Original Paper

Novel Insights into Oxidative Stress and Antioxidant Enzymes in Acute Antibody-Mediated Rejection of Renal Allografts

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Keywords. Enzyme;Graft rejection;Kidney;Oxidative Stress; Oxidoreductases

Introduction. Antibody mediated rejection (AMR) is a major challenge in kidney transplantation and adversely affects allograft survival. Oxidative stress (OS) is implicated in AMR pathogenesis by triggering inflammation, apoptosis and fibrosis in the graft tissue. However, the status of OS and antioxidant defense in AMR patients remains unclear. We aimed to evaluate the levels of OS markers and antioxidant enzymes in AMR patients.

Methods. We conducted a case-control study involving 22 biopsy-proven AMR patients (test group) and 14 kidney recipients with stable graft function (control group). Serum total oxidant status (TOS), total antioxidant capacity (TAC), total thiol groups, nitric oxide (NO), 8-isoprostane (8-IP) were determined and activities of superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx) were measured by spectrophotometric methods.

Results. Data analysis showed significant increases in TOS, TAC and 8-IP levels together with marked reductions in NO and total thiol groups in AMR patients. CAT and GPx activities did not differ between groups, however SOD activity was significantly lower in AMR patients.

Conclusion. Our study showed increased OS and impaired antioxidant defense in AMR patients. NO level may serve as a potential biomarker of OS severity and immune response in AMR. Further studies are required to elucidate the mechanisms and consequences of OS in AMR and to explore the therapeutic potential of antioxidants.

IJKD 2024;18:227-35
www.ijkd.org
DOI: 10.52547/ijkd.7822

INTRODUCTION

Oxidative stress (OS) refers to an imbalance between the generation of oxidizing agents and the antioxidant defense mechanisms, which could result in cellular damages, apoptosis, and cell death.[1] Organs with high metabolic activity, such as kidneys, are particularly susceptible to OS.[2]

The involvement of OS is well-documented in various age-related chronic and degenerative diseases.[3] Research into oxidative stress is a burgeoning field, and kidney disorders such as chronic kidney disease (CKD) and end-stage kidney disease (ESKD) have been identified as conditions with heightened oxidative stress levels.[4,5] While some studies have reported an improvement in OS following successful kidney transplantation in ESKD patients, [6] there remains a lack of consensus on the precise nature of OS in kidney

transplantation.[7] Several studies have reported increased activities of superoxide dismutase (SOD) and glutathione peroxidase (GPx) in patients with allograft rejection,[8,9] while others have observed decreased GPx enzyme activities.[10,11]

Previous investigations in kidney transplant recipients have shown that OS triggers immune responses, leading to neutrophil recruitment, renal damage, and a reduction in allograft survival, ultimately resulting in chronic allograft dysfunction.[12] Immunosuppressive agents have been proposed to reduce the risk of rejection and enhance graft survival, although their impact on OS status remains a subject of debate.[13,14] Therefore, it is crucial to elucidate the intricate role of each component within the renal oxidative/reductive system.

Antibody-mediated rejection (AMR), a significant complication that occurs following kidney transplantation and affects approximately 20% of patients within the first-year post-transplantation. It is responsible for almost 30% of all cases of acute rejection.[15] Oxidative stress is believed to contribute to cell death and allograft dysfunction in AMR. This occurs when immunoglobulins bind to the graft tissue; activate the complement system and lead to the formation of the membrane attack complex (MAC). The MAC, in turn, triggers ion flux, elevates intracellular calcium ions, and generates reactive oxygen species (ROS), resulting in oxidative stress and cell death.[16] We hypothesized that the imbalance in the activity of enzymes involved in the oxidation/oxidation system, and the changes in the level of the main factors of oxidative stress may play a role in tissue damage in AMR. Therefore, understanding the changes in OS markers might be useful for predicting allograft survival and choosing an effective treatment.[17,18] Oxidative stress plays a pivotal role in the pathogenesis of kidney diseases, but there is limited knowledge regarding the OS status in patients with acute antibody-mediated rejection, a severe form of allograft rejection. Although previous research has underscored the role of oxidative stress in both chronic and acute rejections, no study has specifically investigated oxidative stress in AMR patients. Given that, the presence of antibodies in patients can induce oxidative stress, this study aimed to discern the intensity and patterns of oxidative stress in AMR patients.

MATERIALS AND METHODS

Patients

This case-control study was conducted in the transplant center of Labafinejad hospital, Tehran, Iran. The study included all first-time kidney transplant recipients aged between 15 to 65 years old. The exclusion criteria were multiple organ transplantation, HLA-incompatible transplants, graft loss during the first three months after transplantation, hepatitis, infections and pregnancy.

All patients were on triple immunosuppressive treatment consisting of tacrolimus, mycophenolate mofetil, and methylprednisolone. Renal biopsy specimens were obtained from patients with unexplained graft dysfunction, in the absence of obstruction, and with serum creatinine levels higher than 25% above baseline in two consecutive measurements. Biopsy specimens were analyzed by two independent nephropathologists, and patients with antibody-mediated rejection (AMR) were considered as the patient group according to the 2018 Banff classification reference guide.[19] Recipients with stable graft function including no increase in serum creatinine, no proteinuria and no rejection episode for at least 6 months were considered as the control group.

Twenty-two recipients with AMR were identified according to Banff criteria (the AMR group), and 14 recipients with stable graft function in follow- up visits were included in the control group. Of these individuals, 24 (67%) were males and 12 (33%) were females.

Ethical Issues

All procedures performed in the study were in accordance with the version 2013 of Declaration of Helsinki (1967) and the study was approved (IR.UMSHA.REC.1396.902) by Research Ethics Committee of Hamadan University of Medical Sciences (Hamadan-Iran) and by the Ethics Board of Shahid Beheshti University of Medical Science (Tehran-Iran). This retrospective case- control study evaluated oxidative status in kidney transplant recipients. The nature of the study was explained and informed consents were obtained from all participants. Demographic characteristics and clinical data were collected by using patient records. Blood and urine samples were collected on the day of biopsy from all transplant patients who were candidates for renal biopsy and at follow

up visits from stable patients.

Serum collection and preparation

Blood samples of fasting participants were collected in gel and clot activator tubes, centrifuged at 3000 rpm for 8 minutes, and the separated serum samples were stored at -80℃ for the further procedures.

Urine protein assay

Bicinchoninic acid protein assay kit (Thermo Scientific, USA) was used to measure the 24-hour urinary protein level of samples. Purple color formed by the reaction of BCA and Cu^{+1} in alkaline solution is detectable. The absorbance was read at 562 nm and bovine serum albumin was used as a standard.

Determination of serum total antioxidant capacity (TAC)

Antioxidant capacity of serum samples was measured by the FRAP method, which detects the color change of a Fe3+-TPTZ (2,4,6-tripyridyl-S-triazine) complex when it is reduced by antioxidants. Results expressed as µmol/L Fe2+.[20]

Determination of serum total oxidant status (TOS)

Serum TOS was measured by using FOX (ferrous oxidation-xylenol orange) reagent which changes color when it reacts with ferric ion and xylenol orange. Ferric ion is produced by the oxidation of ferrous ion by hydrogen peroxide in acidic conditions.[21] Ten µl of serum and 190 µl of FOX reagent were used for each measurement. The absorbance was read at 560 nm and TOS values were calculated by using a standard curve of H_2O_2 and expressed as µmol/L H_2O_2.

Determination of serum oxidative stress index (OSI)

Serum OSI was defined as the ratio of TOS/TAC with an arbitrary unit (TOS (µM)/ TAC (mM)).[22]

Determination of serum 8-isoprostane (8-IP)

Human 8-Isoprostane ELISA kit (MyBioSource, San Diego, CA, USA) was used to measure serum 8-IP level as manufacturer's instructions. The serum 8-IP concentration was calculated by using a standard curve of 8-IP. The results were expressed the as pg/ml.

Determination of serum total thiol groups

DTNB (2,2-dithiobisnitrobenzoic acid) assay was used to measure the free thiol groups in the sample. This assay detects the yellow color of TNB, which is produced by the reaction of DTNB and free thiol groups.[23] The absorbance was read at 412 nm and results were expressed the as nmol/ml.

Determination of serum nitric oxide (NO)

Serum NO level was measured by the Griess assay, which detects the color change of Azo dye when it reacts with NO2- in acidic conditions.[24] The absorbance was recorded at 560 nm and the concentration of NO was determined by using nitrate standard curve and expressed as µmol/L.

Determination of serum antioxidant enzyme activities

Determination of serum catalase (CAT) activity. Serum catalase activity was measured by using Catalase Activity Kit (Kiazist, Hamadan, Iran) which detects the color change of Purpald reagent when it reacts with formaldehyde. Formaldehyde is produced by the reaction of methanol and H2O2 with CAT. Activity of CAT was calculated by using a standard curve of formaldehyde. The results were expressed as mU/ml using the following formula:

$$\text{CAT activity}\left(\frac{\text{mU}}{\text{ml}}\right)=\frac{\text{sample valume}}{20}\times 12\times\text{sample dilution}$$

Determination of serum superoxide dismutase (SOD) activity

Serum SOD activity was assessed by using SOD activity Kit (Kiazist, Hamadan, Iran) which detects the color change of a chromogen when it reacts with xanthine and xanthine oxidoreductase. SOD inhibits this reaction by scavenging superoxide radicals. The absorbance was read at 560 nm and SOD activity was calculated on the basis of the inhibition rate using the following formula:

$$\text{inhibition rate}=\frac{(\text{B2 absorbance}-\text{B1 absorbance})-(\text{sample absorbance}-\text{control absorbance})}{(\text{B2 absorbance}-\text{B1 absorbance})}\times 100$$

Determination of serum glutathione peroxidase (GPx) activity

Serum GPx activity was measured by detecting the NADPH reduction in a coupled reaction of glutathione peroxidase and reductase. GPx

Activity kit (Kiazist, Hamadan, Iran) was used. The absorbance change was recorded at 340 nm over time and calculated GPx activity using the following formula:

$$\text{GPx activity}\left(\frac{mU}{ml}\right) = \frac{\Delta 340 \,/\, min}{0.00216\ M} \times \frac{110}{sample\ valume} \times sample\ dilution$$

Statistical analysis

Statistical analysis was performed by using the Statistical Package for Social Sciences version 16 (SPSS Inc., Chicago-USA). Kolmogorov-Smirnov test was used to check normality of data. Independent -samples t-test was performed to compare mean values between groups. Values were reported as M mean ± SD and significance level was set at $P < .05$. Mann Whitney U test was used to analyze SOD data, which was not normally distributed, and results were expressed as median and interquartile range (IQR).

RESULTS

Three hundred and two kidney transplant recipients underwent renal biopsy during two years from 2019 to 2021 in Labbafinejad hospital; Tehran, Iran. Twenty-two AMR patients and 14 stable patients (control group) were included in our study. The AMR group had a shorter duration between transplantation and enrollment compared to the control group (1 month vs 21 months),

Table 1. Demographic and clinical data of AMR patients and patients with stable graft function

Parameters	AMR (n = 22) Mean ± SD	Stable (n = 14) Mean ± SD	*P*
Age (year)	41.32 ± 14.61	37.43 ± 13.46	> .05
Dialysis prior to transplantation (month)	6.6 4 ± 8.67	12.14 ± 15.36	> .05
Cause of ESRD#			
Diabetes	1 (4.54%)	2 (14.29%)	
HTN	4 (18.18%)	2 (14.29)	
GN	4 (18.18%)	4 (28.57%)	
ADPKD	3 (13.63%)	1 (7.14%)	
Others	10 (45.45%)	5 (35.71%)	
Time from transplantation to enrollment (month)†	1 ± 64	21 ± 62	< .05
eGFR (ml/min/1.73 m^2)	30.36 ± 14.72	77.21 ± 15.21	< .05
Urine total protein (mg/ml)	14.88 ± 12.48	11.03 ± 15.13	< .05
Hb (g/dl)	10.72 ± 1.54	14.18 ± 1.04	< .05
Hct (%)	32.95 ± 4.89	42.44 ± 3.03	< .05
WBC (×10^3/μl)	8.51 ± 3.34	6.69 ± 1.61	> .05
Plt (×10^3/μl)	206.00 ± 68.33	180.14 ± 34.66	> .05
FBS (mg/dl)	111.35 ± 36.03	79.35 ± 25.35	> .05
BUN (mg/dl)	62.75 ± 26.79	22.24 ± 9.45	< .05
Uric acid (mg/dl)	5.46 ± 1.17	5.8 2 ± 1.39	> .05
Creatinine (mg/dl)	2.77 ± 1.73	1.14 ± 0.23	< .05
SGOT (U/L)	25.52 ± 17.00	25.92 ± 12.25	> .05
SGPT (U/L)	35.63 ± 38.89	24.69 ± 16.83	> .05
Ca (mg/dl)	8.76 ± 1.01	9.52 ± 0.54	< .05
P (mg/dl)	4.41 ± 1.04	3.85 ± 1.07	> .05
Na (mEq/L)	138.00 ± 3.94	138.27 ± 2.57	> .05
K (mEq/L)	4.41 ± 0.77	4.12 ± 0.36	> .05
Total Cholesterol (mg/dl)	158.50 ± 56.30	169.00 ± 95.06	> .05
LDL-C (mg/dl)	82.57 ± 19.48	105.50 ± 37.00	> .05
HDL-C (mg/dl)	41.50 ± 9.72	44.14 ± 12.72	> .05
VLDL-C (mg/dl)	26.90 ± 18.10	24.00 ± 14.80	> .05
TG (mg/dl)	134.50 ± 90.50	120.00 ± 73.75	> .05
ALP (U/L)	211.44 ± 103.13	231.17 ± 54.34	> .05

*These data are presented in the form of number of patients and percentage. ADPKD: Autosomal dominant polycystic kidney disease, BUN: blood urea nitrogen, eGFR: estimated glomerular filtration rate, ESRD: end-stage renal disease, FBS: fasting blood sugar, GN: Glomerulonephritis, HN: hypertension, Plt: platelet, SGOT: serum glutamate-oxaloacetate transaminase, SGPT: serum glutamate-pyruvate transaminase, ALP: alkaline phosphatase.
†These data are presented as median ± interquartile range and resulted from Mann Whitney U test.

which means they developed rejection in the early months after transplantation. The majority of AMR patients (68%) experienced allograft rejection within the first year of transplantation, with only five cases happened thereafter. Neither group had any previous episodes of rejection before the trial, as indicated by their medical records. Glomerulonephritis and hypertension were the main most common causes of kidney failure requiring transplantation in both groups, followed by other reasons.

Renal function parameters were significantly different between the groups. Renal function of participants was evaluated with the estimated glomerular filtration rate (eGFR), blood urea nitrogen (BUN) and creatinine. The AMR group exhibited significantly worse renal function than the control group, as shown by lower eGFR (30.36 vs 77.21 ml/min/1.73 m^2), higher BUN (62.75 ± 26.79 vs 22.24 ± 9.45 mg/dl) and creatinine (2.77 ± 1.73 vs 1.14 ± 0.23 mg/dl). However, no significant difference was observed in urinary protein levels between the groups. The AMR group also had lower hemoglobin, hematocrit and calcium levels. The liver function and lipid profile did not differ significantly within the groups (Table 1).

Markers of oxidative stress in AMR

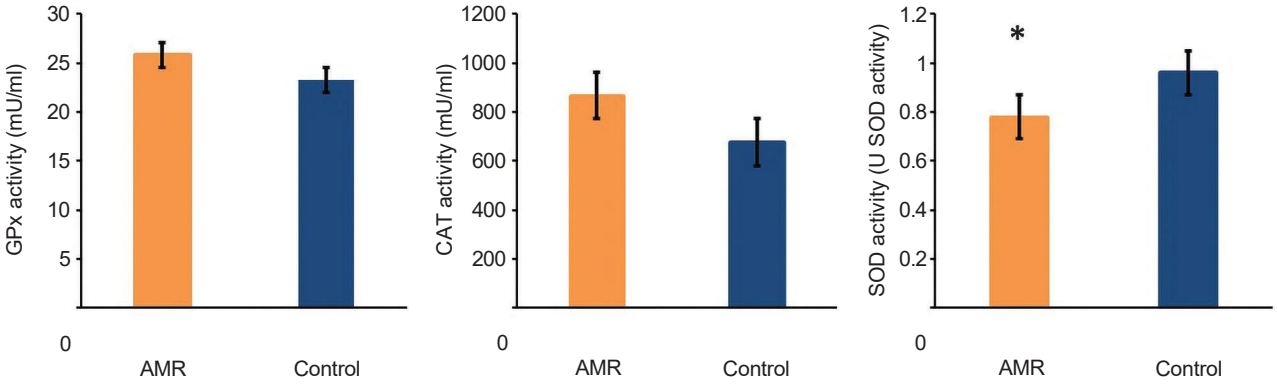
The levels of oxidative stress markers were compared between patients with acute antibody-mediated rejection (AMR) of kidney transplants and patients with stable graft function (control group). Total oxidant status (TOS), total antioxidant capacity (TAC), oxidative stress index (OSI), 8-isoprostane (8-IP), total thiol groups, nitric oxide (NO), activity of catalase (CAT), glutathione peroxidase (GPx) and superoxide dismutase (SOD) were measured as indicators of oxidative stress. We found that AMR group, compared with the control group, had higher TOS, TAC and 8-IP levels, lower total thiol groups and NO levels, and similar OSI levels (Table 2).

GPx, CAT and SOD activities were measured as the main enzymes involved in oxidative stress. Although CAT and GPx activities were higher in AMR patients, analysis showed no significant changes in CAT (866.76 ± 509.44 vs 676.20 ± 548.78 mU/ml, $P > .05$) and GPx (25.84 ± 5.68 vs 23.29 ± 4.36 mU/ml, $P > .05$) activities between groups; while, as presented in Figure, median

Table 2. summarized comparison of oxidant and non-enzymatic antioxidant parameters in study population

Parameters	AMR (n = 22) Mean ± SD	Stable (n = 14) Mean ± SD	*P*
TOS (μM)	55.00 ± 11.91	42.54 ± 14.58	< .05*
TAC (mM)	0.81 ± 0.31	0.58 ± 0.27	< .05
OSI	67.86 ± 27.04	69.80 ± 59.91	> .05
8-Isoprostane (pg/ml)	31.98 ± 0.53	29.77 ± 1.01	< .05
Total thiol group (nm/ml)	0.2 ± 0.1	0.40 ± 0.94	< .05
NO (μM)	54.34 ± 13.33	66.79 ± 19.40	< .05

*Statistical significance was set as < .05
TOS: total oxidant status, TAC: total antioxidant capacity, OSI: Oxidative stress index, NO: nitric oxide



Activities of antioxidant enzymes involved in antioxidant defense system. (A) Glutathione peroxidase, (B) Catalase, and (C) Superoxide dismutase activity in AMR patients and patients with stable graft function. For glutathione peroxidase and catalase data is represented as mean ± SD while for superoxide dismutase data is expressed as median and interquartile range (IQR). "*" indicates significant difference compared with control group ($P < .05$ for AMR versus stable group)

and IQR (interquartile range) of SOD activity was markedly lower in AMR group [0.78 (0.11)] compared to control group [0.96 (0.22)].

DISCUSSION

In this study, we conducted an assessment of various oxidative stress markers and antioxidant enzymes in acute AMR patients, comparing them with recipients who maintained stable graft function. Although we matched the AMR and control groups by age, there was a significant difference in allograft age, with most AMR patients diagnosed within one year of transplantation, while most control group participants were included in the study one- year post-transplantation to ensure graft stability. Both groups had no history of rejection up to the time of sampling. As anticipated, the AMR group exhibited poor renal function and lower levels of hemoglobin, hematocrit, and calcium, which are influenced by kidney function.

We hypothesized that the AMR group would show higher oxidative stress levels and lower antioxidant capacity compared with the control group. We measured levels of nitric oxide (NO), 8-isoprostane (8-IP), and thiol groups as oxidative stress markers in AMR and control patients. It was found that AMR patients had significantly lower NO levels than the control group. Previous research has produced conflicting results regarding NO levels in renal transplantation and rejection, which may depend on factors such as the timing of measurement, the type of rejection, and the effects of immunosuppressive drugs.[25-27] The fact that most of our AMR patients had rejection at a later stage after transplantation, and that we measured oxidative stress markers on the day of the biopsy may have contributed to our results. NO has a complex and dual role in allograft rejection. On one hand, NO reacts with ROS to form peroxynitrite, a cytotoxic agent that damages the allograft, and on the other hand, NO inhibits leukocyte adhesion and platelet aggregation, which are involved in allograft injury.[28,29] The balance between these effects may be influenced by several factors, such as inflammatory cytokines, NF-κB, iNOS, BH4 and superoxide.[30-32] We suggest that the immune and oxidative responses in AMR patients may lead to lower NO levels and higher oxidative stress in the allograft.

8-Isoprostane (8-iso-PGF2α) is a stable isomer of prostaglandin F2 that is formed by lipid peroxidation.[33] Previous studies have shown that patients with end stage kidney disease (ESKD) on long-term hemodialysis have elevated levels of 8-IP compared to the patients with normal kidney function.[34] However, serum 8-IP levels decrease rapidly after transplantation, although they do not reach the levels of healthy individuals.[35] In contrast, urinary F2-isoprostane levels do not change significantly after transplantation.[36] This study also revealed that the AMR patients had higher serum 8-IP levels than the control group, indicating that transplantation does not normalize serum 8-IP levels in kidney disease patients and that AMR may trigger abnormal lipid oxidation processes.

Thiol groups are sulfur-containing compounds that scavenge free radicals and oxidants. They are mainly produced by erythrocytes and are indicative of oxidative stress status.[37] Previous studies have shown that plasma thiol levels are lower in dialysis patients and higher in transplant patients, compared to healthy individual.[38] There is no data on thiol levels in acute rejection episodes, although chronic kidney rejection has been associated with reduced thiol levels[9]. Thiol groups were lower in AMR patients compared to the control group, suggesting that they correlate with decreased GFR and hematocrit.[39,40] This indicates that impaired kidney function and erythropoietin production may impact thiol availability in erythrocytes in AMR patients.

The activities of antioxidant enzymes were assessed, and showed a significant decrease in SOD activity and non-significant increases in GPx and CAT activities in AMR patients. The reports on alterations of antioxidant enzymes in renal transplantation are inconsistent . Studies have reported increased,[8,41] decreased[10,17] or unchanged[42] activities of these enzymes after transplantation. However, there is limited evidence on the activities of these enzymes in renal rejection. Fonseca *et al.*, demonstrated that SOD activity did not increase after kidney transplantation,[41] but reduced mitochondrial SOD activity was observed in chronic rejection.[43] Our results indicate a significant reduction of both mitochondrial and cytosolic SOD activity during acute rejection. The reduction in SOD activity is unclear but could be related to tyrosine nitration and mitochondrial SOD inactivation.[43]

Although the mean CAT and GPx activities

were slightly higher in AMR group than in control patients, the difference was not statistically significant. This is consistent with a previous study that found similar GPx activity levels in patients with acute renal rejection and stable graft function.[11] However, another study reported higher GPx activity in chronic rejection compared to stable kidney function.[9] Although CAT and GPx are responsible for degradation of hydrogen peroxide produced by SOD, it should be noted that immunoglobulin light chains can also generate hydrogen peroxide and proinflammatory agents in proximal tubular epithelium,[44] which could account for the elevated CAT and GPx activities in AMR patients.

Total antioxidant capacity (TAC) and total oxidant status (TOS) were both significantly higher in AMR patients than the control group, with no significant difference in the oxidative stress index (OSI). Previous studies have shown that the cytochrome P450-mediated metabolism of calcineurin inhibitors, which are immunosuppressive drugs, generates reactive oxygen species (ROS) and disrupts the redox balance.[13] However, previous studies have indicated that oxidative stress levels after transplantation are not significantly affected by the type of treatment protocol.[45] Both groups of patients and controls were on immunosuppressive therapy, but the initial dose of the drugs was higher in the early months of transplantation. Our AMR group, wtih an acute rejection episode shortly after transplantation, received an even higher dose of the drugs and antioxidant capacity than the other transplant recipients did. While previous studies have suggested that kidney transplantation improves inflammation and antioxidant status (at least six months after transplantation)[46] and increases oxidative stress in acute and chronic rejection,[47] Antolini *et al.* reported that kidney transplant patients had higher antioxidant power in the rejection groups than in the control groups, which was contrary to their expectation. They suggested that renal transplantation might induce an increase in antioxidant capacity, but this would decline as the transplanted kidney function normalized.[48] Therefore, an increase in antioxidant capacity does not necessarily indicate an improvement in the immune system, but it could signal an early stage of kidney dysfunction and failure. Thus, TAC might be a marker of oxidative stress in the initial phase of renal transplantation.[49] Furthermore, TAC is a comprehensive indicator of antioxidant capacity and it could be influenced by not only the improvement of renal function but also the reduction of oxidative stress after transplantation.[18]

This study showed some interesting results, but they are preliminary and need further investigations. Serum samples were used to measure OS status in allograft tissue, because tissue sampling could endanger the graft survival. However, other OS parameters such as malondialdehyde, carbonyl groups, free thiol groups, glutathione, acute phase proteins, and myeloperoxidase activity could give more reliable information about OS status in AMR patients. Urine samples and a larger sample size could also help to validate the serum results.

CONCLUSION

In conclusion, this study showed that AMR patients exhibit higher oxidative stress levels and lower antioxidant enzyme activity, suggesting a role for oxidative stress in AMR pathogenesis and allograft injury. Further research is needed to understand the mechanisms of oxidative stress in AMR and evaluate the potential benefits of antioxidant therapy for allograft survival and function.

ACKNOWLEDGMENT

Authors are grateful for both Hamadan University of Medical Sciences and Shahid Beheshti University of Medical Sciences for financial support of the project (UMSHA-9612228430).

CONFLICT OF INTERESTS

Authors declare no conflict of interest.

FUNDING

The study was funded by Vice-chancellor for Research and Technology, Hamadan University of Medical Sciences, Iran (UMSHA-9612228430).

ETHICAL APPROVAL

The study was approved by the Ethics committee of Hamadan University of Medical Sciences (IR.UMSHA.REC.1396.919) and was conducted in accordance with the Declaration of Helsinki.

INFORMED CONSENT

Informed written consent was obtained from the study participants.

AUTHORSHIP

MN and IK were responsible for designing the research. S-SH and SS prepared the manuscript. S-SH conducted the sample preparation and laboratory analysis. All authors participated in reviewing the manuscript.

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Received July 2023
Revised October 2023
Accepted November 2023