm cell leading to the increase in its volume resulting in the plasma membrane swelling and twisting of the tail of the sperm [17,18]. Those sperm cells whose membrane integrity is compromised do not show the phenomena of the swelling. However, the optimal physical integrity of the membrane does not exclude the possibility of a disturbed otherwise normal biochemical composition of the membrane. The sperm cell membrane is very rich in polyunsaturated fatty acids and, by being very fluid, it provides the favorable condition for the fertilization; however, due to this fact it is also highly vulnerable for the effect of free radicals and lipid peroxidation. It has been recently emphasized that one of the possible causes of the male infertility is the presence of oxidative stress, i.e. the imbalance between the production and detoxification of free radicals in



Graph 2. Values of determined parameters in seminal plasma (distribution of patients according to the score of HOS test)
A – activity of superoxide dismutase (sod) [U/mL]; B – concentration of malondialdehyde (mda) [nmol/mL]
Legend: *p<0.05; **p<0.01; ***p<0.001

Grafikon 2. *Vrednosti određivanih parametara u seminalnoj plazmi (raspodela pacijenata prema rezultatima uspešnosti HOS testa)*
A – aktivnost superoksid dizmutaze (sod) [U/mL]; B – koncentracija malondialdehida (mda) [nmol/mL]
*Legenda: *p<0,05; **p<0,01; ***p<0,001*

the seminal plasma [19]. Oxygen free radicals originate mainly from different semen components, including both immobile or morphologically abnormal sperm cells, leukocytes and morphologically normal, but functionally impaired sperm cells [20]. It has been shown that oxygen radicals might lead to the defective sperm cell functioning through the process of lipid peroxidation [21,22].

The activity of superoxide dismutase in the seminal plasma was significantly lower in all examined groups of infertile patients compared to the fertile controls, which is in accordance with the results of other researchers [23,24]. The lowest activity of superoxide dismutase was observed in the group of olygozoospermic patients and it was confirmed by the correlation between the number of sperm cells and the total enzyme activity. In addition, the decreased activity of superoxide dismutase in the seminal plasma resulted in a reduced ability of this enzyme to convert superoxide radical to hydrogen peroxide. The increased concentration of superoxide anion radicals could damage the vital macromolecules. However, the spontaneous dismutation of superoxide anion radical into hydrogen peroxide induces the lipid peroxidation and impairs sperm membrane integrity, leading to the complete inhibition of motility and energy metabolism of sperm cells [25]. Since the activity of glutathione peroxidase in the seminal plasma is low, catalase plays the most important role in detoxification of hydrogen peroxide in the seminal plasma. The literature data indicate that catalase provides the most effective antioxidative protection against the effects of free radicals when the sperm cells are incubated in the systems producing oxygen radicals profusely [26, 20,33]. By examining the catalase activity in the seminal plasma and in sperm cells it was demonstrated that the activity of catalase was significantly higher in those samples in which the sperm cells and other cellular elements of the ejaculate produced reactive oxygen radicals [27]. Our results showed that the catalase activity was significantly lower in all infertile patients compared to the fertile controls. The most pronounced decrease of the catalase activity was observed in the group of normozoospermic subjects. Polish researchers [24] have also shown that the catalase activity decreased in the seminal plasma of infertile patients. In this study, the activity of catalase was lower in the seminal plasma of oligozoospermic patients compared to the fertile controls, and it was confirmed by the statistically significant correlation between the number of sperm cells and enzyme activity.

The consequences of reduced activities of antioxidative enzymes are intensified effects of free radicals and increased oxidative damage to the important macromolecules [28]. There are numerous literature data indicating the lipid peroxidation as the main mechanism of the oxidative damage to the sperm cells, and the cause of their defective function in infertile men [29-35]. The results of our study showed that the concentration of malondialdehyde, a specific marker of lipid peroxidation, was significantly higher in the seminal plasma of the oligo-, asteno-, and teratozoospermic patients compared to the fertile controls and normozoospermic men. The highest concentration of malondialdehyde was recorded in the seminal plasma of the oligozoospermic group suggesting that the activity of oxygen radicals in these samples was the most damaging. This finding confirms the important correlation between the number of sperm cells in the ejaculate and the concentration of malondialdehyde in the seminal plasma. In addition, our results demonstrated the significant increase in the content of lipid peroxide in the seminal plasma as well as in the sperm cells [35]. Moreover, our study, as well as the previous one [35], showed the highest concentration of malondialdehyde in the oligozoospermic patients.

On the other hand, when measuring the activity of superoxide dismutase in the groups formed according to the success of the HOS test, there was an increase in the enzyme activity with the decreased

success of the HOS test. The highest activity of superoxide dismutase was recorded in the group with the lowest success of the HOS test. This finding can be explained by the possible increased antioxidative effect of superoxide dismutase in respond to the damaging effects of free radicals. There was no significant difference in the activity of catalase among the tested groups, which is in accordance with the results of Indian researchers [36]. The reduction of the HOS test performance reflects the impaired integrity of the sperm membrane, as well as the increased lipid peroxidation, including the increased content of malondialdehyde in the seminal plasma, as demonstrated by other researchers [36, 37]. It appears that the increased effect of superoxide dismutase in our samples was not enough to overcome the devastating effects of radicals subsequently leading to the increased lipid peroxidation. Our research has shown that the measuring of activity of superoxide dismutase might give highly informative data on the antioxidative defense mechanism. Moreover, the concentration of malondialdehyde was increased in all groups showing some disorders of the spermogram; there was also an increase in the concentration of malondialdehyde in all groups formed according to the performance of the HOS test. These findings suggest that the process of lipid peroxidation is the main mechanism of damaging the sperm cells and disturbing their functions. The HOS test, as an additional parameter performed while making the spermogram,, correlates well with the degree of lipid peroxidation and therefore it is justifiable to introduce it into the routine diagnostics of male fertility.

Conclusion

According to our results, it could be concluded that the determination of antioxidative parameters and concentration of malondialdehyde, in addition to the routine parameters of spermogram and the HOS test, may help in establishing the clinical diagnosis of infertility in men with the underlying oxidative stress.

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Sažetak

Uvod

Reaktivni kiseonični radikali, visokoreaktivni metaboliti kiseonika, imaju važnu ulogu u normalnom funkcionisanju i metabolizmu spermatozoida. Svoj fiziološki efekat ostvaruju u zavisnosti od svoje prirode i koncentracije, kao signalni molekuli u procesima hiperaktivacije i akrozomske reakcije spermatozoida a takođe, bitnu ulogu imaju i u pričvršćivanju spermatozoida za jajnu ćeliju. Fiziološke efekte radikali ostvaruju samo dok postoji ravnoteža između produkcije i degradacije, kada produkcija radikala nadmaši antioksidativne kapacitete spermatozoida i seminalne plazme dolazi do oksidativnog oštećenja membranskih lipida i proteina, kao i do oštećenja DNK praćenog fragmentacijom i dekondenzacijom. Cilj rada je bio da se ispita da li postoji oksidativni stres i kakav ja antioksidativni kapacitet seminalne plazme kod infertilnih pacijenata.

Materijal i metode

Određivanje osnovnih parametara spermograma vršeno je po proceduri koju propisuje Svetska zdravstvena organizacija, a za sva ostala merenja korišćene su savremene spektrofotometrijske metode.

Rezultati

Aktivnost antioksidativnih enzima, superoksid dizmutaze i katalaze znatno je snižena kod infertilnih pacijenata u odnosu na kontrolne vrednosti, dok je koncentracija malondijaldehida povišena. Postoji dobra korelacija između ispitivanih parametara i broja spermatozoida.

Diskusija

*Zbog svoje građe izgleda da su spermatozoidi veoma osetljivi na oksidativni **insult. Plazma membrana spermatozoida je jako bo**gata polinezasićenim masnim kiselinama koje lako podležu peroksidaciji. Kiseonični slobodni radikali uglavnom potiču iz različitih semenih komponenti, uključujući nepokretne i morfološki abnormalne spermatozoide, leukocite i morfološki normalne ali funkcionalno abnormalne spermatozoide.*

Zaključak

Određivanje antioksidativnih parametara i malondijaldehida, pored parametara spermograma i hypoosmotic sweelling testa, može da pomogne u postavljanju dijagnoze infertiliteta kod muškaraca u čijoj etiologiji učestvuje oksidativni stres.

Ključne reči: *Oksidativni stres; Infertilitet; Superoksid dizmutaza; Katalaza, Malondijaldehid; HOS test*

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