MEDICAL SCIENCE MONITOR

ANIMAL STUDY

e-ISSN 1643-3750
© Med Sci Monit, 2019; 25: 8242-8247
DOI: 10.12659/MSM.915230

Received: 2019.01.18
Accepted: 2019.03.26
Published: 2019.11.02

The Comparison of Parameters of Oxidative Stress in Native Rat Livers Between Different Immunosuppressive Regimens

Authors' Contribution:
Study Design A
Data Collection B
Statistical Analysis C
Data Interpretation D
Manuscript Preparation E
Literature Search F
Funds Collection G

ACDEF 1 Aleksandra Wilk
CDE 1 Dagmara Szypulska-Koziarska
BDEFG 2 Karolina Kędzierska-Kapuza
DEF 3 Jerzy Sieńko
DE 1 Agnieszka Kolasa-Wołosiuk
BCE 2 Kazimierz Ciechanowski
ABDEF 1 Barbara Wiszniewska

1 Department of Histology and Embryology, Pomeranian Medical University, Szczecin, Poland
2 Department of Nephrology, Transplantology, and Internal Medicine, Pomeranian Medical University, Szczecin, Poland
3 Department of General Surgery and Transplantology, Pomeranian Medical University, Szczecin, Poland

Corresponding Author: Dagmara Szypulska-Koziarska, e-mail: dagmara.koziarska@pum.edu.pl
Source of support: Departmental sources

Background: It is thought that immunosuppressive treatment, besides anti-rejection properties, leads to pathological changes within the organ due to activation of mechanisms associated with oxidative stress. The aim of this study was to examine the parameters of oxidative stress in the livers of rats treated with the most commonly used transplant recipient drug regimens.

Material/Methods: The rat livers were obtained from archival material obtained from the previously performed experiment. Malondialdehyde (MDA), reduced glutathione (GSH) concentrations, superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPx) activities were analyzed.

Results: Only the group treated with tacrolimus (T), mycophenolate mofetil (M), and prednisone (P), the TMP group, showed a slight increase in lipid peroxide concentration compared to the control group, though the difference was not statistically significant. Comparison of lipid peroxide concentration between the other treatment combinations and the control group showed a significant decrease. Additionally, a difference in lipid peroxide concentrations in the livers was observed between the cyclosporine A (C) group and tacrolimus (T) group. Alterations of other oxidative stress parameters were also observed in different regimens.

Conclusions: Long-lasting immunosuppressive treatment does indeed affect redox status; however, the antioxidant defenses of the liver against the effects of excess hydrogen peroxide are efficient, so the superoxide dismutase/glutathione peroxidase (SOD/GPx) and superoxide dismutase/catalase (SOD/CAT) ratios were not significant.

MeSH Keywords: Abnormalities, Drug-Induced • Immunosuppression • Immunosuppressive Agents • Oxidative Stress

Full-text PDF: https://www.medscimonit.com/abstract/index/idArt/915230

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Background

The mechanism of immunosuppressive drugs toxicity, including nephrotoxicity and hepatotoxicity, remains unclear; however, it is thought to be associated with the induction of oxidative stress [1,2]. Molecular methods are therefore needed to further explain the mechanism. The production of reactive oxygen species (ROS) – including superoxide radicals ($O_2^{\cdot -}$), hydrogen peroxide (H_2O_2), and hydroxyl radicals (·OH) – injures the cell by (among other mechanisms) damaging membrane integrity and cellular metabolism [3,4]. One indicator of oxidative stress is malondialdehyde (MDA), a lipid peroxidation product (LPO) responsible for altering the permeability of the cell membrane. Furthermore, enzymes such as superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), and glutathione reductase (GR), as well as nonenzymatic components such as glutathione (GSH), are activated following the generation of ROS. This is related to the activation of the antioxidant protective capacity of cells [3,5].

The choice of immunosuppressive treatment depends on the disease etiology, including the presence of the hepatitis C virus, and general health status. The medicines used by organ recipients (including liver recipients) include calcineurin inhibitors (CNIs) like cyclosporine A (C) and tacrolimus (T), which are the mainstays of immunosuppression therapy [6]. The hepatotoxicity of CNIs are involve the production of ROS and the depletion of the liver's antioxidant system [7–13]. The second group of drugs administered following organ transplantation include the mammalian target of rapamycin inhibitors (mTORs) like sirolimus [14–16]. Another drug, mycophenolate mofetil (M), is an immunosuppressor that inhibits inosine monophosphate dehydrogenase [17,18].

These drugs are currently used in transplant recipients to prolong the functioning of the graft. Additionally, patients who have undergone transplantation need to use the glucocorticosteroid, prednisone, which also has immunosuppressive properties. The therapy is generally based on a combination of immunosuppressive medicines. In the current study, the most commonly used protocols of immunosuppressive medicines were used.

Based on the available literature on the toxicity mechanism of immunosuppressive medicines and on the role of the liver in maintaining homeostasis throughout the body, the goal of this research was to analyze the effect of the most commonly used immunosuppressive regimens on the oxidative stress status of the liver, by determining the concentration of malondialdehyde (MDA), which is commonly used as an indicator of oxidative stress in various cells and tissues, and the concentration of glutathione (GSH), as well as the activity of the antioxidant enzymes superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPx). Expanding our knowledge of the effect of immunosuppressive drugs on the oxidative stress reactions that lead to liver injury can certainly be expected to help optimize the treatment of the patients who have undergone transplantation of the liver and other organs.

Material and Methods

The biological material of the current study was rat livers that were the archival material obtained from the experiment previously performed by Kedzierska et al. [19]. This study was approved by the Szczecin Local Ethical Committee for Experiments on Animals (decision no. 24/2008, dated 24 Nov 2008).

Analyses were performed according to Directive 2010/63/EU of the European Parliament and of the Council of September 22, 2010 on the protection of animals used for scientific purposes, in order to limit the number of animals in one experiment. Kędzierska et al. performed research regarding rat renal tissues and the rats received the drugs regimens in the doses shown in Table 1.

Tissue sample preparation was performed as previously described [20]. After preparation, the supernatants were stored at –80°C and used for the analysis of the parameters of oxidative stress. All biochemical analyses, including oxidative stress parameters, were performed according to manufacturer's

Table 1. The study design. Abbreviations of the drugs that are used to name the study groups in brackets.

Group	Prednisone (P)	Tacrolimus (T)	Cyclosporin A (C)	Sirolimus (S)	Mycophenolate mofetil (M)
Control (n=6)	–	–	–	–	–
TSP (n=6)	+	+	–	+	–
CSP (n=6)	+	–	+	+	–
MSP (n=6)	+	–	–	+	+
CMP (n=6)	+	–	+	–	+
TMP (n=6)	+	+	–	–	+

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Table 2. The lipid peroxidation products concentration (LPO); activity of antioxidant enzymes (superoxide dismutase – SOD, catalase – CAT, glutathione peroxidase – GPx and glutathione concentration (GSH)) in rat livers.

Group	Parameters	LPO (nM/mg protein)	SOD (U/mg protein)	CAT (U/mg protein)	GPx (U/mg protein)	GSH (nM/mg protein)
Control	Mean ±SD Median Range	1.35±0.42 1.116 0.80–2.07	6.69±2.48 7.87 2.15–9.2	109.23±2.15 109.48 106.97–112.48	20.99±18.25 12.05 6.45–49.74	1.47±0.47 1.27 1.08–2.43
TSP	Mean ±SD Median Range	0.80±0.32* 0.81 0.29–1.17 *vs. control	7.28±2.66 7.87 2.08–10.01	108.44±3.74 108.61 103.35–113.58	53.64±39.56 41.59 17.14–126.15	1.02±0.36 1.17 0.59–1.47
CSP	Mean ±SD Median Range	0.67±0.28* 0.59 0.45–1.05 *vs. control	7.36±1.47 7.48 5.52–8.96	109.66±2.7 109.6 106.97–112.48	36.63±17.23 31.41 22.75–60.96	1.96±0.46* 1.86 1.53–2.6 *vs. TSP
MSP	Mean ±SD Median Range	0.6±0.11* 0.61 0.49–0.7 *vs. control	6.38±2.98 6.56 2.13–9.24	107.37±0.46 107.37 106.97–107.77	42.11±39.11 32.42 7.98–95.59	1.46±0.46 1.43 0.85–2.01
CMP	Mean ±SD Median Range	0.63±0.15** 0.7 0.39–0.77 **vs. control	7.39±2.19 7.87 4.49–10	106.41±2.09* 106.97 102.72–108.61 *vs. control	22.32±29.18 8.74 1.35–73.69	1.46±0.26* 1.39 1.12–1.8 *vs. TSP
TMP	Mean ±SD Median Range	1.79±1.17* 1.8 0.5–3.01 *vs. CMP	8±1.4 8.5 5.91–9.24	108.31±2.89 107.77 104.7–113.58	16.47±20.9 4.41 1.36–44.65	1.3±0.26* 1.3 0.95–1.67 *vs. CSP

* Significantly different at p<0.05; ** significantly different at p<0.005.

instruction, as described previously [3,20]. The product of the lipid peroxidation MDA was estimated using a Bioxytech LPO-586 Assay Kit (Oxis Research, Poland). For enzyme assays, the total (Cu-Zn and Mn) SOD (EC 1.15.1.1) activity was measured using the Bioxytech SOD-525 Assay Kit (OxisResearch, Poland). The activity of CAT (EC 1.11.1.6) was measured using the Bioxytech Catalase-520 Assay Kit (OxisResearch, Poland). The activity of cellular GPx (EC 1.11.1.9) was measured using the Bioxytech GPx-340 Assay Kit (OxisResearch, Poland). The concentration of glutathione (GSH) was measured using the Bioxytech GSH-400 Assay Kit (OxisResearch, Poland).

Statistical analysis

The obtained results were analyzed statistically using Statistica 6.1. software. Arithmetical mean and standard deviation were calculated for each of the examined parameters. As most of the distributions deviated from the normal distribution (Shapiro-Wilk test), non-parametric tests were used. The differences between the parameters were shown by using Kruskal-Wallis ANOVA followed by Mann-Whitney tests. The level of significance was $P<0.05$.

Results

We compared the results regarding parameters of oxidative stress between the control group and all the treatment groups, and additionally did comparisons between every treatment group.

The concentration of MDA in all examined groups

The greatest concentration of MDA was found in the liver treated with TMP (tacrolimus, mycophenolate mofetil, prednisone) and it was almost 25% greater than in the livers from the control group, and it was significantly higher compared to the CMP (cyclosporin A, mycophenolate mofetil, prednisone) livers. We observed a statistically significant decrease of MDA concentration compared to the control group for rats treated with TSP (tacrolimus, sirolimus, prednisone), CSP (cyclosporin A, sirolimus, prednisone), MSP (mycophenolate mofetil, sirolimus, prednisone), and CMP (Table 2).

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Table 3. The superoxide dismutase (SOD) to catalase (CAT) ratio and superoxide dismutase (SOD) to glutathione peroxidase (GPx) ratio of activity of control and treatment groups.

Group	Parameters	SOD/CAT	SOD/GPx	U M-W Test
Control	Mean ±SD Median Range	0.06±0.02 0.07 0.02–0.09	0.48±0.34 0.40 0.15–0.93	Control *vs.*
TSP	Mean ±SD Median Range	0.07±0.02 0.07 0.02–0.09	0.22±0.18 0.21 0.02–0.54	*NS*
CSP	Mean ±SD Median Range	0.07±0.01 0.07 0.05–0.08	0.22±0.07 0.21 0.15–0.31	*NS*
MSP	Mean ±SD Median Range	0.06±0.03 0.07 0.02–0.09	0.38±0.35 0.35 0.02–0.82	*NS*
CMP	Mean ±SD Median Range	0.07±0.02 0.08 0.04–0.09	2.66±2.99 2.32 0.12–7.41	*NS*
TMP	Mean ±SD Median Range	0.07±0.01 0.08 0.05–0.09	2.50±2.62 1.69 0.16–6.79	*NS*

NS –statistically insignificant, p>0.05.

SOD activity

The differences in activity of SOD was not statistically significant; however, comparisons between the control group and the treatment groups showed increase of SOD activity in every group treated with immunosuppressive drugs, except the MSP group which showed a slight decrease of SOD activity (Table 2).

CAT activity

When we compared the control group with rats treated with immunosuppressive drugs, the catalase activity slightly decreased; however, a statistically significant decrease was observed only in CMP group (Table 2).

GPx activity

The glutathione peroxidase activity increased over 150% in livers treated with TSP compared with the control rat livers, but the difference was not statistically significant. The lowest activity was observed in TMP group and compared to the control group, the GPx activity had about a 20% decrease (Table 2).

GSH concentration

No statistical differences were noted for glutathione concentration in the control group compared with the treatment groups; however, we found significant higher concentration of GSH in the CSP group versus the TSP group; higher concentration of GSH in the CMP group versus the TSP group; and increased GSH concentration in the CSP group versus the TMP (Table 2).

SOD/CAT and SOD/GPx ratio

To check if the antioxidant defense mechanism is sufficient within the cytosol or plasma membrane, we examined SOD/CAT and SOD/GPx ratios. No statistical differences were noted between all the treatment groups and the control group taking into account SOD/CAT and SOD/GPx ratio. The highest SOD/GPx ratio was observed in the CMP group and the TMP group. The values of ratios are shown in Table 3 and the comparison of mean values of ratios are presented in Figures 1 and 2.

Discussion

Among the many health-affecting factors to which people are exposed, environmental factors play a significant role. Such factors may be present in the air, water, or soil, or in the diet – and this last category includes pharmaceuticals. Immunosuppressive therapy plays an extremely important role for transplant recipients, undoubtedly prolonging their lives; however, these medicines exhibit a wide range of toxic properties and affect not only immune elements, but also epithelial cells, the endothelium of blood vessels, and cells of mesenchymal origin [2,19,21,22]. It is very important to choose the optimal drug protocol so as

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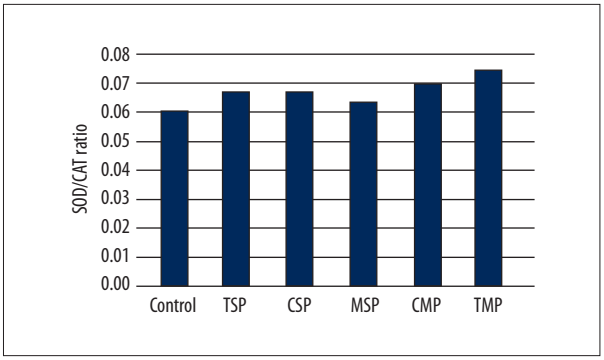

Figure 1. SOD/CAT ratio in examinated regimens (TSP – tacrolimus, sirolimus, prednisone; CSP – cyclosporine A, sirolimus, prednisone; MSP – mycophenalote mofetil, sirolimus, prednisone; CMP – cyclosporine A, mycophenalote motefil, prednisone; TMP – tacrolimus, mycophenalote motefil, prednisone).

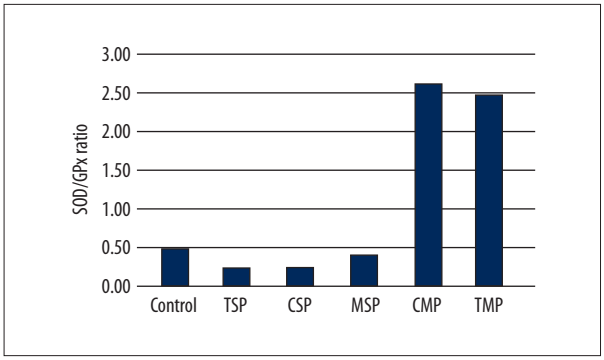

Figure 2. SOD/GPx ratio in examinated regimens (TSP – tacrolimus, sirolimus, prednisone; CSP – cyclosporine A, sirolimus, prednisone; MSP – mycophenalote mofetil, sirolimus, prednisone; CMP – cyclosporine A, mycophenalote motefil, prednisone; TMP – tacrolimus, mycophenalote motefil, prednisone).

to prolong the lifespan of the graft while avoiding or minimizing side effects. The mechanism of toxicity of immunosuppressive drugs is mostly associated with the generation of ROS and the induction of oxidative stress [17,23–27]. Oxidative stress injures many organs, including the liver, which is an intensely metabolic organ whose physiology is very complex. The liver performs several major metabolic functions, but also plays a key role in the detoxification process. It is moreover involved in the delivery of many proteins and metabolites to the blood and other tissues [28,29].

Our study was based on the comparison of the most commonly used immunosuppressive treatment by transplant recipients, and the emphasis reflects the influence of the regimens, not the single drug on redox status. It is known that some immunosuppressive drugs, including cyclosporine (C), tacrolimus (T), and sirolimus (S), lead to the excessive production of radicals, which is associated with the generation of lipid peroxidation products, including malondialdehyde (MDA) which is the main indicator of oxidative stress. MDA is a product of lipid peroxidation [2–4,28,30]. On the other hand, mycophenolate mofetil inhibits ROS production [17].

All the groups of animals used in this study followed different pharmaceutical protocols, and the highest concentration of MDA was seen in the TMP rats; this was the only group where the MDA level increased over that of the control rats, though the difference was not statistically significant. The standard deviation was also very high, which may suggest differing individual sensitivities. Furthermore, in the physiological state, the toxic agents can lead to an increase in the activity of antioxidant enzymes; interestingly, however, the TMP group showed only a gentle tendency towards an increase in SOD activity and a decrease in CAT and GPx activity. It is possible that the combinations of immunosuppressive drugs used in this group had a deleterious effect on the CAT and GPx proteins, thus affecting their activity; however, further experimentation is needed. We also observed a reduction in the concentration of GSH, which is a cofactor for GPx. Reduced glutathione is a nonenzymatic antioxidant, mostly present in the hepatocytes, where it performs functions that include delivering electrons to free radicals, neutralizing them [4,28]. It thus seems reasonable that the activity of GPx fell with the decrease in GSH level. Catalase neutralizes hydrogen peroxide, including that generated by SOD. The level of generated hydrogen peroxide was not sufficiently high to stimulate to action of CAT.

The concentrations of LPO in the other experimental groups (TSP, CSP, MSP, and CMP) were significantly lower than in the control group, possible as a result of the adaptation of the liver to the long-term use of these immunosuppressant protocols. We also found a significant difference between the TMP and CMP groups in terms of the level of LPO, which may be related to the hundred-times stronger prooxidative action of tacrolimus compared to cyclosporine A [23].

Superoxide dismutase is the first line of defense against cell injury by ROS generation. It catalyzes the neutralization of the superoxide anion, releasing hydrogen peroxide [31]. In the current experiment, SOD activity was not affected, hydrogen peroxide was not generated, and this probably resulted in the lack of change in the activity of the other antioxidant enzymes, such as CAT and GPx, compared to control group. The CMP group was an exception, showing a significant decrease in the activity of CAT compared to the control group. Perhaps mycophenolate mofetil inhibits oxidative stress by decreasing ROS generation. Due to the fact that mycophenolate mofetil also inhibits iNOS (inducible nitric oxide synthase) expression and the synthesis of NO, it is used as a codrug, commonly with CNIs, to prolong the functioning of the graft [17].

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The products of lipid peroxidation react with GSH; when oxidative stress is intense, the ATP and GSH in cells is utilized [28]. In our study, we also found that the concentration of GSH did not significantly change in any of the treatment groups when compared with the control. The lack of changes in the level of GSH corresponds with the lack of changes in the activity of GPx. Interestingly, we noted a statistically significant reduction in GSH concentration in TSP versus CSP and in TSP versus CMP. This can again be explained by the fact that tacrolimus (T) is a much more toxic medicament than cyclosporine (C) thus, in the TSP rats, the concentration of GSH was reduced, as it was oxidized to GSSG, lowering the GSH/GSSG ratio.

Conclusions

In summary, our experiments found the following: 1) immunosuppressive treatment affected the oxidative stress in liver. 2) The highest concentration of LPO was noted in the TMP rats. 3) In the TMP group, we also observed only a gentle increase in SOD activity, while the activity of CAT and GPx decreased. 4) The concentration of LPO in the other experimental groups significantly decreased compared to the control group. 5) The antioxidant defense of the liver against the results of excess hydrogen peroxide is efficient, both in the cytosol and the cellular membrane, what was additionally indicated by the SOD/CAT and SOD/GPx ratios.

Our results suggest that immunosuppressive regimens influence the parameters of redox balance. Despite the fact that this study used animal tissue, our obtained results could be considered as the indication for clinicians.

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