Research Article

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The effect of immunosuppressive treatment during pregnancy on the activity of antioxidant enzymes in selected visceral organs among the offspring of wistar rats.

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ABSTRACT

Kidney transplantation remains the treatment of choice in end-stage kidney disease. The introduction of new immunosuppressive drugs has significantly extended survival time in individuals after KTx, together with improving their quality of life. As a consequence, the number of women planning motherhood after kidney transplantation is also increasing. Despite strict specialist control, such pregnancies are still considered high-risk, as the impact of immunosuppressive drugs on fetal development is very significant.

This study evaluates the effect of most common immunosuppressive treatment schemes on the indicators of oxidative stress in in the intestines and spleen in an animal model, using Wistar rats. All drugs were administered to pregnant females by a gastric tube in weight- adjusted doses. Initially, a full dose was used, but this resulted in severe fetal damage in the majority of rats, grouped according to drug regimens. The experiment was then repeated with the doses reduced by half, finally obtaining a sufficient number of progeny animals for the study.

The research demonstrated alterations in the activity of antioxidant enzymes and concentrations of reduced glutathione in all groups of offspring rats whose mothers received immunosuppressive treatment during pregnancy. Results varied depending on the regimen and drug doses 27 used. Within the group treated with the full dose of cyclosporine A, mycophenolate mofetil, and prednisone a significant increase in the activity of antioxidant enzymes in the spleen occured. In the tacrolimus and mycophenolate mofetil (reduced-dose) group, a variety of changes were observed in all tissues and organs examined. In the group receiving cyclosporine A, mycophenolate mofetil and prednisone at a reduced dose as well as in the group receiving cyclosporine A, everolimus and prednisone at a reduced dose, an increase in the activity of antioxidant enzymes was demonstrated, mainly in the small intestine.

KEYWORDS

Immunosuppressive Drugs,Pregnancy, Kidney Transplantation, Wistar Rats, Free radicals, Antioxidants

INTRODUCTION

Kidney transplantation remains the method of choice in the treatment of patients with end-stage kidney disease. This procedure prolongs the life of patients and improves its quality [1, 2, 3]. The principle of transplantation is to restore patients' ability to function in society unhindered by the disease: they should be able to work, play sports, or have children. The choice of optimal immunosuppressive treatment aims for long-term survival without graft rejection and for maintaining immunocompetence by avoiding severe infections and carcinogenesis, which are the major complications of chronic immunosuppression [4, 5]. A typical immunosuppressive treatment regimen after kidney transplantation consists of

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at least three medications and requires the use of three different drug groups. Classes of drugs differ in their mechanism of action, pharmacokinetics and the spectrum of side effects - skillful adjustment of the regimen and dosage allows therapy

optimalization [6, 7, 8, 9]. Majority of women of childbearing age, after successful kidney transplantation, will have ovarian cycles normalized and fertility regained after 6 months. Maintaining pregnancy requires a good baseline creatinine level (optimally below 1.5mg%) [10, 11], no proteinuria, and well-controlled hypertension, so it is optimal to get pregnant with a stable function of the graft. However, pregnancy after solid organ transplantation poses a threat to the health of the mother and the child, as well as further graftfunction. In 12% of patients, the function of the graft deteriorates despite its satisfactoryfunction at fertilization [12].

The risk of acute kidney rejection is estimated at 8% [13], and pregnancy is a state ofimmunotolerance [14]. As high as 70-80% of all pregnancies result in a live birth (up to90% after the first trimester) [13]. Pregnancy is advised no sooner than 1-2 years after the transplantation. Following that time, maximum reduction of immunosuppressive drugs dose can be allowed [11, 15]. In the group of patients treated with tacrolimus (Tc), the incidence of spontaneous miscarriage is estimated at 22-33%, while the risk of miscarriage in the general population of healthy women is about 15% [16, 17]. More than a half of babies are born pre-term, with a birth weight below 2500 g [13, 16]. Due to limited data,breastfeeding has generally not been recommended so far, although new studies indicate the possibility of safe breastfeeding while on glucocorticosteroids, azathioprine, cyclo-sporine (CsA), and Tc [18, 19].

Many novel drugs are considered harmful to the fetus (such as everolimus, sirolimus,mycophenolate mofetil MMF). Their impact, due to the impossibility of conducting prospective studies in pregnant women, is not fully understood or defined. Thus, these immunosuppressants should be discontinued 6 weeks before planned pregnancy. Based on currently available papers, we have data regarding the use of immunosuppressive drugs during pregnancy in laboratory animals and humans, but mainly in monotherapy. MMF in experimental models affected organogenesis by causing numerous malformations(anophthalmia, agnathia, hydrocephalus, ectopia of the heart and kidneys, umbilical and diaphragmatic hernias), intrauterine fetal death, and fetal hypotrophy. In humans (severalreported cases, including unplanned pregnancies, lacking adequate medical supervision)multiple malformations occurred, including microtia, hypertelorism, micrognathia, cranial deformity, and cleft palate. The risk of aforementioned congenital defects among theoffspring of patients treated with MMF is significantly higher (26.7%) than in those remaining on properly selected immunosuppressive treatment (4%-5%) [20]. Rapamycinan mTOR inhibitor, was embryotoxic and fetotoxic in rats (hypotrophy and resorption offetuses, impaired ossification), especially in combination with cyclosporine [21].

Many side effects of immunosuppressants are related to the gastrointestinal tract they affect up to 80% of people undergoing immunosuppressive therapy [22, 23, 24]. The etiology of this phenomenon is multifactorial. Due to excessive production of free radicals,along with the concomitant insufficiency of antioxidant processes, an overt oxidative stress results in damage to cells, tissues, and organs [25]. In certain situations, the destructive capability of free radicals is purposely used by the organism - e.g. they are produced by leukocytes to destroy microorganisms in an inflammatory focus [26]. In a limited amount and under strictly defined conditions, free radicals support the function of the human body by participating in chemical reactions galore, e.g. they activate receptors that stimulate or inhibit the secretion of hormones, and participate in muscle contraction or stress response. When the balance of production and reduction of free radicals is not sustained, their amount increases and their destructive effect is exposed through the damage of nucleic acids and proteins.The aforementioned process is called oxidative stress. Its presence is responsible, among others, for DNA damage, which may be associated with the aging process. It also has a proven role in carcinogenesis [27].

The formation of new free radicals is normally counterbalanced by the action of the so-called antioxidants, either enzymatic or non-enzymatic. Superoxide dismutase (SOD),an example of an enzymatic antioxidant, reduces hydrogen peroxide. Other enzymes that are part of the "protective shield" are glutathione peroxidase (glutathione peroxidase[GPx]), glutathione reductase (GR), glutathione S-transferase (GST) and catalase (CAT).Among non-enzymatic antioxidants, vitamin C,vitamin E, carotenoids, or glutathione (inthe reduced form - GSH) are worth mentioning. Glutathione is one of the most important intracellular antioxidants. It has been shown that the intracellular increase of oxidized form of glutathione is associated with excessive activation of enzymes leading to hepato cyte apoptosis in immunosuppressed patients [28]. The association between the excessive oxidative stress and reduced fertility has also been described [29, 30].

The study aimed to compare the effect of immunosuppressive treatment regiments (in three-drug combinations, commonly used in clinical practice) on the activity of redox processes in selected visceral organs in the offspring of immunosuppressed, pregnant female Wistar rats. Obviously, it was not possible to perform such studies prospectively in humans for ethical reasons. Wistar rats were selected based on available data supporting their suitability to monitor the effects of immunosuppressive drugs on tissue and organ damage similar to humans. Choosing the animal model made it possible to

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assess the impact of the medications on the fetus and further development of the offspring after birth.We examined the activity of oxidative stress in the small intestine, large intestine, and spleen of the progeny rats by measuring the activity of CAT, GR, GPx, and SOD and de-termining the concentration of GSH.

MATERIALS AND METHODS

Animals and Treatment

The study was carried out using biological material obtained from the Wistar rats specifically, the offspring of mothers subjected to immunosuppressive treatment during pregnancy. The biological material was frozen at -80 oC at the Department of Biochemis try and Medical Chemistry of the Medical University of Warsaw. This experiment and sections of laboratory animals were carried out as part of a research project in 2013-2014.

The first part of the experiment was performed on 32 female rats and 8 male rats (used only for breeding purposes). The rats were bred at the Center for Experimental Medicine of the Medical University of Bialystok. At the baseline, all animals were 12 weeks old and had an average body weight of 230 grams. All animals obtained veterinary records certifying good health and no known genetic burden. The study was approved by the Local Ethical Committee for Animal Experiments (No. 12/2013, decision date: October 24,2013) and was conducted in compliance with the ethical standards of the facility where itwas conducted (Pomeranian Medical University animal quarters). Initially, the animals received a full, weight-adjusted dose of drugs, but few offspring were obtained due to the teratogenicity of the drugs used. In the next part of the experiment, the dose of immunosuppressive drugs was reduced by 50%, only the dose of prednisone was sustained. This allowed us to obtain more live births from each treated female and significantly improved the survival of young rats. The second part of the study with half of the doses of drugs used was also approved by the Local Ethical Committee for Animal Experiments (No.10/2014 and No. 11/2014, decision date: 06/06/2014).

Throughout the experiment, the animals were kept in separate cages with a 12-hour day and night cycle. They were fed with Labofeed H and given unlimited water. Before fertilization, the animals were divided into 4 groups - a control group and 3 study groups.Each study group received a treatment that reflected the most common immunosuppressive regimens used after kidney transplantation in humans. The CMG group received CsA, MMF, and prednisone, the TMG group - Tc, MMF, and prednisone, and the CEG group - CsA, everolimus, and prednisone. Each group consisted of 8 individuals. All the substances used were administered in pharmaceutical form through a gastric tube, dissolved to a volume of 5 ml/kg b.w./24h. In the control group, an equivalent volume of pure saline was administered. Drug doses have been calculated to reflect human doses,with consideration to known differences in the metabolism of each drug, based on dataavailable in the specialist literature [31,32,33,34,35]: tacrolimus (Prograf) at a dose of 4 mg/kg b.w., mycophenolate mofetil (CellCept) at a dose of 20 mg/kg b.w., cyclosporine A(Sandimmun Neoral) at a dose of 5 mg/kg b.w., prednisone (Encorton) 4 mg/kg b.w., everolimus (Certican) 0.5 mg/kg b.w. Drugs were administered at 24-hour intervals. Animals were weighed weekly, following appropriate dose modification. After 2 weeks of initial therapy (pre-conception), each female was placed in a cage with the male, then,after pregnancy confirmation, moved to a separate cage, where assigned treatment was administered for 3 weeks. The drug administration was stopped at delivery. After completing the first part of the study, the entire group consisted of 31 mothers (1 control female died during probing due to esophageal perforation). The number of offspring obtained in the control group was 69, in the CMG group - 13, and in the CEG group - one.There was no live progeny in the TMG group. One individual from the CMG group died at 3 days of age, and another at 28 days. Six animals from this group were euthanized due to significant phenotypic defects such as anophthalmia or hydrocephalus at the age of 19 days, as they had no prognosis for further survival. The rest of the animals from the CMG group was euthanized 8 weeks old, according to the original study criteria (rats reached adulthood at 8 weeks). At the same time, the appropriate number of rats from the control group (12 individuals) was euthanized.

In the second part of the study, with lower doses of drugs administered, 8 females were used and divided into 3 groups: CMG 1/2 - 2 females, CEG 1/2 - 3 females, and TMG 1/2 - 3 females. The size of the second group was reduced for ethical reasons. Following the use of half the doses of drugs, a greater number of offspring was obtained: in the CMG 1/2 group - 24 rats, in the CEG 1/2 group - 7, in the TMG group 1/2 - 32. After 8 weeks, the offspring rats were anesthetized with 40 mg/kg b.w. of pentobarbital administered intraperitoneally. Twelve animals from the CMG 1/2 group, 12 from the TMG 1/2 group, and 7 from the CEG 1/2 group were finally tested. Fragments of their small intestine, large intestine, and spleen were collected during section and frozen for further research.

METHODS

The activity of antioxidant enzymes in the collected organs was determined at the Department of Biochemistry and Medical Chemistry of the Medical University of Warsaw.

Catalase activity (CAT)

CAT activity was determined using a Cayman Chemical Company kit and an ASYS UVM 340 spectrometer (Biogenet). The method is based on the reaction of the enzyme with methanol in the presence of H2O2. The formaldehyde

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produced is measured calori metrically using a chromogen that forms a specific bicyclic heterocycle with the aldehydes and changes their color [https://www.caymanchem.com/pdfs/707002.pdf].

Superoxide dismutase (SOD) activity

SOD activity was measured using the Superoxide Dismutase Assay Kit (Cayman Chemical Company, USA), according to the manufacturer's instruction, and the ASYS UVM 340 spectrophotometer (Biogenet). In this method, the tetrazolium salt is used by SOD to detect superoxide radicals generated by xanthine oxidase and hypoxanthine [https://www.caymanchem.com/pdfs/706002.pdf].

Glutathione peroxidase (GPx) activity

GPx activity was measured using the Glutathione Peroxidase Assay Kit (Cayman Chemical Company USA), according to the manufacturer's instruction, and the ASYS UVM 340 spectrophotometer (Biogenet). Gpx catalyzes the reduction of hydroperoxides, including hydrogen peroxide, via reduced glutathione. Selenocysteine, present in the active site of the enzyme, participates directly in the two-electron reduction of the superoxide substrate. The enzyme uses glutathione as the final electron donor to regenerate the reduced form of selenocysteine. The kit measures Gpx activity indirectly via a coupled reaction with GR (glutathione reductase). Oxidized glutathione, produced for hydroperoxide reduction by Gpx, is restored to its reduced state by GR and NADPH [https://www.caymanchem.com/pdfs/703102.pdf]

Glutathione Reductase (GR) Activity

GR activity was measured using a glutathione reductase assay kit (Cayman Chemical Company, USA) according to the manufacturer's instruction and an ASYS UVM 340 spectrophotometer (Biogenet). The kit measures GR activity by measuring the oxidation rate of NADPH. The oxidation of NADPH to NADP+ is accompanied by a decrease in absorbance and is directly proportional to the activity of GR in the sample [https://www.cay-manchem.com/pdfs/703202.pdf].

Reduced glutathione (GSH) content

GSH activity in the test sample was determined based on the Glutathione Assay Kit (Cayman Chemical Company, USA). Glutathione content was determined in the supernatant obtained after centrifugation of tissue previously homogenized in PBS solution according to the instruction manual included in the kit. The basis for the assay is the formation of a colored TNB compound, the amount of which, measured spectrophotometrically at a wavelength of 405-414 nm, is inversely proportional to the concentration of GSH in the sample and allows to accurately determine the total concentration of glutathione (oxidized and reduced form) [https://www.caymanchem.com /product/703002/glutathione-assay-kit].

Statistical analysis

Mean, median, minimum and maximum values, as well as standard deviations,were calculated for the control group and each of the study groups. Statistical differences between the groups were assessed using non-parametric Kruskal-Wallis and U-Mann Whitney tests. These tests were selected after analyzing the data distribution with the ShapiroWilk test. For most of the data, distribution was not normal, taking into account the number of rats. P-values were considered significant when $p<0.05$. Analysis was made using the Statistica 10 software.

RESULTS

With full doses of drugs, only the CMG regimen resulted in obtaining enough off spring to allow comparison with the control group and draw statistically significant conclusions. Using the results from the next part of the study, the data obtained from the offspring of mothers treated with the full dose of drugs in the CMG regimen were compared with the group of offspring of mothers receiving 50% of the dose in this regimen (CMG 1/2). Then, the CMG 1/2 group and the other study groups using half doses of drugs (TMG 1/2, CEG 1/2) WERE COMPARED WITH THE CONTROL GROUP.

3.1. CMG scheme - a full dose of drugs. Comparison to the control group.

Differences in the activity of antioxidant enzymes and the concentration of reduced glutathione in individual organs are presented in Table 1. A statistically significant difference in the activity of superoxide dismutase and glutathione peroxidase in the spleen was demonstrated (Figures 1 and 2).

Statistically significant results ($p<0.05$) are marked in red, results on the verge of statistical significance are marked in green.

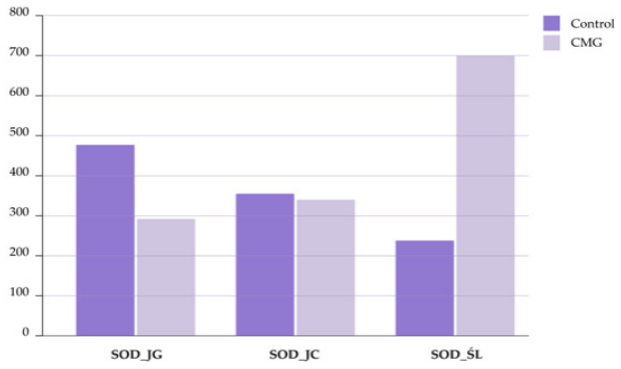


Figure 1. Activity of superoxide dismutase in individual organs in the control group and CMG.

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Table 1. Differences in the activity of antioxidant enzymes and the concentration of reduced gluta- 249 thione in individual organs in the control group and CMG (mean values+SD).

Antioxidant enzyme / reduced glutathione	Organ	Control group, mean + SD	Control group, median	CMG group, mean + SD	CMG group, median	p-value
CAT	LI	187,16 ± 49,24	181,65	189,13 ± 87,88	139,08	0,7990
CAT	SI	128,19 ± 38,58	116,62	141,49 ± 32,81	145,40	0,6461
CAT	SP	177,81 ± 61,26	163,85	296,66 ± 165,25	199,48	0,16
SOD	LI	476,88 ± 157,82	489,19	293,52 ± 335,70	62,61	0,3827
SOD	SI	356,37 ± 83,66	349,19	341,25 ± 73,72	358,25	0,9593
SOD	SP	236,13 ± 207,02	165,5	698,78 ± 579,94	449,19	0,0094
GPx	LI	751,67 ± 713,21	683,44	361,30 ± 324,60	165,79	0,5743
GPx	SI	585,74 ± 475,71	396,46	766,90 ± 906,14	344,54	0,9593
GPx	SP	628,37 ± 489,40	497,45	2657,43 ± 1676,41	1701,31	0,0023
GSH	LI	406,62 ± 107,35	426,86	431,53 ± 173,82	382,38	0,9593
GSH	SI	224,95 ± 67,99	209,12	232,04 ± 32,45	234,27	0,3284
GSH	SP	325,83 ± 114,17	306,12	457,02 ± 141,39	401,70	0,0637
GR	LI	1,21 ± 0,61	1,19	1,24 ± 0,84	0,98	0,6461
GR	SI	0,23 ± 0,20	0,18	0,40 ± 0,52	0,14	0,9593
GR	SP	1,26 ± 0,60	1,19	1,32 ± 0,66	1,17	0,8269

1 CMG – regimen with cyclosporine, mycophenolate mofetil and prednisone in full dose, CAT – catalase, SOD- superoxide dismutase, GPx – glutathione peroxidase, GSH – reduced glutathione, GR – glutathione reductase, SI – small intestine, LI – large intestine, SP – spleen.

Figure 2

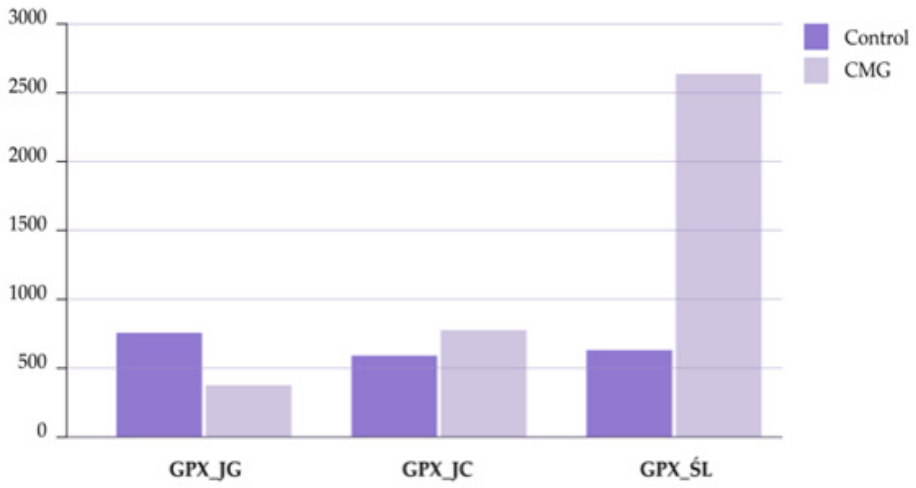


Figure 2. Activity of glutathione peroxidase in individual organs in the control group and CMG.

3.2. CMG half dose regimen. Comparison to the control group

Differences in the activity of antioxidant enzymes and the concentration of reduced glutathione in individual organs are presented in Table 2. A statistically significant difference in the activity of catalase and the concentration of reduced glutathione in the small intestine was shown (Figures 3 and 4).

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Table 2. Differences in the activity of antioxidant enzymes and the concentration of reduced glutathione in individual organs in the control group and CMG 1/2 (mean values+SD).

Antioxidant enzyme / reduced glutathione	Organ	Control group, mean + SD	Control group, median	CMG ½ group, mean + SD	CMG ½ group, median	p-value
CAT	LI	187,16 ± 49,24	181,65	198,79 ± 39,73	199,26	0,5387
CAT	SI	128,19 ± 38,58	116,62	179,21 ± 67,40	158,93	0,0425
CAT	SP	177,81 ± 61,26	163,85	178,87 ± 35,11	165,41	0,7223
SOD	LI	476,88 ± 157,82	489,19	514,05 ± 106,16	509,56	0,6744
SOD	SI	356,37 ± 83,66	349,19	453,12 ± 213,27	386,90	0,3463
SOD	SP	236,13 ± 207,02	165,5	284,50 ± 123,82	268,45	0,1802
GPx	LI	751,67 ± 713,21	683,44	1343,60 ± 1186,38	740,14	0,2543
GPx	SI	585,74 ± 475,71	396,46	488,27 ± 557,05	360,13	0,4965
GPx	SP	628,37 ± 489,40	497,45	684,58 ± 377,32	558,17	0,5387
GSH	LI	406,62 ± 107,35	426,86	512,30 ± 179,30	491,77	0,0804
GSH	SI	224,95 ± 67,99	209,12	344,87 ± 154,70	305,69	0,0206
GSH	SP	325,83 ± 114,17	306,12	314,85 ± 58,23	294,26	0,9229
GR	LI	1,21 ± 0,61	1,19	0,93 ± 0,64	0,79	0,2030
GR	SI	0,23 ± 0,20	0,18	0,56 ± 0,48	0,41	0,0503
GR	SP	1,26 ± 0,60	1,19	1,37 ± 0,31	1,38	0,5573

[2] CMG ½ – regimen with cyclosporine, mycophenolate mofetil and prednisone in a reduced dose, CAT – catalase, SOD superoxide dismutase, GPx – glutathione peroxidase, GSH – reduced glutathione, GR – glutathione reductase, SI small intestine, LI – large intestine, ŚL – spleen. Statistically significant results ($p<0.05$) are marked in red, results on the verge of statistical significance are marked in green.

Figure 3

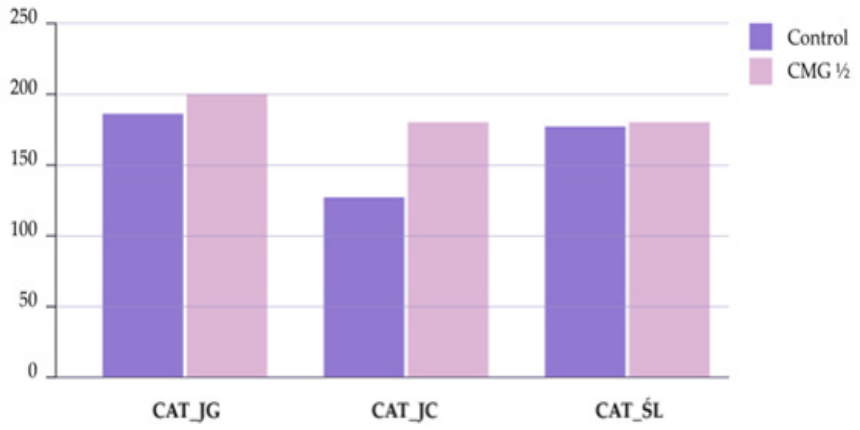


Figure 3. Catalase activity in individual organs in the control group and CMG ½.

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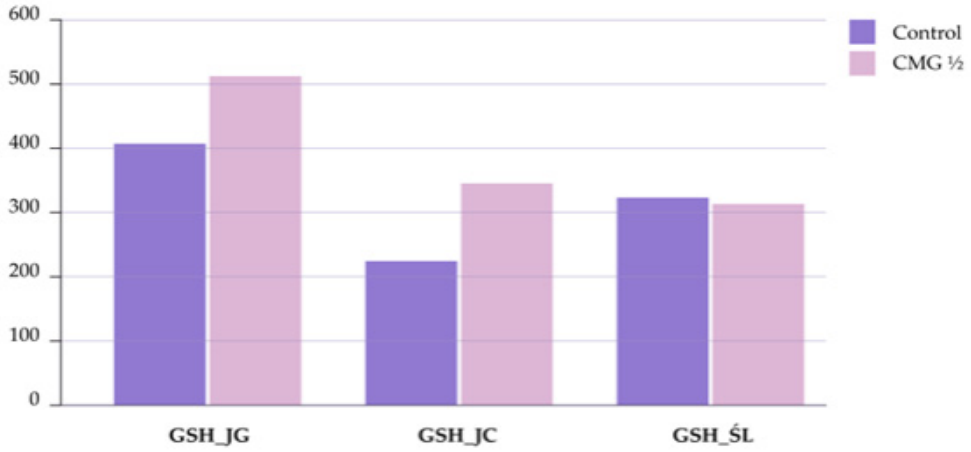


Figure 4. Concentration of reduced glutathione in individual organs in the control group and CMG ½.

3.3. CMG regimen - comparison of full dose and ½ dose

Differences in the activity of antioxidant enzymes and the concentration of reduced glutathione in individual organs are presented in Table 3. A statistically significant difference in the activity of glutathione peroxidase and in the concentration of reduced glutathione in the spleen was shown (Figures 5 and 6).

Table 3. Differences in the activity of antioxidant enzymes and the concentration of reduced glutathione in individual organs in the CMG and CMG 1/2 groups (mean values + SD).

Antioxidant enzyme / reduced glutathione	Organ	Control group, mean + SD	Control group, median	CMG ½ group, mean + SD	CMG ½ group, median	p-value
CAT	LI	189,13 ± 87,88	139,08	198,79 ± 39,73	199,26	0,5135
CAT	SI	141,49 ± 32,81	145,40	179,21 ± 67,40	158,93	0,2065
CAT	SP	296,66 ± 165,25	199,48	178,87 ± 35,11	165,41	0,0992
SOD	LI	293,52 ± 335,70	62,61	514,05 ± 106,16	509,56	0,4395
SOD	SI	341,25 ± 73,72	358,25	453,12 ± 213,27	386,90	0,2544
SOD	SP	698,78 ± 579,94	449,19	284,50 ± 123,82	268,45	0,0553
GPx	LI	361,30 ± 324,60	165,79	1343,60 ± 1186,38	740,14	0,1645
GPx	SI	766,90 ± 906,14	344,54	488,27 ± 557,05	360,13	0,6787
GPx	SP	2657,43 ± 1676,41	1701,31	684,58 ± 377,32	558,17	0,0027
GSH	LI	431,53 ± 173,82	382,38	512,30 ± 179,30	491,77	0,3097
GSH	SI	232,04 ± 32,45	234,27	344,87 ± 154,70	305,69	0,1292
GSH	SP	457,02 ± 141,39	401,70	314,85 ± 58,23	294,26	0,0127
GR	LI	1,24 ± 0,84	0,98	0,93 ± 0,64	0,79	0,3710
GR	SI	0,40 ± 0,52	0,14	0,56 ± 0,48	0,41	0,5135
GR	SP	1,32 ± 0,66	1,17	1,37 ± 0,31	1,38	0,5941

[3] CMG – regimen with cyclosporine, mycophenolate mofetil and prednisone in full dose; CMG ½ – regimen with cyclosporine, mycophenolate mofetil and prednisone in reduced dose, CAT – catalase, SOD – superoxide dismutase,GPx – glutathione peroxidase, GSH – reduced glutathione , GR – glutathione reductase, SI – small intestine, LI – largeintestine, SP – spleen. Statistically significant results ($p<0.05$) are marked in green, results on the verge of statistical significance are marked in green.

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Figure 5

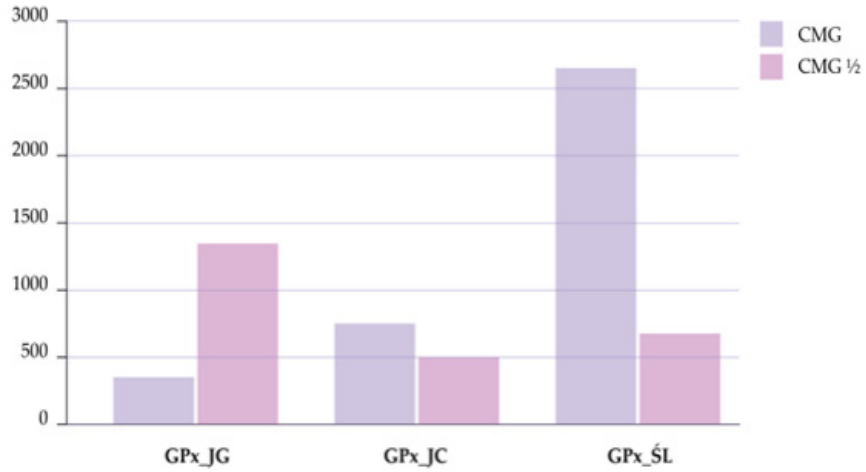


Figure 5. Glutathione peroxidase concentration in individual organs in the CMG and CMG ½ groups.

Figure 6

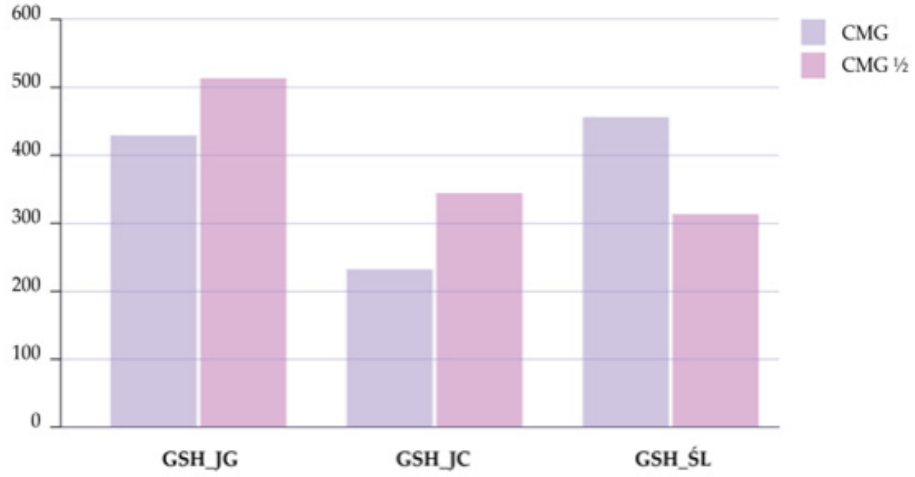


Figure 6. Concentration of reduced glutathione in individual organs in the CMG and CMG ½ groups.

3.4. Half dose TMG regimen. Comparison to the control group

Differences in the activity of antioxidant enzymes and the concentration of reduced glutathione in individual organs are presented in Table 4. A statistically significant difference was found in the activity of catalase in the small intestine and large intestine, in the activity of glutathione reductase in the large intestine and spleen, and in the activity of superoxide dismutase in the spleen. Differences in the concentration of reduced glutathi one in the small intestine and spleen were also found (Figures 7, 8, 9 and 10).

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Table 4. Differences in the activity of antioxidant enzymes and the concentration of reduced glutathione in individual organs in the control group and TMG 1/2 (mean values + SD).

Antioxidant enzyme / reduced glutathione	Organ	Control group, mean + SD	Control group, median	TMG ½ group, mean + SD	TMG ½ group, median	p-value
CAT	LI	187,16 ± 49,248	181,65	117,98 ± 29,27	113,41	0,0008
CAT	SI	128,19 ± 38,58	116,62	220,23 ± 175,78	146,38	0,0278
CAT	SP	177,81 ± 61,26	163,85	209,34 ± 61,17	213,02	0,2276
SOD	LI	476,88 ± 157,82	489,19	436,58 ± 193,69	398,56	0,5824
SOD	SI	356,37 ± 83,66	349,19	500,96 ± 385,30	358,75	0,7021
SOD	SP	236,13 ± 207,02	165,5	372,61 ± 154,08	319,50	0,0090
GPx	LI	751,67 ± 713,21	683,44	825,90 ± 692,29	735,00	0,8212
GPx	SI	585,74 ± 475,71	396,46	752,51 ± 780,84	497,33	0,6511
GPx	SP	628,37 ± 489,40	497,45	777,44 ± 373,57	616,94	0,3463
GSH	LI	406,62 ± 107,35	426,86	371,79 ± 52,39	364,53	0,4176
GSH	SI	224,95 ± 67,99	209,12	386,51 ± 292,45	297,17	0,0056
GSH	SP	325,83 ± 114,17	306,12	430,93 ± 118,32	437,15	0,0426
GR	LI	1,21 ± 0,61	1,19	0,52 ± 0,35	0,58	0,0034
GR	SI	0,23 ± 0,20	0,18	0,55 ± 0,74	0,20	0,5079
GR	SP	1,26 ± 0,60	1,19	1,91 ± 0,64	1,73	0,0357

4 TMG – regimen with tacrolimus, mycophenolate mofetil and prednisone in a reduced dose, CAT – catalase, SOD – superoxide dismutase, GPx – glutathione peroxidase, GSH – reduced glutathione, GR – glutathione reductase, SI – small intestine, LI – large intestine, SP – spleen. Statistically significant results ($p<0.05$) are marked in red.

Figure 7

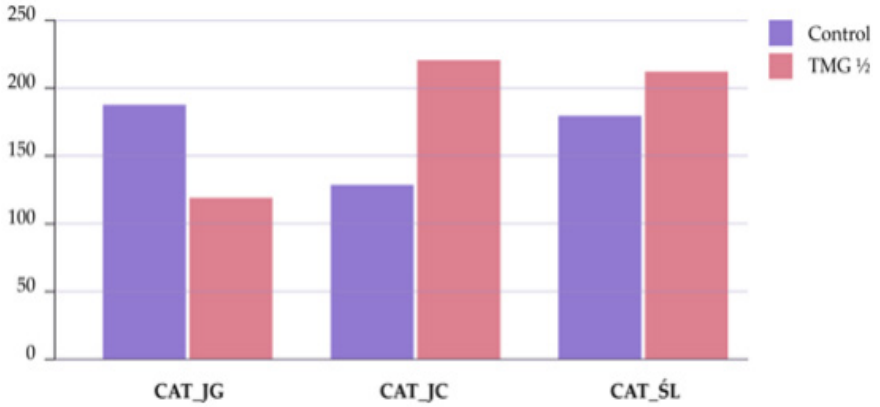


Figure 7. Catalase activity in individual organs in the control group and TMG ½.

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Figure 8

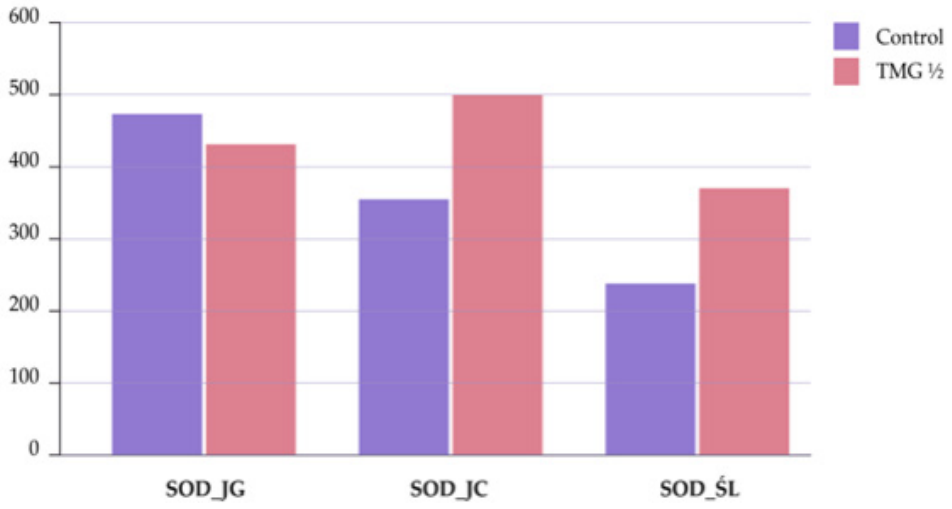


Figure 8. Superoxide dismutase activity in individual organs in the control group and TMG ½.

Figure 9



Figure 9. Concentration of reduced glutathione in individual organs in the control group and TMG ½.

Figure 10

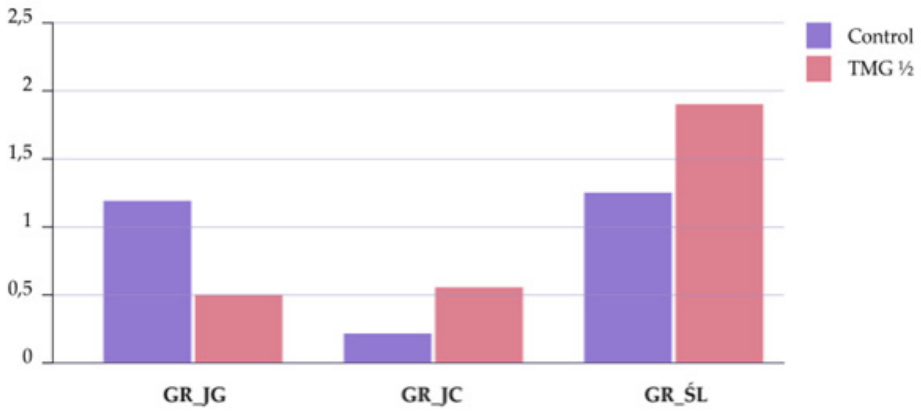


Figure 10. Activity of glutathione reductase in individual organs in the control group and TMG ½.

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3.5. Half dose CEG regimen. Comparison to the control group

Differences in the activity of antioxidant enzymes and the concentration of reduced glutathione in individual organs are presented in Table 5. A statistically significant difference was found in the activity of catalase and glutathione reductase in the small intestine.A statistically significant difference in the concentration of reduced glutathione in the small intestine was also shown (Figures 11, 12 and 13).

Table 5. Differences in the activity of antioxidant enzymes and the concentration of reduced gluta thione in individual organs in the control group and CEG ½ (mean values + SD).

Antioxidant enzyme / reduced glutathione	Organ	Control group, mean + SD	Control group, median	CEG ½ group, mean + SD	CEG ½ group, median	p-value
CAT	LI	187,16 ± 49,248	181,65	212,78 ± 67,42	198,61	0,3845
CAT	SI	128,19 ± 38,58	116,62	191,98 ± 55,82	183,10	0,0221
CAT	SP	177,81 ± 61,26	163,85	138,17 ± 23,20	129,02	0,1198
SOD	LI	476,88 ± 157,82	489,19	492,23 ± 96,73	486,88	0,8369
SOD	SI	356,37 ± 83,66	349,19	391,01 ± 161,06	459,17	0,6504
SOD	SP	236,13 ± 207,02	165,5	197,85 ± 49,22	170,83	0,8369
GPx	LI	751,67 ± 713,21	683,44	615,24 ± 496,36	517,27	0,9018
GPx	SI	585,74 ± 475,71	396,46	453,04 ± 369,41	441,95	0,7108
GPx	SP	628,37 ± 489,40	497,45	476,28 ± 145,12	438,56	1,0329
GSH	LI	406,62 ± 107,35	426,86	363,23 ± 137,29	375,43	0,5918
GSH	SI	224,95 ± 67,99	209,12	334,00 ± 124,54	282,05	0,0171
GSH	SP	325,83 ± 114,17	306,12	271,44 ± 45,86	262,53	0,4320
GR	LI	1,21 ± 0,61	1,19	0,74 ± 0,43	0,72	0,1003
GR	SI	0,23 ± 0,20	0,18	0,64 ± 0,55	0,46	0,013
GR	SP	1,26 ± 0,60	1,19	1,19 ± 0,25	1,31	0,8601

[5] CEG ½ – regimen using cyclosporine A, mycophenolate mofetil and prednisone in a reduced dose, CAT – catalase, SOD – superoxide dismutase, GPx – glutathione peroxidase, GSH- reduced glutathione, GR – glutathione reductase,SI – small intestine, LI – large intestine, SP – spleen. Statistically significant results ($p<0.05$) are marked in red.

Figure 11

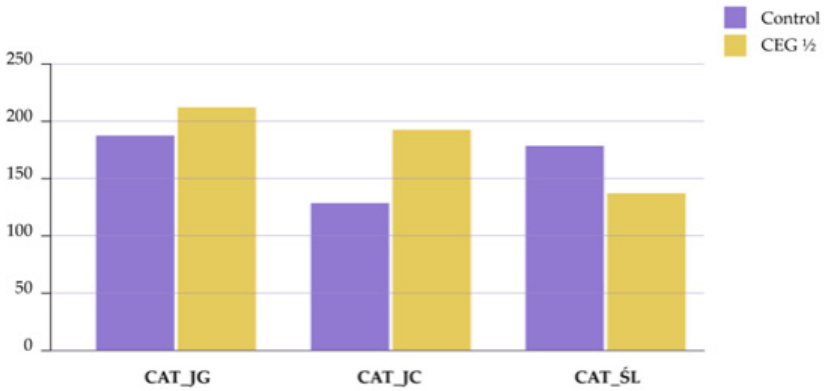


Figure 11. Catalase activity in individual organs in the control group and CEG ½.

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Figure 12

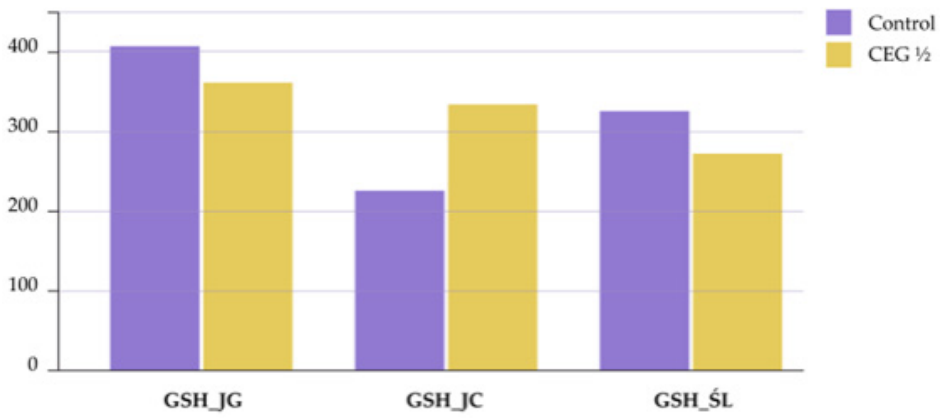


Figure 12. Concentration of reduced glutathione in individual organs in the control group and CEG ½.

Figure 13

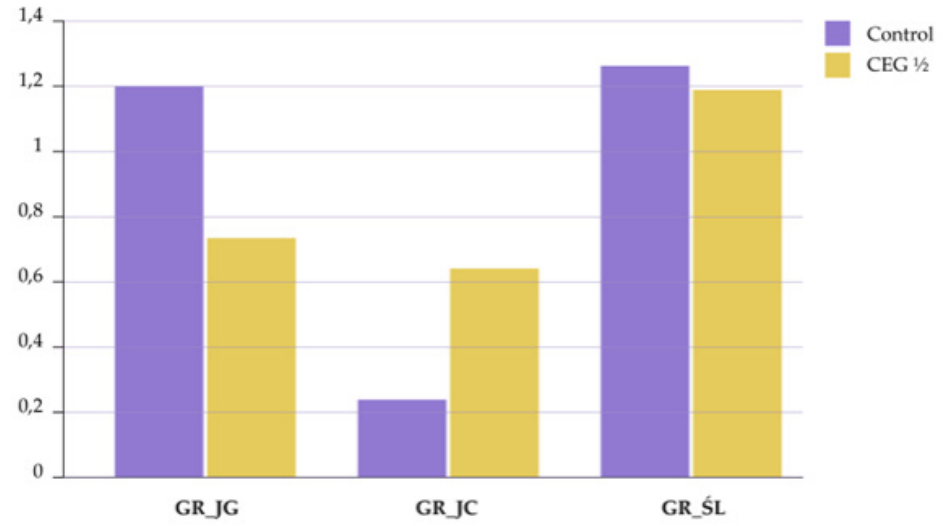


Figure 13. Activity of glutathione reductase in individual organs in the control group and CEG ½.

Table 6. presents a summary of observed differences between the studied groups, which is also a summary of the research. Both the names of the enzyme/reduced glutathione and the organ affected were given (changes on the verge of statistical significance were not included).

Table 6. Summary of the study results

Groups compared	Enzyme / reduced	Organ
CMG vs control	CAT	spleen
	GPx	spleen
CMG ½ vs control	CAT	small intestine
	GSH	small intestine
CMG vs CMG ½	GPx	spleen
	GSH	spleen

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TMG ½ vs control	CAT	small intestine, large intestine
	SOD	spleen
	GSH	small intestine, spleen
	GR	small intestine, spleen
CEG ½ vs control	CAT	small intestine
	GSH	small intestine
	GR	small intestine

[6] SOD – Superoxide dismutase; GPx – Glutathione peroxidase; GSH – Reduced glutathione concentration; GR – Glutathione reductase; CMG – Regimen with cyclosporine, mycophenolate mofetil and prednisone; TMG – Regimen with tacrolimus, mycophenolate mofetil and prednisone;CEG – Regimen with cyclosporine, everolimus and prednisone.

4. DISCUSSION AND CONCLUSIONS

Along with the growing number of patients after kidney transplantation, it comes as no surprise that the percentage of transplanted individuals wishing to become a parent also increases. It is therefore crucial to thoroughly investigate the effect of immunosuppressive treatment on the fetus. There is an urgent need to develop transparent guidelines and a unified model of comprehensive care for a transplant patient planning pregnancy.Cooperation of specialists in the field of transplantology, nephrology and gynecology is required. It is also necessary to educate patients extensively about preparing for transplantation. Full identification of possible risks associated with usage of immunosuppressants in pregnant patients (or those planning pregnancy) is an issue too broad to be explored within a single study. The animal model used in this study aimed to focus on selected visceral organs and showed differences in the activity of catalase, glutathione peroxidase, superoxide dismutase, glutathione reductase, and reduced glutathione concentration in the spleen, small intestine, and large intestine in all considered immunosuppressive treatment regimens vs the control group. The study also uncovered significant variations in the activity of free radical scavenging systems depending on the dose of drugs used (full dose according to the CMG regimen vs the group with 50% of the dose). The visceral organs chosen for this study are known to be immunologically active and thus,susceptible to the impact of immunosuppressants. The spleen is the largest organ of the lymphatic system, participating in a whole range of hematopoietic and immune processes.In rats, the spleen is considered fully developed and functional in the 3rd week of life already [36]. There are some differences between the structure of the spleen in humans and mice - for example, the white pulp division zones are much more marked in mice, but the receptors and the ligands necessary for their activation are similar in both species [37].The intestines perform numerous functions in the human body. In addition to the basic,nutritional function, the immune function is also vital - the intestines are a specialized barrier between the interior of our body and the outside world, they participate in the detection, identification, and elimination of pathogens. In recent years, there have been several publications linking the activity of free radicals with inflammatory bowel diseases,showing their relationship with intestinal microbiota disorders as well as the damage to intestinal endothelium [38, 39, 40, 41, 42]. Disturbances in the structure of the gastrointestinal tract in the course of chronic inflammatory bowel diseases are similar to those observed in substance-driven oxidative stress, reports are available regarding methotrexate [43] or aluminum [44]. There are few studies on the effect of immunosuppressive treatment on free radical generation in the gut and spleen. More often, scientific research focused on describing this issue in other organs [28, 41, 45, 46], and the results obtained were difficult to interpret unequivocally. Duru et al. found a reduction in GPX and SOD activity in the kidney and erythrocytes of animals with concomitant increase in oxidative stress indicators (nitric oxide + MDA (malondialdehyde)) during CsA treatment and a decrease in catalase activity in the plasma of animals receiving MMF [47]. Another study found a decrease in the activity of SOD and catalase in the blood of patients treated with CsA and MMF vs the control group and the group receiving rapamycin+MMF. The authors of the aforementioned study did not determine whether these differences resulted from the use of rapamycin or MMF [48]. The activity of catalase, superoxide dismutase, glutathione peroxidase, and the concentration of reduced glutathione in the spleen of tested animals were significantly higher in those receiving full doses of CsA, MMF and prednisone vs control group.Those changes probably indicate a significant increase in oxidative stress in this organ and are a substantial proof of the influence of immunosuppressive drugs on the structure and function of the spleen. It is known that the activity of immunological processes in the spleen increases with growing exposure to harmful factors or pathogens found in the bloodstream. The increase in the activity of the investigated enzymes in the spleen may prove that their synthesis is not disrupted, but on the other hand, it may be an indirect proof of the escalation of reactions involving free radicals.

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Regarding TMG ½ regimen, a statistically significant increase in the activity of superoxide dismutase and glutathione reductase was observed along with the increase in the concentration of reduced glutathione in the spleen of the examined individuals. Interestingly, these alterations affected not only the spleen, but all of the examined organs. This may suggest the presence of various derangements of homeostasis in these organs, resulting from this specific combination of drugs. In a previous study, Vural et al. found an increase in superoxide dismutase activity in the erythrocytes of patients after kidney transplantation undergoing immunosuppression with tacrolimus-based regimens [49].Interestingly, in our study, in contrast to the increase in the activity of antioxidant reactions in the spleen and small intestine, the TMG regimen showed a decrease in the activity of catalase and glutathione reductase in the large intestine. Dalmarco et al. observed a decrease in GPX activity in the kidneys of mice receiving MMF according to the authors,this was probably due to the nephrotoxicity of the drug leading to the proximal tubule damage, where glutathione peroxidase synthesis occurs [50,51]. We hypothesize that in the TMG regimen, the synthesis of catalase and glutathione reductase in the large intestine was also disturbed; however, there might be a potentially beneficial effect of dampened activity of free radicals in the lower parts of the GI tract. A comparison of the full-dose CMG group vs the half-dose CMG group revealed a significant increase in the activity of catalase and glutathione peroxidase in the spleen in the first group. This may indicate that the toxicity of CMG on the spleen is dose-dependent.

In response to the production of free radicals, the body activates the mechanisms intended to eliminate them in a specific order. One of the scavengers that act first are peroxidases when their utilization capacity reaches its limit, the activity of other enzymes such as catalase and dismutase increases [52]. In our study, GPX activity in the spleen was higher in the full-dose CMG group, both in comparison to the control group and the reduced-dose CMG group. In the subset of animals in the reduced-dose CMG regimen vs the control group, catalase activity and the concentration of reduced glutathione in the small intestine substantially increased, while no significant changes were observed in the spleens of the tested animals. This may indicate that the reduced doses of drugs did not induce extensive oxidative stress in this lymphatic organ, though the apparent increase in antioxidant activity in the small intestine most likely implies an increase in local inflammatory response in this area.

Among the offspring of females receiving the CEG regimen, changes in the small intestine were mainly observed: a significant increase in the concentration of reduced glutathione, catalase activity, and glutathione reductase. In this case, a damaging impact on the intestinal barrier typical of everolimus is possible (inhibition of quickly proliferating cells). The mTOR kinase (blocked by mTOR inhibitors: rapamycin and everolimus) is a part of the complex mechanism regulating transcription, translation, and proliferation of many different proteins involved in the regulation of metabolism, growth, or initiation of apoptosis processes in mammalian cells. In clinical practice, everolimus is more often used than rapamycin due to easier administration (no need for a loading dose) and fewer side effects [53]. The most frequently described side effects of mTOR inhibitors include aggravation of natural wound healing process. Wounds after kidney transplantation are no exclusion, hence the use of mTOR inhibitors immediately after the procedure is uncommon they are usually introduced as conversion treatment at a later stage [54]. A study by Shing et al. focused on rapidly proliferating (such as vascular endothelial cells) and showed an increase in catalase activity in the aorta of animals treated with rapamycin, relative to controls.There were no statistically significant differences in catalase activity in animals receiving other immunosuppressants, compared to the placebo group[55].

To summarize the results obtained, alterations in the activity of antioxidant enzymes and concentrations of reduced glutathione were demonstrated in all groups of offspring rats whose mothers were immunosuppressed during pregnancy. The results varied de pending on the regimen and drug doses used. The group treated with the full dose of CsA, MMF, and prednisone (CMG regimen) showed a substantial increase in the activity of antioxidant enzymes in the spleen. In the group receiving Tc, MMF, and prednisone at a reduced dose (TMG ½ regimen), various changes were observed in all investigated tissues and organs. In both groups: the reduced-dose CMG (CMG ½ regimen) and the reduced dose CsA, everolimus, and prednisone (CEG ½ regimen), an increase in the activity of dose CsA, everolimus, and prednisone (CEG ½ regimen), an increase in the activity of antioxidant enzymes was demonstrated, mainly in the small intestine.

Supplementary Materials:

The following supporting information can be downloaded at: www.mdpi.com/xxx/s1, Figure 1: Activity of superoxide dismutase in individual organs in the con trol group and CMG; Figure 2: Activity of glutathione peroxidase in individual organs in the contro group and CMG; Figure 3: Catalase activity in individual organs in the control group and CMG ½.; Figure 4: Concentration of reduced glutathione in individual organs in the control group and CMG ½; Figure 5: Glutathione peroxidase concentration in individual organs in the CMG and CMG ½ groups; Figure 6: Concentration of reduced glutathione in individual organs in the CMG and CMG ½ groups; Figure 7: Catalase activity in individual organs in the control group and TMG ½; Figure 8: Superoxide dismutase activity in individual organs in the control group and TMG ½; Figure 9: Concentration of reduced glutathione in individual

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organs in the control group and TMG ½; Figure 10: Activity of glutathione reductase in individual organs in the control group and TMG ½; Figure 11: Catalase activity in individual organs in the control group and CEG ½; Figure 12: Concentra tion of reduced glutathione in individual organs in the control group and CEG ½.; Figure 13: Ac tivity of glutathione reductase in individual organs in the control group and CEG ½.

Author Contributions: Conceptualization Joanna Kabat-Koperska,Irena Baranowska-Bosiacka Methodology Mateusz Bosiacki, Małgorzata Dunaj. Software Krzysztof Safranow. Validation and Formal Analysis Joanna Kabat-Koperska, Krzysztof Safranow. Investigation Joanna Kabat Koperska. Data curation Joanna Kabat-Koperska, Grzegorz Marcinkowski. Writing-Original Draft Preparation Grzegorz Marcinkowski, Joanna Kabat-Koperska. Writing-Review and Editing Grze gorz Marcinkowski, Joanna Kabat-Koperska. Supervision Irena Baranowska-Bosiacka, Joanna Kabat-Koperska.

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Informed Consent Statement: Not applicable.

Data Availability Statement: Tekst pracy doktorskiej jest dostępny w bibliotece PUM w Szczecinie.

Conflicts of Interest: The authors declare no conflict of interest.

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