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OXIDATIVE AND NITROSATIVE STRESS IN STABLE RENAL TRANSPLANT RECIPIENTS WITH RESPECT TO THE IMMUNOSUPPRESSION PROTOCOL – DIFFERENCES OR SIMILARITIES?

OKSIDATIVNI I NITROZATIVNI STRES U ODNOSU NA IMUNOSUPRESIVNI PROTOKOL KOD PACIJENATA SA STABILNOM FUNKCIJOM PRESAĐENOG BUBREGA – RAZLIKE I SLIČNOSTI

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Summary

Background: The aim of the study was to evaluate parameters of oxidative and nitrosative stress as well as antioxidative parameters in a group of renal transplant recipients with stable graft function and no clinical signs of cardiovascular disease. We also aimed to determine the correlations among these parameters and to evaluate potential differences in all the biomarkers with regard to the immunosuppression protocol.
Methods: We enrolled 57 renal transplant recipients and 31 controls who were age and sex matched with the renal transplant recipients. All of the patients included in this study had post-renal transplant surgery at least 12 months earlier and were on standard immunosuppressive therapy. In this study, we determined thiobarbituric acid-reactive substances in plasma and red blood cells and advanced oxidation protein products, nitrosative stress parameters (asymmetric and symmetric dimethylarginine – ADMA and SDMA), and antioxidative parameters (total SH groups and catalase activity).
Results: The results of our study demonstrated that the levels of oxidative and nitrosative stress were significantly increased compared to the healthy population ($p<0.01$

Kratak sadržaj

Uvod: Transplantacija bubrega sama po sebi popravlja bubrežnu funkciju, ali ne dovodi do potpunog oporavka. Cilj ovog rada bio je da se odrede parametri oksidativnog i nitrozativnog stresa kao i parametri antioksidativne zaštite u populaciji pacijenata sa presađenim bubregom sa stabilnom funkcijom grafta i bez kliničkih znakova kardiovaskularne bolesti. Takođe, naš cilj je bio da se utvrdi povezanost među ispitivanim parametrima i procene potencijalne razlike između svih ispitivanih biomarkera u odnosu na imunosupresivni protokol.
Metode: U istraživanje je uključeno 57 pacijenata sa presađenim bubregom i 31 kontrolni subjekt, koji su po godinama i polu odgovarali pacijentima sa presađenim bubregom. Svi pacijenti uključeni u istraživanje imali su transplantaciju bubrega najmanje 12 meseci pre početka istraživanja i bili su na standardnoj imunosupresivnoj terapiji. U ovom radu određivali smo reaktivne supstance tiobarbituratne kiseline (TBARS) u plazmi i eritrocitima, uznapredovale produkte oksidacije proteina (AOPP), parametre nitrozativnog stresa (asimetrični i simetrični dimetilarginin – ADMA i SDMA) i antioksidativne zaštite (ukupne SH grupe i aktivnost katalaze).

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except for plasma catalase activity $p<0.05$). Correlation analysis showed significant positive correlations between: ADMA and SDMA ($p<0.01$); ADMA and nitrates ($p<0.05$); SDMA and nitrates ($p<0.05$); between OS parameters in the experimental group; AOPP and SH groups ($p<0.05$) and TBARS in plasma and SH groups ($p<0.01$), SDMA and AOPP ($p< 0.05$); SDMA and TBARS in plasma ($p<0.05$); SDMA and SH groups ($p<0.01$); nitrates and SH groups ($p<0.05$).

Conclusion: There was no significant difference in oxidative and nitrosative stress parameters with respect to the immunosuppressive protocol.

Keywords: ADMA, SDMA, oxidative stress, nitrosative stress, renal transplantation

Rezultati: Rezultati naše studije su pokazali znatno više vrednosti parametara oksidativnog i nitrozativnog stresa kod pacijenata sa presađenim bubregom u odnosu na zdrave dobrovoljce ($p<0,01$ osim za aktivnost katalaze u plazmi, kada je $p<0,05$). Korelaciona analiza pokazala je značajnu pozitivnu korelaciju između ADMA i SDMA ($p<0,01$); ADMA i nitrata ($p<0,05$); SDMA i nitrata ($p<0,05$); između parametara oksidativnog stresa u eksperimentalnoj grupi; AOPP i SH grupe ($p<0,05$), kao i TBARS u plazmi i SH grupe ($p<0,01$), SDMA i AOPP ($p<0,05$); SDMA i TBARS u plazmi ($p<0,05$); SDMA i SH grupe ($p<0,01$); nitrata i SH grupe ($p<0,05$).

Zaključak: Naši rezultati nisu ukazali na statistički značajnu razliku u ispitivanim parametrima među pacijentima na ciklosporinu A i takrolimusu.

Ključne reči: ADMA, SDMA, oksidativni stres, nitrozativni stres, transplantacija bubrega

Introduction

Kidney transplantation improves long-term survival compared to maintenance dialysis and is the treatment of choice for patients with end stage renal disease (1). The most frequent cause of late allograft loss is cardiovascular disease (CVD) which constitutes the leading cause of death (2). In fact, when compared with the general population, renal transplant recipients show a four-fold greater risk for CVD and a two-fold higher risk for cardiovascular death (3). Renal transplant recipients mainly have a history of uremia and dialysis, usually associated with increased oxidative stress (OS), and therefore carry the burden of accelerated atherosclerosis already at the time of transplantation (4, 5). Uremia itself could be a condition associated with increased OS (6) or a pathological condition able to induce an accumulation of oxidant species (7). Reactive oxygen species (ROS) highly induce tissue damage which results in accumulation of reactive aldehydes, lipid peroxidation, increased plasma thiol oxidation and DNA damage (7, 8). The recovery of renal function after transplantation results in amelioration of the biomarkers of inflammation and OS (4). This improvement might be explicated by the restored clearance of uremic toxins, the regression of left ventricular hypertrophy and better nutritional status (9). But various studies have shown that even after transplantation there is still imbalance between ROS and antioxidative mechanisms that results in OS (10).

ROS may directly alter proteins which results in formation of oxidized amino acids (11). Proteolysis of proteins containing methylarginine residues results in an increased plasma concentration of asymmetric dimethylarginine (ADMA) (12). ADMA is a competitive inhibitor of nitric oxide synthase (NOS) and may be an intermediate mechanism whereby OS impairs endothelial function (13). Finally, it has been demonstrated that ADMA predicts incident cardiovascular events in patients with renal diseases (14). Besides ADMA, there are also elevated plasma levels of symmetric dimethylarginine (SDMA) in patients suffering from renal disease (15, 16). Transplantation by itself reduces SDMA levels, but its effect on ADMA is still questioned (17, 18).

Advanced oxidation protein products (AOPP) are protein biomarkers for oxidative stress for patients with uremia (19). Plasma concentrations of AOPP increase with the progression of chronic renal failure and are able to trigger the synthesis of inflammatory cytokines acting as inflammatory mediators in renal patients (20).

Standard immunosuppressive therapy usually consists of triple drug therapy using three drug classes: calcineurin inhibitors (cyclosporine or tacrolimus), antiproliferative agents and corticosteroids. Some studies reported that increased levels of malondialdehyde are a consequence of immunosuppressive therapy and that OS is induced mostly by cyclosporine A therapy (21).

The aim of the study was to evaluate parameters of oxidative stress (OS); lipid peroxidation markers; thiobarbituric acid-reactive substances (TBARS) in plasma and red blood cells (RBC), as well as advanced oxidation protein products (AOPP) and nitrosative stress (NS) parameters (ADMA, SDMA and NOx) and antioxidative parameters (total SH groups and catalase activity) in a group of renal transplant recipients with stable graft function and no clinical signs of cardiovascular disease. We also aimed to determine the correlations among all these parameters and to evaluate potential differences in all the biomarkers with regard to the immunosuppression protocol.

Materials and Methods

The study was conducted between March and November 2012 at the Clinic for Nephrology, Dialysis and Transplantation, Clinical Centre Nis. During this period, 57 renal transplant recipients were enrolled,

all of whom had undergone post-renal transplant surgery at least 12 months prior to enrollment and were on standard immunosuppressive therapy. Patients with any signs of graft rejection or overt cardiovascular disease were deferred. The control group (12 men and 19 women), mean age 45±8.53, was recruited from the medical staff who were age and sex matched with the renal transplant recipients.

The study was approved by the Ethics Committee of Medical Faculty Nis and informed written consent was obtained from each patient. The study included 38 men and 19 women, mean age 44.08± 11.32 years, with 4 years median time from transplantation (range 1–24 years). Regarding the type of transplantation, 26.02% were from a deceased donor and 73.97% were living donor related. Among the patients, 32.88% were smokers. Regarding the history of primary disease, 31.5% had diabetes mellitus (type 1+2) and 86.3% had hypertension (or had an antihypertensive therapy). Immunosuppressive therapy included: calcineurin inhibitors, mycophenolate mofetil (MMF) and corticosteroids. The first group, 40.35% of patients, were treated with cyclosporine A (3 mg/d) in combination with MMF (1.5–2 g/d) and prednisone (range 5–20 mg/d) and the second group, 59.65% of patients, were treated with tacrolimus (0.05–0.1 mg/d) with MMF and prednisone as a standard regimen.

Biochemical measurements were obtained using standard clinical laboratory methods and analyses performed on the Erba XL600, Germany. C-reactive protein (CRP) was measured using immunonephelometric assays (Olympus AU400). Serum, plasma and isolated RBC were used for the determination of OS and NS parameters.

TBARS content was assayed in plasma and RBC according to the methods of Andreeva and Jain, respectively (22, 23). The catalase activity was determined by the spectrophotometric method, based on the ability of hydrogen peroxide to form a stable stained complex with molybdenum salts (24). AOPP were determined in plasma mixed with H_2O, acetic acid and potassium iodide. The absorbance was read spectrophotometrically at 340 nm and compared with a solution of chloramine T dissolved in the same buffer. The data were expressed as μmol/L of chloramine equivalents and related plasma total protein (19).

The amount of total (protein and non-protein) sulfhydryl (SH) groups was estimated in plasma by the spectrophotometric assay based on reduction of 2,2-dithiobisnitrobenzoic acid (DTNB), the absorbance was read at 412 nm, and the results were expressed as μmol/L (25, 26). After deproteinization, the production of NO• was evaluated by measuring nitrite and nitrate concentrations. Nitrites were assayed directly spectrophotometrically at 543 nm, using the colorimetric method of Griess (Griess reagent: 1.5% sulfanilamide in 1 mol/L HCl plus 0.15% N-(1-naphthyl) ethylenediamine dihydrochloride in distilled water). However, nitrates were previously transformed into nitrites by cadmium reduction (27). Chemicals were purchased from Sigma (St. Louis, MO, USA).

ADMA and SDMA were evaluated by high-performance liquid chromatography on an apparatus (Agilend) using fluorimetric detection with fluorescence detection according to the method developed by Paroni et al. (28).

Statistical Analysis

Characteristics of the study group were expressed as mean ± SD for normal distribution or median (interquartile range) for non-normal distribution, or with frequency and percentage for categorical data. Clinical and biochemical data of the renal transplant recipients and the control group were compared by using Student t-test for normally distributed data and Mann-Whitney U test for data that were not normally distributed. The relationship between two variables was determined by Pearson's correlation coefficient (r). All analyses were performed with SPSS statistical analysis software, version 10.0 (SPSS, Chicago, IL, United States) at a significance level set at $p<0.05$.

Results

Clinical and biochemical data of the renal transplant recipients are presented in *Table I*. Significant differences were found between experimental and control group in BMI ($p=0.045$), hemoglobin ($p=0.043$), white blood cells ($p=0.006$), total cholesterol ($p<0.001$), LDL-cholesterol ($p<0.001$), triglycerides ($p<0.001$), urea ($p=0.043$) and creatinine ($p<0.001$). *Table II* presents differences between oxidative and nitrosative stress parameters: plasma catalase ($p=0.013$), SH groups ($p<0.001$), AOPP ($p=0.003$), ADMA ($p<0.001$), SDMA ($p<0.001$), nitrates ($p=0.005$) and TBARS in plasma ($p<0.001$) and in RBC ($p<0.001$).

Correlation analysis showed a significant positive correlation between ADMA and SDMA ($r=0.650$, $p<0.001$); ADMA and nitrates ($r=0.453$, $p=0.020$); SDMA and nitrates ($r=0.508$, $p=0.008$). There was also a statistically significant positive correlation between OS parameters in experimental group: AOPP and SH groups ($r=0.401$; $p=0.038$) and TBARS in plasma and SH groups ($r=0.575$, $p=0.001$) (*Table III*), SDMA and AOPP ($r=0.412$, $p=0.037$); SDMA and TBARS in plasma ($r=0.413$, $p=0.036$); SDMA and SH groups ($r=0.537$, $p=0.005$); nitrates and SH groups ($r=0.376$, $p=0.049$) (*Table III*). There was no significant difference in NS parameters (ADMA, SDMA and nitrates) in the group of renal transplant recipients with respect

Table I Clinical and biochemical data of the renal transplant recipients and the control.

	Renal transplant recipients	Control group	p
Number of participants	57	31	
Age	44±11	45±9	
Sex (male/female)	38/19	12/19	0.044
BMI (kg/m^2)	26±4	24±3	0.045
Hemoglobin (g/L)	129±17	136±12	0.043
WBC (total count) × 10^9/L	8.33±3.01	6.80±1.63	0.006
Neutrophils (%)	66±10	61±7	0.013
Total cholesterol (mmol/L)	6.18±2.13	4.72±0.90	<0.001
LDL-cholesterol (mmol/L)	3.65±1.05	2.82±0.72	<0.001
HDL-cholesterol (mmol/L)	1.33±0.33	1.39±0.33	0.507
Triglycerides (mmol/L)	2.48±1.15	1.18±0.48	<0.001
Urea (mmol/L)	8.87±3.72	4.52±1.18	<0.001
Creatinine (μmol/L)	140.55±83.34	70.81±14.15	<0.001

Data are expressed as mean ± SD.

BMI, Body mass index; RBC, red blood cells; WBC, white blood cells; LDL, low density cholesterol; HDL, high density cholesterol.

Table II Oxidative and nitrosative stress parameters in renal transplant recipients compared to control group.

Parameter	Renal transplant recipients	Control group	p
Catalase (plasma) (U/L)	397±187	294 ±146	0.013
SH groups (μmol/L)	213±122	142±26	<0.001
AOPP (μmol/L)	46.56±38.06	27.49±16.21	0.003
ADMA (μmol/L)	0.75±0.22	0.46±0.15	<0.001
SDMA (μmol/L)	2.32±0.90	0.77±0.14	<0.001
Nitrates (NO_2/NO_3) (μmol/L)	25.41±13.40	17.31±9.81	0.005
TBARS (plasma) μmol/L	10.75±3.21	7.48±1.64	<0.001
TBARS (RBC) nmol/mL RBC	5.95±2.43	4.01±1.71	<0.001

Data are expressed as mean ± SD.

ADMA, Asymmetric dimethylarginine; SDMA, symmetric dimethylarginine; AOPP, advanced oxidation protein products; TBARS, thiobarbituric acid-reactive substances; RBC, red blood cells.

Table III Correlation analysis between dimethylarginines and oxidative stress parameters in renal transplant recipients (results are expressed as correlation coefficients).

	ADMA	SDMA	AOPP	Nitrates	TBARS (plasma)	TBARS (RBC)	Catalase (plasma)	SH groups
ADMA	–	0.650**	0.125	0.453*	0.313	0.134	0.028	0.346
SDMA		–	0.412*	0.508*	0.413*	0.259	0.083	0.537**
AOPP			–	0.172	0.347	–0.121	–0.047	0.401*
Nitrates				–	0.254	0.198	0.124	0.376*
TBARS plasma					–	0.267	–0.011	0.575**
TBARS (RBC)						–	0.349	0.293
Catalase plasma							–	0.367

ADMA, Asymmetric dimethylarginine; SDMA, symmetric dimethylarginine; AOPP, advanced oxidation protein products; TBARS, thiobarbituric acid-reactive substances; RBC, red blood cells.

**p<0.01; *p<0.05

Table IV Levels of ADMA, SDMA and nitrates with respect to immunosuppressive therapy (CyA/MMF/prednisone-TAC/MMF/prednisone vs. control).

Parameter	Control group	Tacrolimus	Cyclosporine A
Nitrates	17.04±4.39	25.01±9.92*	21.97±7.51*
ADMA	0.464±0.15	0.754±0.23*	0.66±0.13*
SDMA	0.77±0.14	2.08±0.78*	2.49±0.78*

ADMA, Asymmetric dimethylarginine; SDMA, symmetric dimethylarginine; CyA, cyclosporine A; TAC, tacrolimus; MMF, mycophenolate mofetil.

* $p<0.001$ vs. control

Table V Comparison of the measured oxidative stress parameters between subgroups with respect to therapy (CyA/MMF/prednisone vs. TAC/MMF/prednisone).

	TBARS (plasma)	TBARS (RBC)	Catalase	SH groups	AOPP
CyA	11.25 [3.17]	5.19 [2.35]	268.38 [219.58]	211.91 [65.51]	35.63 [14.90]
TAC	9.41 [2.08]	5.38 [1.83]	313.69 [256.18]	166.61 [65.51]	29.45 [18.15]
Z	–1.666	–0.183	–0.285	–2.462	–1.712
Asymp. Sig. (2-tailed)	0.096	0.855	0.775	0.014	0.087

Results are expressed as median [interquartile range].

AOPP, Advanced oxidation protein products; TBARS, thiobarbituric acid-reactive substances; RBC, red blood cells; CyA, cyclosporine A; TAC, tacrolimus; MMF, mycophenolate mofetil.

to the immunosuppressive protocol (*Table IV*). Finally, there was no significant difference in the measured OS parameters between subgroups with respect to the immunosuppressive treatment (CyA vs. TAC). The only significant increase was found in the concentration of SH groups in the group of patients treated with cyclosporine compared to patients treated with tacrolimus ($p=0.014$) (*Table V*).

Discussion

Transplantation *per se* ameliorates kidney function, but it does not recover it completely. Renal transplant patients seem to have less oxidative stress compared with routinely dialyzed patients. However, factors such as immune response to allograft, ischemia reperfusion injury, opportunistic infections and immunosuppressive therapy may trigger OS in these patients (29, 30). Oxidative stress parameters, further, may have not been removed from plasma because of insufficient excretion and may continue to rediffuse in circulation (31). In addition, there is some evidence that changes in plasma TBARS levels are accompanied by an increase in renal TBARS levels in rats with renal mass reduction suggesting that higher plasma ROS levels could reflect local ROS production in the kidneys, and it may be that in our model the kidneys are the main place of ROS generation (32). Lipid peroxides degrade to reactive aldehydes such as MDA that react with proteins, nucleic acids and lipids triggering off further tissue and organ damage (33). In our model, renal transplant recipients had significantly increased concentrations of TBARS (both plasma and RBC) and AOPP (in plasma), which was not only a result of their higher production, but may be attributed to their extensive half-lives and the ability to diffuse to various tissues (34).

The patients in our study showed a significant increase in AOPP levels compared to the control and these results are opposite to the data about normalization of oxidative stress parameters after kidney transplantation (35). Our findings correlate with the results of some other investigators suggesting that continuous immunosuppressive therapy probably contributes to enhanced formation of AOPP, even if the graft function is normal (36).

Many studies have confirmed that plasma levels of ADMA in the healthy population are related to age, blood pressure, insulin resistance and carotid intima-media thickness (37, 38). These findings suggest that ADMA can be an early biomarker of atherosclerotic lesion and that it can be used for the assessment of cardiovascular risk (39). We demonstrated that our experimental group, with no clinically present cardiovascular disease, also had higher concentrations of ADMA, indicating that they have increased risk for atherosclerosis and possibly declining renal function. A significant increase in plasma ADMA levels could inhibit NO production with further development of cardiovascular disease (40). Nitrosative stress biomarkers were statistical-

ly higher in our experimental group, so we could propose that they are at higher risk of all the conditions connected with adverse vascular effects (especially when taking into account that our study group already had some comorbidities associated with vascular wall damage, namely high blood pressure, smoking, diabetes and obesity) (41). Increased plasma levels of ADMA have been demonstrated in patients with both kidney and heart failure and have been shown to decrease a few months after kidney transplantation, remaining still higher compared to healthy volunteers (42–44). ADMA has been proposed as a predictor of mortality in dialysis patients (45). Renal transplant recipients also demonstrate upregulation of the nitric oxide (NO) system, probably by increased endothelial nitric oxide synthase (NOS) gene expression and nitrite/nitrate levels (46). ADMA levels are also associated with OS and hypercholesterolemia through the reduction in DDAH activity (47, 48). Besides, in our previous article we demonstrated that ADMA may be a more significant marker in men with kidney allografts than in women, concerning oxidative stress control of its level and function (49).

SDMA is almost completely excreted by the kidneys and correlates strongly with different parameters of renal function (50). Plasma SDMA levels increase with creatinine and could be a better marker of renal dysfunction compared to this compound (51). Our renal transplant recipients had higher SDMA levels than controls, indicating that they had worse renal function than the healthy population. In addition, it was demonstrated that SDMA levels increased rapidly after total nephrectomy in healthy living related kidney donors and it was suggested that SDMA could be an early biomarker of change in glomerular filtration (52).

Immunosuppressive therapy also seriously affects the endothelium. Oxidative stress is one of the main contributors to endothelial damage and toxic effects (53). Corticosteroids can inhibit the activation of nuclear factor kappa B (NFkB), whose synthesis is stimulated by oxidative stress (54). In an animal model, the use of prednisone had a protective role, by increasing the synthesis of catalase, dismutase and glutathione peroxidase and reducing MDA concentration in the glomeruli, suggesting that corticosteroids are a class of immunosuppressive drugs that provide nephroprotection, the effect opposite to calcineurin inhibitors (55). Both calcineurin inhibitors increase the production of ROS in cultured rat renal mesangial cells and their administration results in the production of ROS in glioma cells, which constitutes the side effects of these drugs (56, 57).

Oxidative stress after kidney transplantation is mostly associated with a higher TBARS concentration, which represents a direct link with cyclosporine A therapy (58). Tacrolimus is a calcineurin inhibitor associated with tissue protection from the ischemia/reperfusion phenomenon particularly when administered before ischemia (59). Tacrolimus also inhibits the activation of NFkB, which is strongly connected with the generation of ROS (60). Some animal models showed no effects of tacrolimus on oxidative stress, and in *in vitro* models tacrolimus reduced the induction of OS (61, 62).

In line with this, we demonstrated that there was no significance in all the evaluated parameters of oxidative or nitrosative stress among the groups of patients treated with tacrolimus or cyclosporine A. Similar findings were obtained in the early post-transplant period and in patients with post-transplant hypertension (46, 63). In a group of patients with stable renal function administration of cyclosporine A was associated with high levels of MDA and those treated with tacrolimus had significantly lower MDA levels, but our findings do not support these results (58). Possible explanation for our results may be the fact that both drugs are similarly metabolized by the CYP3A4 member of the cytochrome P450 superfamily and therefore have similar levels of cellular damage. We did not confirm a significant difference in the values of AOPP between the patients in relation to therapy.

The concentration of SH groups was the only parameter that was higher in the patients receiving cyclosporine A, which conforms to the findings of a positive correlation between cyclosporine therapy and hyperhomocysteinemia (64).

Conclusion

The results of our study demonstrated that in the group of patients with stable graft function and no overt cardiovascular disease the levels of oxidative and nitrosative stress were significantly increased compared to the healthy population. Transplantation probably decreases their levels, but they do not reach the normal range even if the graft function is normal.

We did not confirm a statistically significant difference in the levels of oxidative and nitrosative stress parameters evaluated between the patients treated with cyclosporine A or tacrolimus. Continuous immunosuppressive therapy contributes to enhanced formation of ROS after transplantation, but the particular type of calcineurin inhibitor, probably due to the similar metabolic pathway, does not seem to have an impact. Finally, we could suggest that the post-transplant immunosuppression therapy should focus on the nephrotoxicity of the medication (or some other criteria), rather than their influence on oxidative stress or impaired arginine metabolism.

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Conflict of interest statement

The authors stated that have no conflicts of interest regarding the publication of this article.

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