Chinese Journal of Physiology 58(2): 124-133, 2015
DOI: 10.4077/CJP.2015.BAC243

Assessment of Oxidative Status in Patients with Acute Kidney Injury: A Pilot Study

Snezana Pesic[1], Marija Milinkovic[2], Milena Vuletic[3], Nevena Barudzic[4], Vladimir Zivkovic[5], Vladimir Jakovljevic[5], Dragan Djuric[6], and Biljana Stojimirovic[2]

[1]*Center for Hemodialysis, Medical Center, Obrenovac*
[2]*Institute of Urology and Nephrology, Clinical Center of Serbia, Belgrade*
[3]*Department of Pathology, Faculty of Medical Sciences, University of Kragujevac, Kragujevac*
[4]*Department of Pharmacy, Faculty of Medical Sciences, University of Kragujevac, Kragujevac*
[5]*Department of Physiology, Faculty of Medical Sciences, University of Kragujevac, Kragujevac*
and
[6]*Institute of Medical Physiology "Richard Burian", School of Medicine, University of Belgrade Belgrade, Republic of Serbia*

Abstract

Extensive experimental evidence confirms the role of oxidative stress as a major contributor to the pathogenesis of acute kidney injury (AKI). However, less information is available on the evolution of prooxidant-antioxidant parameters from early to end-phase renal function decline in humans. This study aimed to determine the oxidative status in dynamic throughout the evolutionary phases of the disease. The study included patients with cardiovascular pathology and AKI hospitalized in the intensive care unit (n = 69) and age-matched healthy controls (n = 30). They were followed through three phases of AKI; first phase was the phase of diagnosis, which is characterized by oliguria/anuria, second phase was established diuresis, and third phase was the polyuric phase. In these phases of the disease, blood samples were taken from the patients for biochemical analysis. From the collected whole blood, we measured spectrophotometrically prooxidants: index of lipid peroxidation, measured as Thiobarbituric acid reactive substances (TBARS), nitrite (NO_2^-), superoxide anion radical (O_2^-) and hydrogen peroxide (H_2O_2), and antioxidants: activity of superoxide dismutase (SOD), catalase (CAT) and reduced glutathione (GSH) from erythrocyte lysate. Comparing the results of the three measurements, a significant difference was found in the levels of NO_2^- and GSH, both of which increased in the second phase ($P < 0.05$) and then decreased in the third phase, and a significant increase in TBARS, which was elevated in the second phase ($P < 0.05$) and did not change significantly until the third phase. Our results showed phase-dependent modification in 3 parameters of the oxidative status (TBARS, NO_2^- and GSH). Whether these changes contribute to the deterioration of renal function in AKI remains to be established.

Key Words: acute kidney injury, nitrite, oxidative stress

Introduction

Oxidative stress is a condition in which the delicate balance that exists between the production of prooxidants and their subsequent amelioration *via* the antioxidant defense system (ADS) becomes skewed in favor of prooxidants (18). Prooxidants, *i.e.* reactive oxygen and nitrogen species (RONS), are

Corresponding author: Prof. dr Vladimir Jakovljevic, Department of Physiology, Faculty of Medical Sciences, University of Kragujevac, 69 Svetozara Markovica, 34000 Kragujevac, Serbia. Tel: +381-34-342-944, Fax: +38134306800, ext. 112, E-mail: drvladakgbg@yahoo.com.tw
Received: September 16, 2013; Revised (Final Version): July 14, 2014; Accepted: July 31, 2014.
 ISSN : 0304-4920. http://www.cps.org.tw

constantly being generated in the body to a small extent and, since they have a potential to react with a variety of chemical species, they have multiple functions in cell signaling and enzymology (11, 23, 24). On the other hand, excessive ROS production, that appears to be induced by both psychological and physical stress, may lead to oxidative damages and numerous pathological processes (7, 13, 36, 41, 45, 48).

Considering the biological effects of ROS, in the past decades, numerous experimental and clinical studies have addressed the role of oxidative stress in the setting of renal ischemia/reperfusion injury (22, 39, 49). Acute kidney injury (AKI) is a sudden reduction in glomerular filtration rate of individual nephrons with tubular function disorder that occurs due to ischemia-reperfusion or toxic damage (9, 10).

In recent years, there are several published studies that discuss the prevalence of various parameters of oxidative stress in patients with AKI such as uric acid (29), and proinflammatory cytokines were significantly increased in patients with AKI as compared to healthy subjects (18). Several experimental studies have been done using animal models (ischemic, toxic and several subtypes), which confirmed the reduction of glutathione in the renal parenchyma in AKI (14, 51). Patients with AKI commonly suffer from heart disease. Both cardiac and renal diseases are present in the same patient and these diseases have been associated with increased costs of care, complications and mortality (32). Cardiorenal syndromes (CRS) have been further classified in five defined entities which represent clinical circumstances in which both the heart and the kidney are involved in bidirectional injury and dysfunction *via* a final common pathway of cell-to-cell death and accelerated apoptosis mediated by oxidative stress (32). On the other hand, studies addressing the contribution of oxidative stress in different phases of AKI are lacking. Determination of the phase characterized by the most important oxidative damage will become the major therapeutic target. Accordingly, the present study aimed to dynamically assess the redox status in patients with AKI throughout the evolution of the disease.

Materials and Methods

Study Population

The study group consisted of 69 patients with AKI (average age 65 years, SD ± 12.86) with cardiovascular pathology, usually after cardiovascular surgery, who were recruited in intensive care units at the Clinical Center Serbia. The diagnosis of AKI was based on the increase in serum creatinine by 50% compared to the reference values according to the RIFLE criteria, recommended by Acute Dialysis Quality Initiative. At the time of the study, all patients were treated with appropriate therapy depending of the phase of disease. Control group included 30 age-matched healthy individuals. The study was approved by the Ethical Committee, Clinical Center Serbia. This study was performed in accordance with the principles of the Declaration of Helsinki.

Patients were followed through three phases of AKI: [1] at admission, which is characterized by oliguria/anuria (first phase); [2] established diuresis (second phase), and [3] polyuric phase (third phase). In these phases of the disease, blood samples were taken from the patients for biochemical analysis for the following parameters of oxidative stress: index of lipid peroxidation, measured as TBARS, nitrite (NO_2^-), superoxide anion radical (O_2^-) and hydrogen peroxide (H_2O_2), while antioxidants: activity of superoxide dismutase (SOD), catalase (CAT) and glutathione (GSH) were measured from erythrocyte lysate, using a spectrophotometer.

Biochemical Assays

Superoxide Anion Radical (O_2^-) Determination

The level of superoxide anion radical (O_2^-) was measured using NBT (nitro blue tetrazolium) reaction in TRIS-buffer combined with plasma samples and read at 530 nm (1).

H_2O_2 Determination

The protocol for measurement of H_2O_2 was based on oxidation of phenol red in the presence of horseradish peroxidase (42). Two hundred microliter sample with 800 μl PRS (Phenol Red Solution) and 10 μl POD (Horseradish Peroxidase) were combined at 1:20. The level of H_2O_2 was measured at 610 nm.

NO_2^- Determination

Nitric oxide (NO) decomposes rapidly to form stable metabolite nitrite/nitrate products. NO_2^- was determined as an index of NO production with Griess reagent (21). 0.1 ml 3N PCA (perchloric acid), 0.4 ml 20 mM EDTA (ethylenediaminetetraacetic acid) and 0.2 ml plasma were put on ice for 15 min, then centrifuged 15 min at 6,000 rpm. After pouring off the supernatant, 220 μl K_2CO_3 was added. Nitrites were measured at 550 nm. Distilled water was used as a blank probe.

Index of Lipid Peroxidation (TBARS) Determination

The degree of lipid peroxidation in plasma was estimated by measuring TBARS using 0.4 ml 1%

thiobarbituric acid (TBA) in 0.05 NaOH mixed with 0.8 ml of plasma, incubated with plasma at 100°C for 15 min and read at 530 nm. Distilled water was used as a blank probe. TBA extract was obtained by combining 0.8 ml plasma and 0.4 ml trichloroacetic acid (TCA), and the samples were then put on ice for 10 min, and centrifuged for 15 min at 6,000 rpm. This method was described by Ohkawa *et al*. (38).

Determination of CAT, SOD, and GSH

Isolated RBCs were washed three times with 3 volumes ice-cold 0.9 mmol/l NaCl and hemolysates containing about 50g Hb/l, prepared according to McCord and Fridovich (31), were used for the CAT. CAT activity was determined according to Beutler (6). Lysates were diluted in distilled water (1:7 v/v) and treated with chloroform-ethanol (0.6:1 v/v) in order to remove haemoglobin. Then, 50 μl catalase buffer, 100 μl sample and 1 ml 10 mM H_2O_2 were added. Measurement was done at 360 nm. Double distilled water was used as a blank probe. SOD activity was determined by the epinephrine method of Misra and Fridovich (35) in which 100 μl lysate and 1 ml carbonate buffer were mixed, and then 100 μl epinephrine was added. Determination was done at 470 nm. Level of GSH was determined according to Beutler (5) based on GSH oxidation *via* 5,5 dithiobis-6,2-nitrobenzoic acid (DTNB). GSH extract was obtained by combining 0.1 ml 0.1% EDTA, 50 μl erythrocyte lysate and 750 μl precipitation solution. After vortexing and extraction on cold ice for 15 min, centrifugation at 4,000 rpm was performed for 10 min. Three hundred microliter lysate, 750 μl Na_2HPO_4 and 100 μl DTNB was pipetted into test tubes. As a blank probe, distilled water was used. Concentration and the amount of reduced glutathione were determined on the basis of a standard curve for each assay. For standard curve construction, standard stock-solution of GSH at a concentration 1, 5 mmol/l was used. Measuring of absorbance was performed at λ_{max} = 412 nm.

Statistical Analysis

Results are expressed as the mean (X) ± standard deviation (SD). To check whether a variable had a normal distribution, the Kolmogorov-Smirnov test and the Shapiro-Wilk test were used. After checking data distribution, the appropriate parametric or non-parametric test was used. The differences between two groups were assessed using *t*-test or Mann-Whitney test. For testing according to two categorical variables were used in Pearson's *Chi*-square test and Fisher's test. Multiple linear regression analyses were used to identify variables associated with pro- and antioxidans. $P < 0.05$ were considered statistically significant. The statistical analysis was performed with SPSS for Windows version 10.0 (SPSS Inc, Chicago, IL, USA).

Results

Level of Prooxidant Parameters Through Various Phases of Disease

TBARS Values

The mean value of TBARS in the oliguric phase was 2.91 ± 0.22 nmol/ml plasma, with 95% confidence interval from 2.38 to 3.43 nmol/ml and a median of 2.27 nmol/ml. The empirical distribution of the TBARS values in the oliguric phase was highly significantly different from the theoretical normal curve ($P < 0.05$). In the phase of established diuresis, there was an increase in the mean values of TBARS 4.00 ± 0.44 nmol/ml and a median of 2.54 nmol/ml, which was confirmed by a statistically significant increase (Z = 2.116, $P < 0.05$) and 95% confidence interval ranged from 2.96 to 5.04 nmol/ml of plasma. Empirical distribution of TBARS in the phase of established diuresis was significantly different from normal ($P < 0.05$). In the polyuric phase, there was an increase in the mean values of TBARS 4.62 ± 0.88 nmol/ml and a median of 2.64 nmol/ml, which was confirmed as a statistically significant increase (Z = 1.864, $P < 0.05$) and 95% confidence interval ranged from 2.49 to 6.76 nmol/ml of plasma. Empirical distribution of TBARS in the polyuric phase was significantly different from normal ($P < 0.05$), between the oliguric and polyuric phases was statistically significant increase (Z = 2.623, $P < 0.05$) (Fig. 1).

NO_2^- Values

The mean value of NO_2^- in the oliguric phase was 14.95 ± 1.79 nmol/ml, with 95% confidence interval of 12.97 to 16.93 nmol/ml and a median of 15.16 nmol/ml of plasma. The empirical distribution of NO_2^- values in the oliguric phase was on the verge of significance from a theoretical normal curve ($P > 0.05$). In the phase of established diuresis, there was an increase in mean NO_2^- 19.41 ± 0.88 nmol/ml and a median of 19.48 nmol/ml plasma, which was confirmed by a statistically significant increase (Z = 2.326, $P < 0.05$) and 95% confidence interval ranged from 16.33 to 22.50 nmol/ml of plasma. Empirical distribution of NO_2^- in the phase of established diuresis was not statistically significantly different from normal ($P > 0.05$). In the polyuric phase, there was a decrease in the mean NO_2^- to

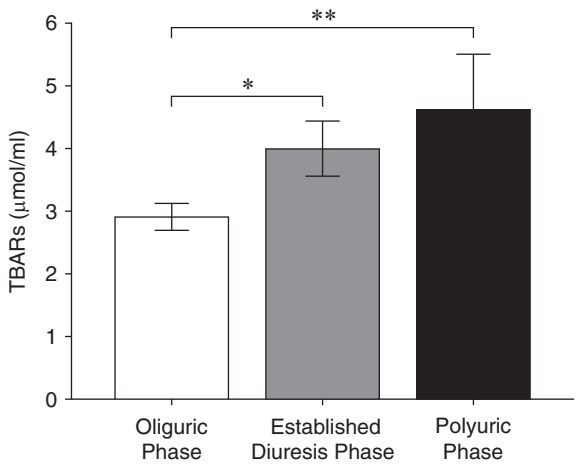

Fig. 1. TBARs dynamics during different phases of AKI (*$P < 0.05$, **$P < 0.01$).

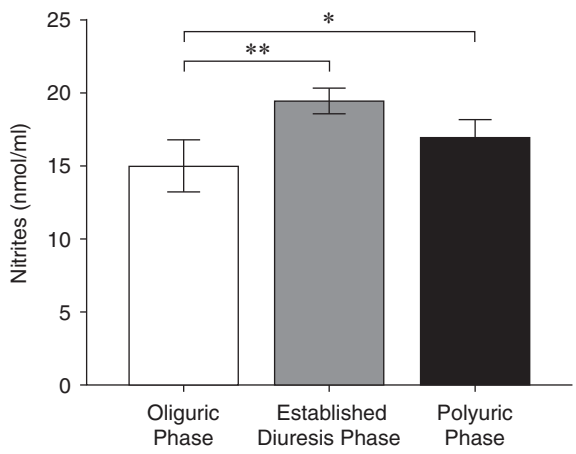

Fig. 2. Nitrites dynamics during different phases of AKI (*$P < 0.05$, **$P < 0.01$).

16.90 ± 1.21 nmol/ml and a median of 15.16 nmol/ml, which was confirmed as statistically significant decrease ($Z = 1.925$, $P < 0.05$) comparing to the oliguric phase, and the 95% confidence interval ranged from 13.86 to 22.50 nmol/ml. Empirical distribution of NO_2^- in the polyuric phase was not significantly different from normal ($P > 0.05$) (Fig. 2).

H_2O_2 Values

The mean value of H_2O_2 in the oliguric phase was 5.47 ± 1.28 nmol/ml, with 95% confidence interval of 3.81 to 7.01 nmol/ml and a median of 2.76 nmol/ml of plasma. The empirical distribution of the values of H_2O_2 in the oliguric phase was significantly different from the theoretical normal curve ($P < 0.05$). At the phase of established diuresis, there was an increase in the mean H_2O_2 6.23 ± 1.11 nmol/ml plasma and a median of 3.27 nmol/ml plasma, which

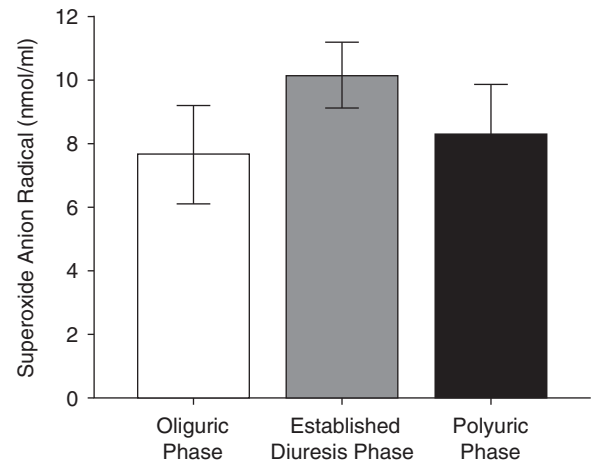

Fig. 3. Superoxide anion radical dynamics during different phases of AKI.

was confirmed as a statistically significant increase ($Z = 1.213$, $P > 0.05$) and 95% confidence interval for the values of H_2O_2 in the phase of established diuresis was from 4.06 to 8.40 nmol/ml plasma. Empirical distribution of H_2O_2 in the phase of established diuresis was significantly different from normal ($P < 0.05$). In the poliuric phase, there was increase of H_2O_2 to 7.89 ± 1.26 nmol/ml plasma, and a median of 2.78 nmol/ml plasma, which was not confirmed as a statistically significant decrease. Comparing values of H_2O_2 through various phases of disease, statistically significant changes were not found (Fig. 3).

O_2^- Values

The mean value of O_2^- in the oliguric phase was 7.65 ± 1.54 nmol/ml, with 95% confidence interval of 5.50 to 9.80 nmol/ml and a median of 4.28 nmol/ml of plasma. The empirical distribution of the values of O_2^- in the oliguric phase was significantly different from the theoretical normal curve ($P < 0.05$). At the phase of established diuresis, there was an increase in the mean of O_2^- 10.16 ± 1.03 nmol/ml plasma and a median of 6.43 nmol/ml plasma, which was not confirmed as a statistically significant increase ($Z = 1.637$, $P > 0.05$) and 95% confidence interval for the values of O_2^- in the phase of established diuresis was from 7.13 to 13.20 nmol/ml plasma. Empirical distribution of O_2^- in the phase of established diuresis was significantly different from normal ($P < 0.05$). In the poliuric phase, there was a decrease of O_2^- to 8.30 ± 1.57 nmol/ml plasma, and a median of 5.11 nmol/ml plasma, which was not confirmed as a statistically significant decrease ($Z = 0.435$; $P > 0.05$) comparing to phase of established diuresis, and the confidence interval was 4.74-11.85 nmol/ml plasma. Comparing the oliguric

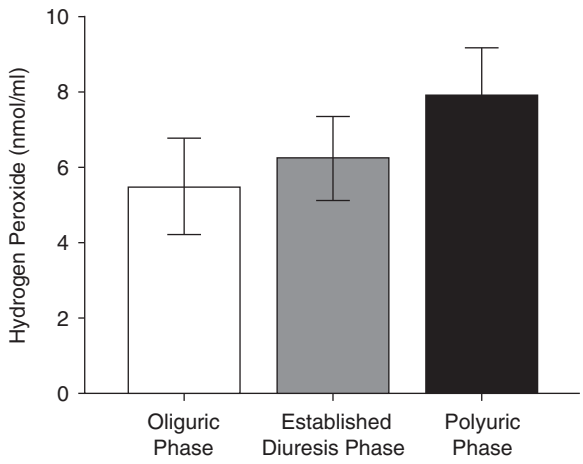

Fig. 4. Hydrogen peroxide dynamics during different phases of AKI.

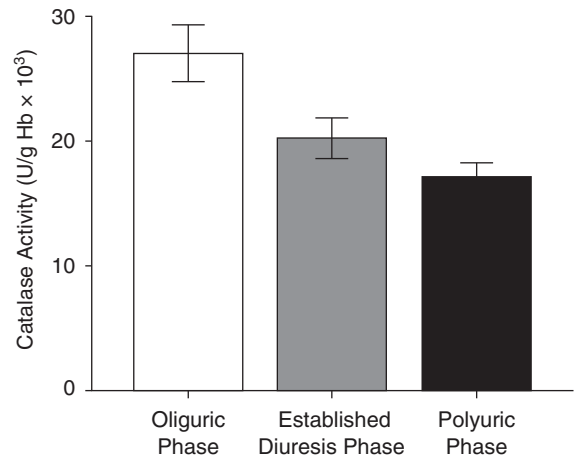

Fig. 5. Catalase activity during different phases of AKI.

and polyuric phases, we did not found statistically significant changes in the values of O_2^- (Z = 1.067; $P > 0.05$) (Fig. 4). Compared to the control group, values of all pro-oxidant markers were significantly increased in our study group, at admission (Table 3).

Level of Antioxidant Parameters Through Various Phases of Disease

CAT Values

The mean value of CAT in the oliguric phase was 27.01 ± 2.29 U/g Hb×10^3, with 95% confidence interval of 20.54 to 33.47 U/g Hb×10^3 and a median of 18. The empirical distribution of the values of CAT in the oliguric phase was significantly different from the theoretical normal curve ($P < 0.05$). At the phase of established diuresis, there was a decrease in the mean of CAT 20.20 ± 1.62 U/g Hb×10^3 and a median of 13.75 U/g Hb×10^3, which was not confirmed as a statistically significant decrease (Z = 1.041 $P > 0.05$) and the 95% confidence interval for the CAT values in the process of establishing diuresis was from 14.64 to 25.75 U/g Hb×10^3. Empirical distribution of CAT in the phase of established diuresis was significantly different from normal ($P < 0.05$). The mean value of CAT decreased in the polyuric phase to 17.13 ± 1.12 U/g Hb×10^3 with a median of 6.13 U/g Hb×10^3, which was not confirmed as a statistically significant decrease (Z = 1.707, $P > 0.05$), comparing to phase of established diuresis, and the 95% confidence interval was 8.07 – 26.19 U/g Hb×10^3 (Fig. 5).

SOD Values

The mean value of SOD in the oliguric phase was 1343.09 ± 145.29 U/g Hb×10^3, with 95% confidence interval of 925.72 to 1760.45 U/g Hb×10^3 and a median of 903.54 U/g Hb×10^3. The empirical distribution of the values of SOD in the oliguric phase was significantly different from the theoretical normal curve ($P < 0.05$). At the phase of established diuresis, there was a decrease in the mean of SOD 1212.13 ± 122.41 U/g Hb×10^3 and a median of 826.21 U/g Hb×10^3, which was not confirmed as a statistically significant decrease (Z = 0.754 $P > 0.05$) while the 95% confidence interval for the SOD values in the phase of established diuresis was from 789.11 to 1643.14 U/g Hb×10^3. Empirical distribution of SOD in the phase of established diuresis was significantly different from normal ($P < 0.05$). Although there was an increase in the value of SOD in the poliuric phase to 1564.69 ± 176.23 U/g Hb×10^3 with a median of 516.89 U/g Hb×10^3, statistically significant difference was not noticed comparing with the polyuric phase, with the 95% confidence interval from 811.08 to 2318.29 U/g Hb×10^3 (Fig. 6).

GSH Values

The mean value of GSH in the oliguric phase was 442.20 ± 9.93 nmol/ml, with 95% confidence interval of 416.87 to 467.53 nmol/ml and a median of 435.48 nmol/ml of erythrocytes. The empirical distribution of the values of GSH in the oliguric phase was significantly different from the theoretical normal curve ($P < 0.05$). At the phase of established diuresis, there was an increase in the mean of GSH 1148.05 ± 147.56 nmol/ml plasma and a median of 469.56 nmol/ml of erythrocytes, which was confirmed as a statistically significant increase (Z = 2.941, $P < 0.05$) and the 95% confidence interval for the values of GSH in the phase of established diuresis was from 258.36 to 2554.47 nmol/ml erythrocytes. Empirical

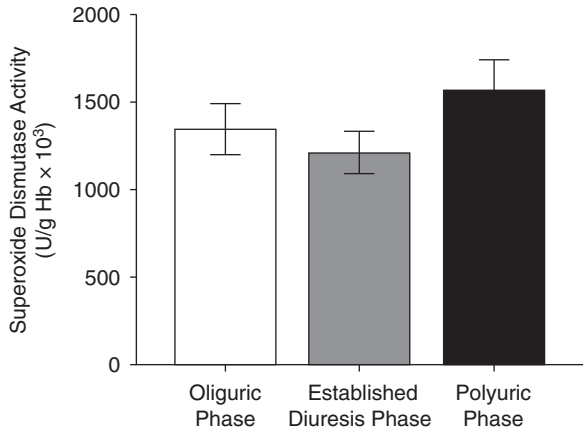

Fig. 6. Superoxide dismutase activity during different phases of AKI.

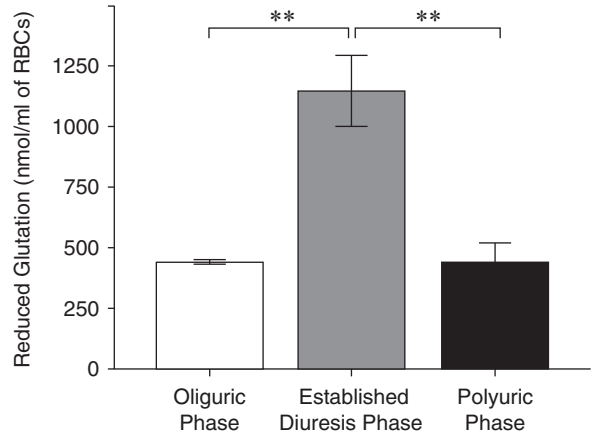

Fig. 7. Reduced glutation activity during different phases of AKI (**$P < 0.01$).

distribution of GSH in the phase of established diuresis was not significantly different from normal ($P > 0.05$), but had low borderline significance ($P > 0.05$). The mean value of GSH decreased in the polyuric phase to 439.98 ± 81.47 nmol/ml of erythrocytes with median of 439.27 nmol/ml of erythrocytes, which was confirmed as a statistically significant decrease ($Z = 2.898$, $P < 0.05$), comparing to phase of established diuresis, and the 95% confidence interval was 405.52 – 474.43 nmol/ml of erythrocytes (Fig. 7). When compared to the control group, the CAT values at admission were significantly decreased whereas the GSH and SOD values increased (Table 3).

Discussion

AKI can be triggered or aggravated by reactive oxygen species (ROS), but established AKI *per se* might also affect the antioxidant defense mechanisms of the organism (33). In that sense, increases in oxidative stress could be an important moment for nutritional and pharmacological effects of therapy in patients with AKI (33). Generation of ROS and NO in hypoxia-reperfusion injury may form a cytotoxic metabolite, peroxynitrite, which is capable of causing lipid peroxidation and DNA damage (28). Revascularization of the ischemic tissues may be followed by microvascular dysfunction, which leads to local and remote cellular and organ failure. In addition to numerous other mechanisms, pathophysiology of ischemia-reperfusion injury concerns impaired NO-mediated relaxation of smooth muscles and NO-O_2^- imbalance in endothelial cells tipped in favor of superoxide (2, 19, 20, 45).

Development of oxidative stress in acute renal ischemia-reperfusion has long been suggested (26, 39). Although, we did not find statistically significant changes in the levels of O_2^- in this study is well accepted that superoxide anions participate in cellular damage in an ischemia-reperfusion model, but the precise molecular species responsible for cellular damage remain uncertain. The relative lack of a detectable effect of SOD (Table 2) may be explained by its consumption in the first line of antioxidant protection, which correlates with statistically insignificant changes in O_2^- and H_2O_2 (Table 1).

NO production in the kidneys can be increased as a consequence of high blood pressure (HBP). Recently, up-regulation of iNOS mRNA in renal medullary tissue has been shown in HBP rats (47). Furthermore, Yu *et al.* (52) first suggested that inhibition of NOS in hypoxic proximal tubules results in improved cell survival. Deleterious effect of iNOS in ischemic acute kidney injury was previously demonstrated using antisense oligonucleotides targeting iNOS (37). These findings were further supported by the establishment of improved tubular cell viability after hypoxic insults in iNOS-deficient mice (27). It has been proposed that cellular damage is attributable to a powerful and cytotoxic oxidant peroxynitrite, which is generated by the diffusion-limited interaction of NO with superoxide. The reactivity of peroxynitrite is reported as pH-dependent (12). It is readily isomerized from stable *cis* to reactive *trans* peroxynitrous acid in acidic conditions like the reperfusion phase in an ischemic kidney. Among oxidative reactions of peroxynitrite, its hydroxyl radical-like reactivity is extremely potent (3) and may lead to the propagated lipid peroxidation. Because hydroxyl radicals convert virtually any organic molecule to the corresponding free radicals, and because they can be particularly damaging to cell membranes rich in polyunsaturated fatty acids, the initiation of free radical chain reactions based on abstraction of the allylic hydrogen is highly plausible (16). These reactions should amplify cell damage. One of the oxidative

Table 1. Values of prooxidant parameters during different phases of AKI

	Oliguric Phase	Established Diuresis Phase	Polyuric Phase
	Mean ± SD	Mean ± SD	Mean ± SD
TBARS	2.91 ± 0.22	4.00 ± 0.44[a]	4.62 ± 0.88[a]
NO_2^-	14.95 ± 1.79	19.41 ± 0.88[b]	16.90 ± 1.21[a]
O_2^-	7.65 ± 1.54	10.16 ± 1.03	8.30 ± 1.57
H_2O_2	5.47 ± 1.28	6.23 ± 1.11	7.89 ± 1.26

Mean ± SD represents average value ± standard deviation. [a]$P < 0.05$ compared to admission – oliguric phase. [b]$P < 0.01$ compared to admission – oliguric phase

reactions initiated by peroxynitrite is the nitration of phenolic rings (4). Both NO and superoxide are required for the generation of peroxynitrite. Inhibition of either molecular species with SOD (antioxidants) was associated with improved renal function and decreased oxidative damage to lipids. An additive effect could be achieved by combining antioxidants and its buffered compounds, further supporting the notion that the observed cytotoxicity was due to the generation of peroxynitrite and other prooxidant species in ischemic-reperfused kidneys.

Apart from scavenging peroxynitrite, GSH and its adjuvant substances have been proposed to inhibit the peroxidation of membrane phospholipid, to inhibit lipoxygenase in the arachidonate cascade, to block the production of superoxide anions from activated leukocytes, inhibit iNOS, and thus exhibits a sustained protective effect against peroxynitrite (50).

Amelioration of renal dysfunction in ischemic kidneys provides not only a strong argument in favor of the proposed prooxidant-driven mechanism of lipid peroxidation and DNA damage but also introduces antioxidants as a potential therapeutic tool in ameliorating reperfusion injury (30). The data presented herein demonstrate the role of oxidative and nitrosative stress, leading to possible peroxynitrite formation in renal damage and confirm the utility of the developed method for the measurement of oxidative stress, escorted to the degree of oxidative/nitrosative damage.

Analyzing the dynamics of oxidative stress parameters (TBARS, O_2^-, H_2O_2 and NO_2^-) as well as the response of the antioxidant system (SOD, CAT and GSH), it is observed that the changes are due to ischemia/reperfusion injury and are characterized by an increased production of free radicals (TBARS and NO_2^-, Table 1) that can lead to damage of cell membranes and increased lipid peroxidation (40). This dynamic for these two parameters is different: TBARS is continuously increased after established diuresis in polyuric phases (Table 1, Fig. 1), while nitrite levels returned to the levels similar to the oliguric phase in the polyuric phase after significant increases in the phase of established diuresis (Table 1, Fig. 2). This nitrite dynamic is more likely to be a consequence of typical ischemia/reperfusion injury (8). Unfortunately, TBARS levels has led us to conclude that the antioxidative defense system cannot scavenge increased production of ROS in polyuric phases, which lead in continuously increased oxidative stress (Table 1, Fig. 1).

In our study, the values of all pro-oxidant markers (TBARS, NO_2^-, O_2^-, and H_2O_2) were significantly increased compared to the control group of patients, which can be expected, and may indicate that in the first (oliguric phase) of AKI, production of free radicals is prominent, compared to both controls (Table 3) and other phases (Table 1) of this pathophysiological state. This result is in correlation with findings of Mishra and coworkers (34), who recorded raised levels of lipid peroxidation products (malondialdehyde) and nitrites in children with AKI in comparison with controls. They also suggested that cutoff levels of plasma nitrite might be most accurate in predicting mortality in ARF patients (34).

Schuck *et al.* reported increased kidney level of TBARS in a rat model of AKI (44). Other authors also showed rises in values of both TBARS and nitrites in the rat model of rat mercuric chloride-induced nephrotoxicity (17). Moreover, studies on human noted increases in values of TBARS, nitrites, nitrate and their sum (NOx) in patients with unilateral nephrectomy (53). Findings of this investigations could, generally, suggest that regardless of human or animal studies, ROS may be increased in both plasma and tissues in AKI patients.

During all phases, the levels of other ROS in the plasma, such O_2^- and H_2O_2, were not significantly changed compared to admission (Table 1, Figs. 3 and 4). These results refer to systemic levels of ROS, but the same could have been generated locally in reliable amounts in a phase-dependent manner and causes tissue damages. Thus, these findings suggest no changes in the plasma values of O_2^- and H_2O_2 in these patients, without data about ROS production in kidneys itself, which may have important role in this pathophysiological phenomenon.

The levels of both measured antioxidative en-

Table 2. Values of antioxidant parameters during different phases of AKI

	Oliguric Phase	Established Diuresis Phase	Polyuric Phase
	Mean ± SD	Mean ± SD	Mean ± SD
CAT	27.01 ± 2.29	20.20 ± 1.62	17.13 ± 1.12
SOD	1343.09 ± 145.29	1212.13 ± 122.41	1564.69 ± 176.23
GSH	442.20 ± 9.93	1148.05 ± 147.56 [b]	439.98 ± 81.47[c]

Mean ± SD represents average value ± standard deviation. [b]$P < 0.01$ compared to admission – oliguric phase. [c]$P < 0.01$ compared to established diuresis

Table 3. Comparison of the status of free radicals and antioxidants in healthy controls and AKI patients

	Controls	AKI Patients at Admission (Oliguric Phase)
	Mean ± SD	Mean ± SD
TBARS	1.06 ± 0.17	2.91 ± 0.22[a]
NO_2^-	25.09 ± 1.64	14.95 ± 1.79[b]
O_2^-	3.04 ± 0.38	7.65 ± 1.54[b]
H_2O_2	2.39 ± 0.13	5.47 ± 1.28[a]
CAT	38.85 ± 2.88	27.01 ± 2.29[a]
SOD	643.46 ± 200.63	1343.09 ± 145.29[b]
GSH	93.290 ± 2.45	442.20 ± 9.93[b]

Mean ± SD represents average value ± standard deviation. [a]$P < 0.05$ control compared to admission (oliguric phase). [b]$P < 0.01$ control compared to admission (oliguric phase)

zymes (SOD and CAT) supported that data, because the dynamic of both enzymes was not significantly changed in our investigation (Table 2, Figs. 5 and 6). On the other hand, only the dynamic changes of GSH may be an indicator of adequate response to the specific disease process and reperfusion phenomenon. In fact, during the second phase of the disease (established diuresis), GSH dramatically increased (Table 2, Fig. 7), with consequent drop in the polyuric phase, what exhibits similar dynamic with nitrites and suggest appropriate response to ischemia/reperfusion injury. Data regarding GSH are particularly interesting when considering that the first line of defense (SOD and CAT) did not change significantly. Our results suggest the contribution of other reactive species to the global oxidative damage from acute kidney injury when considering the 'quiescence' of the cascade O_2^-/SOD/ H_2O_2/CAT.

Compared to controls, patients with AKI at admission had significantly decreased values of CAT, while GSH and SOD values increased (Table 3). One of possible explanations for this result may be compensatory response of anti-oxidative defense system (Table 2) in the first (oliguric) phase of AKI, when oxidative stress is increased (Table 1). Other studies also pointed out changes in activity of antioxidative enzymes during AKI. Metnitz *et al.* have found pronounced depression of antioxidative system activity (CAT, SOD and glutathione-peroxidase) in patients with multiple organ failure (MOF) and associated AKI (33).

Our results clearly suggest that redox (im)balance may in part play role in acute kidney injury, similar with our previous experience in other pathophysiological conditions. Namely, we found increased levels of pro-oxidants and lower antioxidant activity in patients with acute coronary syndromes (ACS) (25), as well as in the maternal and fetal bloods of pregnancies with fetal distress (43).

However, in the present investigation, it seems that the O_2^-/SOD/H_2O_2/CAT cascade was not a major player, while we noticed significant role of lipid peroxidation, nitrites and compensation through increase of GSH level as a major intracellular antioxidant. Relative limitation of the study was the absence of some other measured parameters such peroxynitrite ($ONOO^-$) and oxidized glutathione (GSSG). Results from the present paper may be of clinical importance regarding better understanding the role of oxidative stress in the pathogenesis of acute kidney injury, and thus improvement in the treatment of such complex pathophysiological phenomenon, in the sense of involvement of anti-oxidative adjuvant therapy in these patients.

Acknowledgments

This work was supported by the Grant N°

175043 by the Ministry of Education, Science and Technical Development of the Republic of Serbia and Junior Project N° 04/11 the Faculty of Medical Sciences, University in Kragujevac.

References

1. Auclair, C. and Voisin, E. Nitroblue tetrazolium reduction. In: *Handbook of Methods for Oxygen Radical Research*. Edited by Greenvvald, R.A., Ine: Boka Raton, CRC Press, pp. 123-132, 1985.
2. Banda, M.A., Lefer, D.J. and Granger, D.N. Postischemic endothelium-dependent vascular reactivity is preserved in adhesion molecule-deficientmice. *Am. J. Physiol.* 273: H2721-H2725, 1997.
3. Beckman, J.S., Beckman, T.W., Chen, J., Marshall, P.A. and Freeman, B.A. Apparent hydroxyl radical production by peroxynitrite: implications for endothelial injury from nitric oxide and superoxide. *Proc. Natl. Acad. Sci. USA* 87: 1620-1624, 1990.
4. Beckman, J.S., Ischiropoulos, H., Zhu, L., van der Woerd, M., Smith, C., Chen, J., Harrison, J., Martin, J.C. and Tsai, M. Kinetics of superoxide dismutase- and iron-catalyzed nitration of phenolics by peroxynitrite. *Arch. Biochem. Biophys.* 298: 438-445, 1992.
5. Beutler, E. Reduced glutathione (GSH). In: *Red Cell Metabolism, a Manual of Biochemical Methods*. Edited by Beutler, E. New York: Grune and Stratton, pp. 112-114, 1975.
6. Beutler, E. Catalase. In: *Red Cell Metabolism, a Manual of Biochemical Methods*. Edited by Beutler, E. New York: Grune and Stratton 1982, pp. 105-106.
7. Block, G., Dietrich, M., Norkus, E.P., Morrow, J.D., Hudes, M., Caan, B. and Packer, L. Factors associated with oxidative stress in human populations. *Am. J. Epidemiol*. 156: 274-285, 2002.
8. Bonfoco, E., Krainc, D., Ankarcrona, M., Nicotera, P. and Lipton, S.A. Apoptosis and necrosis: two distinct events induced, respectively, by mild and intense insults with *N* methyl-D-aspartate or nitric oxide/superoxide in cortical cell cultures. *Proc. Natl. Acad. Sci. USA* 92: 7162-7166, 1995.
9. Brady, H.R. and Brenner, B.M. Acite renal failure. In: *Harrison's Principles of Internal Medicine*. Edited by Isselbacher, K.J., Braunwald, E., Wilson, J.D., Martin, J.B., Fauci, A.S., Kasper, D.I. eds. 13th edition. New York, St. Louis, San Francisco: McGraw-Hill Inc., pp. 1265-1274, 1994.
10. Chen, Y.H. and Tarng, D.C. Profound urinary protein loss and acute kidney injury caused by cyclooxygenase-2 inhibitor. *Chinese J. Physiol.* 54: 264-268, 2011.
11. Cooper, D.M., Nemet, D. and Galassetti, P. Exercise, stress, and infammation in the growing child: from the bench to the playground. *Curr. Opin. Pediatr*. 16: 286-292, 2004.
12. Crow, J.P., Spruell, C., Chen, J., Gunn, C., Ischiropoulos, H., Tsai, M., Smith, C.D., Radi, R., Koppenol, W.H. and Beckman, J.S. On the pH-dependent yield of hydroxyl radical products from peroxynitrite. *Free Radic. Biol. Med.* 16: 331-338, 1994.
13. Dalle-Donne, I., Rossi, R., Colombo, R., Giustarini, D. and Milzani, A. Biomarkers of oxidative damage in human disease. *Clin. Chem*. 52: 601-623, 2006.
14. Ejaz, A., Mu, W., Kang, D.H., Roncal, C., Sautin, Y., Henderson, G., Tabah-Fisch, I., Keller, B., Beaver, T.M., Nakagawa, T. and Johnson, R.J. Could uric acid have a role in Acute kidney injury? *Clin. J. Am. Soc. Nephrol.* 2: 16-21, 2007.
15. Fisher-Wellman, K. and Bloomer, R.J. Acute exercise and oxidative stress: a 30 year history. *Dyn. Med.* 8: 1-25, 2009.
16. Fridovich, I. and Freeman, B. Antioxidant defenses in the lung. *Annu. Rev. Physiol.* 48: 693-702, 1986.
17. Gado, A.M. and Aldahmash, B.A. Antioxidant effect of Arabic gum against mercuric chloride-induced nephrotoxicity. *Drug Des. Devel. Ther.* 7: 1245-1252, 2013.
18. Goldberg, R. and Dennen, P. Long-term outcomes of acute kidney injury. *Adv. Chronic. Kidney* 15: 297-307, 2008.
19. Granger, D.N. Ischemia-reperfusion: mechanisms of microvascular dysfunction and the influence of risk factors for cardiovascular disease. *Microcirculation* 6: 167-178, 1999.
20. Granger, D.N. Physiology and pathophysiology of the microcirculation. *Prog. Cardiovasc. Med.* 3: 123-140, 1998.
21. Green, L.C., Wagner, D.A., Glogowski, J., Skipper, P.L., Wishnok, J.S. and Tannenbaum, S.R. Analysis of nitrate, nitrite and [15N] nitrate in biological fluids. *Anal. Biochem.* 126: 131-138, 1982.
22. Ichikawa, I., Kiyama, S. and Yoshioka, T. Renal antioxidant enzymes: their regulation and function. *Kidney. Int.* 45: 1-9, 1994.
23. Jacob, C. and Winyard, P.G. Redox signaling and regulation in biology and medicine. WILETVCH Verlag Gmbh &Co, Weinheim, 2009.
24. Juránek, I. and Bezek, Š. Controversy of free radical hypothesis: reactive oxygen species – cause or consequence of tissue injury? *Gen. Physiol. Biophys.* 24: 263-278, 2005.
25. Koprivica, Z., Djordjevic, D., Vuletic, M., Zivkovic, V., Barudzic, N., Andjelkovic, N., Djuric, D., Iric-Cupic, V., Krkeljic, J. and Jakovljevic, V. Von Willebrand factor and oxidative stress parameters in acute coronary syndromes. *Oxid. Med. Cell. Longev*. 2011: 918312, 2011.
26. Linas, S., Whittenburg, D., Parsons, P. and Repine, J.E. Ischemia increases neutrophil retention and worsens acute kidney injury: role of oxygen metabolites and ICAM 1. *Kidney Int.* 48: 1584-1591, 1995.
27. Ling, H., Gengaro, P.E., Edelstein, C.L., Martin, P.Y., Wangsiripaisan, A., Nemenoff, R. and Schrier, R.W. Effect of hypoxia on proximal tubules isolated from nitric oxide synthase knockout mice. *Kidney Int.* 53: 1642-1646, 1998.
28. Liu, Y. and Chen, L. Mechanisms in adaptation to brain ischemia by ischemic preconditioning. *Adapt. Med.* 4: 20-26, 2012.
29. Lo, L.J., Go, A.S., Chertow, G.M., McCulloch, C.E., Fan, D., Ordonez, J.D. and Hsu, C.Y. Dialysis-requiring acute kidney injury increases the risk of progressive chronic kidney disease. *Kidney Int.* 76: 893-899, 2009.
30. Marnett, L.J. Oxyradicals and DNA damage. *Carcinogenesis* 21: 361-370, 2000.
31. McCord, J.M. and Fridovich, I. The utility of superoxide dismutase in studying free radical reactions. Radicals generated by the interaction of sulfite, dimethyl sulfoxide, and oxygen. *J. Biol. Chem.* 244: 6056-6063, 1969.
32. McCullough, M.A. and Ahmad, A. Cardiorenal syndromes. *World J. Cardiol.* 3: 1-9, 2011.
33. Metnitz, G.H., Fischer, M., Bartens, C., Steltzer, H., Lang, T. and Druml, W. Impact of acute renal failure on antioxidant status in multiple organ failure. *Acta Anaesthesiol. Scand.* 44: 236-240, 2000.
34. Mishra, O.P., Pooniya, V., Ali, Z., Upadhyay, R.S. and Prasad, R. Antioxidant status of children with acute renal failure. *Pediatr. Nephrol.* 23: 2047-2051, 2000.
35. Misra, H.P. and Fridovich, I. The role of superoxide-anion in the autooxidation of epinephrine and a simple assay for superoxide dismutase. *J. Biol. Chem.* 247: 3170-3175, 1972.
36. Møller, P., Wallin, H. and Lisbeth, E.K. Oxidative stress associated with exercise, psychological stress and life-style factors. *Chem-Biol. Interact.* 1: 17-36, 1996.
37. Noiri, E., Peresleni, T., Miller, F. and Goligorsky, M.S. *In vivo* targeting of inducible NO synthase with oligodeoxynucleotides protects rat kidney against ischemia. *J. Clin. Invest.* 97: 2377-2383, 1996.
38. Ohkawa, H., Ohishi, N. and Yagi, K. Assay for lipid peroxides in animal tissues by thiobarbituric acid reaction. *Anal. Biochem.* 95: 351-358, 1979.
39. Palipoch, S. A review of oxidative stress in acute kidney injury: protective role of medicinal plants-derived antioxidants. *Afr. J. Tradit. Complement. Altern. Med.* 16: 88-93, 2013.

40. Paller, M.S., Hoidal, J.R. and Ferris, T.F. Oxygen free radicals in ischemic acute kidney injury in the rat. *J. Clin. Invest.* 74: 1156-1164, 1984.
41. Peter, M. Tiidus radical species in inflammation and overtraining. *Can. J. Physiol. Pharmacol.* 76: 533-538, 1998.
42. Pick, E. and Keisari, Y. A simple colorimetric method for the measurement of hydrogen peroxide produced by cells in culture. *J. Immunol. Methods* 38: 161-170, 1980.
43. Raicević, S., Cubrilo, D., Arsenijević, S., Vukcević, G., Zivković, V., Vuletić, M., Barudzić, N., Andjelković, N., Antonović, O. and Jakovljević, V. Oxidative stress in fetal distress: Potential prospects for diagnosis. *Oxid. Med. Cell. Longev.* 3: 214-218, 2010.
44. Schuck, P.F., Alves, L., Pettenuzzo, L.F., Felisberto, F., Rodrigues, L.B., Freitas, B.W., Petronilho, F., Dal-Pizzol, F., Streck, E.L. and Ferreira, G.C. Acute renal failure potentiates methylmalonate-induced oxidative stress in brain and kidney of rats. *Free Radic. Res.* 47: 233-240.
45. Skalak, R. and Skalak, T.C. Flow behavior of leukocytes in small tubes. In: *Physiology and Pathophysiology of Leukocyte Adhesion.* Edited by Granger, D.N., Schmid-Schonbein, G.W. New York: Oxford University Press, pp. 97-115, 1995.
46. Szocs, K. Endothelial dysfunction and reactive oxygen species production in ischemia/reperfusion and nitrate tolerance. *Gen. Physiol. Biophys.* 23: 265-295, 2004.
47. Tan, D., Tan, S., Zhang, J., Weihua, Z. and Wu, S. Association of sodium induced high blood pressure with up-regulation of inducible nitric oxide synthase (iNOS) expression and inhibition of trimethylation of H3K27 on its promoter region. *Adapt. Med.* 4: 209-215, 2012.
48. Valko, M., Leibfritz, D., Moncol, J., Cronin, M.T., Mazur, M. and Telser, J. Free radicals and antioxidants in normal physiological functions and human disease. *Int. J. Biochem. Cell. Biol.* 39: 44-84, 2007.
49. Witko Sarsat, V., Friendlander, M., Capeillere Blandin, C. Nguyen-Khoa, T., Nguyen, A.T., Zingraff, J., Jungers, P. and Descamps-Latscha, B. Advanced oxidation protein products as a novel marker of oxidative stress in uremia. *Kidney Int.* 49: 1304-1313, 1996.
50. Yamaguchi, T., Sano, K., Takakura, K., Saito, I., Shinohara, Y., Asano, T. and Yasuhara, H. Ebselen in acute ischemic stroke; a placebo-controlled, double-blind clinical trial. *Stroke* 29: 12-17, 1998.
51. Yenilmez, A., Isikli, B., Aral, E., Degirmenci, I., Sutken, E. and Baycu, C. Antioxidant effects of melatonin and coenzyme Q10 on oxidative damage caused by single-dose ochratoxin A in rat kidney. *Chinese J. Physiol.* 53: 310-317, 2010.
52. Yu. L., Gengaro Niederberger, P.M., Burke, T. and Schrier, R.W. Nitric oxide: a mediator in rat tubular hypoxia/reoxygenationinjury. *Proc. Natl. Acad. Sci. USA* 91: 1691-1695, 1994.
53. Zunić, G., Tomić, A. and Spasić, S. Unilateral nephrectomy causes an early abrupt decrease in plasma arginine and simultaneous reduction in glomerular filtration rate in living kidney donors. *Clin. Biochem.* 46: 1394-1398, 2013.