Original Research

Beneficial effects of mycophenolate mofetil on cardiotoxicity induced by tacrolimus in wistar rats

Hanen Ferjani[1], Rim Timoumi[1], Ines Amara[1], Salwa Abid[1], Abedellatif Achour[2], Hassen Bacha[1] and Imen Boussema-Ayed[1]

[1]Laboratory of Research on Biologically Compatible Compounds, Dental Medicine Faculty, University of Monastir, 5019 Monastir, Tunisia; [2]Department of Nephrology, Dialysis and Transplant, University Hospital of Sahloul, 4021 Sousse, Tunisia
Corresponding author: Hassen Bacha. Email: hassen.bacha@fmdm.rnu.tn

Abstract

The immunosuppressive drug tacrolimus (TAC) is used clinically to reduce the rejection rate in transplant patients. TAC has contributed to an increased prevalence of cardiovascular disease in patients receiving solid organ transplantation. Mycophenolate mofetil (MMF), a potent inhibitor of *de novo* purine synthesis, is known to prevent ongoing rejection in combination with TAC. In the present study, we investigated the antioxidant and antigenotoxic effect of MMF on TAC-induced cardiotoxicity in rats. Oral administration of TAC at 2.4, 24, and 60 mg/kg b.w. corresponding, respectively, to 1, 10, and 25% of LD_{50} for 24 h caused cardiac toxicity in a dose-dependant manner. TAC increased significantly DNA damage level in hearts of treated rats. Furthermore, it increased malondialdehyde (MDA) and protein carbonyl (PC) levels and decreased catalase (CAT) and superoxide dismutase (SOD) activities. The oral administration of MMF at 50 mg/kg b.w. simultaneously with TAC at 60 mg/kg b.w. proved a significant cardiac protection by decreasing DNA damage, MDA, and PC levels, and by increasing the antioxidant activities of CAT and SOD. Thus, our study showed, for the first time, the protective effect of MMF against cardiac toxicity induced by TAC. This protective effect was mediated via an antioxidant process.

Keywords: Tacrolimus, mycophenolate mofetil, cardiotoxicity, rats, antioxidant effect, antigenotoxic propriety

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Introduction

Tacrolimus (TAC) is a potent immunosuppressive agent widely used clinically to reduce greatly the incidence and severity of allograft rejection after organ transplantation.[1] TAC belongs to the family of calcineurin inhibitors (CNI), a protein phosphatase with important regulatory effects such as blocking the expression of T cell activation genes.[2] TAC is also a macrolide antibiotic that share potent immunosuppressive and anti-inflammatory properties.[3,4] However, the therapeutic potential of TAC is limited by its cardiovascular risk. Indeed, the chronic use of TAC after organ transplantation leads to endothelial dysfunction, hypertension, arrhythmia, and alterations in cardiac morphology and pathology in most patients receiving cardiac, renal, and liver allografts.[5–11] TAC treatment-induced cardiotoxicity was assessed biochemically and histopathologically. In fact, TAC was found to decrease vascular nitric oxide (NO) production, to increase the release of endothelin-1 and the expression of transforming growth factor (TGF-β) in rats.[8,12,13,14] Moreover, a recent study of Agirbasli *et al.*[15] reported the toxic effects of TAC on cardiovascular system by the blockade of the renin–angiotensin system.

Mycophenolate mofetil (MMF) was isolated from the fungus *Penicillium brevicompactum*.[16] It is a potent non-competitive and reversible inhibitor of inosine monophosphate dehydrogenase (IMPDH); it inhibits the *de novo* pathway of purine nucleotide synthesis, therefore depleting intracellular guanidine nucleotides.[17] This drug was introduced into clinical practice MMF is an immunosuppressive agent treatment of rejection episodes of organ transplant recipients,[18] it was incorporated in to majority of the immunosuppressive combinations,[19] especially with CNIs (TAC or cyclosporine). TAC, in combination with MMF resulted in over-immunosuppression, reduced the rate of acute graft rejection and treatment for relapsing or resistant rejection.[20] During the last year, MMF used also as a potential development for the treatment of lupus,[21] also other reports documents the antitumoral effect of this drug *in vitro* and *in vivo*.[22] On the other hand, MMF reported any cardiotoxic effect[23] and it have been associated with positive effects on atherosclerosis or hypertension in

animal models,[24] however, the impact of MMF treatment on TAC induced heart failure is an area of our interest.

Thus, the aim of the present study was to evaluate the possible anti-genotoxic and antioxidant effects of MMF against TAC-induced genotoxicity and oxidative stress in heart of rats. For this purpose, we measured DNA damage in heat tissues using comet assay. Oxidative stress involvement was assessed by the measure of malondialdehyde (MDA), protein carbonyl (PC) levels, catalase (CAT), and superoxide dismutase (SOD) activities in heart of rats.

Materials and methods

Chemicals

TAC and MMF were purchased from Sigma Aldrich (France).

Animals

This study was conducted on male Wistar rats (weighing 120–150 g), purchased from the Central Pharmacy (SIPHAT, Tunis, Tunisia). Prior to initiation of the experiment, all animals were maintained two weeks under the same laboratory conditions of temperature ($22 \pm 3^{\circ}$C), relative humidity (55 ± 5 %) and a 12/12 h light/dark cycle and received a nutritionally standard diet (SICO, Sfax, Tunisia) and tap water *ad libitum*. The experimental procedures were carried out according to the National Institute of Health Guidelines for Animal Care and approved by the local Ethics Committee.

Acute toxicity (LD_{50}) study

Acute toxicity study was carried out according to method described by Randhawa.[25] The study was carried out in two phases. In the first phase, 100 rats were randomized into 10 groups (10 rats per group) and were given TAC at 50,100, 200, 300, and 400 mg/kg b.w. and MMF at 100, 200, 400,500 and 700 mg/kg b.w orally. Animals were observed for 24 h after treatment for signs of toxicity and mortality. Mortality in animals used for the first phase of the study at 24 h, informed the choice of doses for all experiments.

Animal treatment

After an acclimation period, rats were randomly divided into nine groups of six each. Rats received oral doses of TAC and MMF alone and in combination as follows: Group 1: receiving equivalent amount of water alone; Groups 2, 3, and 4: receiving 2.4, 24, and 60 mg/kg b.w. of TAC corresponding, respectively, to 1, 10, and 25 % of the LD_{50}. Groups 5, 6, 7, and 8: receiving 5, 50, 125, and 250 mg/kg b.w. of MMF corresponding, respectively, to 1, 10, 25, and 50 % of the LD_{50}. Indeed, we know that the MMF has no cardiotoxic effect, for this reason, we suspected that the toxicity of MMF will be at the high doses, in fact, a wide range of doses of MMF has been tested [from a lower dose to a higher dose (50% of LD_{50})] and we observed from what dose the risks appear. Group 9 was given TAC and MMF simultaneously at 60 mg/kg b.w. (TAC) + 50 mg/kg (MMF). To investigate the combined effects, we selected two doses of immunosuppressant drugs for all experimentations in our study. We choose a higher dose of TAC (60 mg/kg b.w.) that increase the cardiotoxicity risk and one dose of MMF (50 mg/kg b.w.) which has no toxic effect on heart of the rats. After 24 h of treatments, animals were euthanized by decapitation. Hearts were excised immediately, washed with ice-cold physiologic saline solution (0.9 %, w/v), cut into small pieces and stored at -80°C until use.

Preparation of heart extracts

Hearts were homogenized with a Potter (glass-Teflon) in the presence of 10 mM Tris–HCl (100 mg: 500 μL), pH 7.4 at 4°C and centrifuged at 4000 rcf for 30 min at 4°C. The supernatant was collected, aliquoted, and stored at -80°C until use for enzyme assays. The protein concentrations were determined according to Protein BioRad assay[26] using bovine serum albumin as standard.

Alkaline single cell gel electrophoresis assay (Comet assay)

The alkaline comet assay was carried out as described by Tice *et al.*[27] with minor modifications.[28] Immediately after euthanasia, each piece of heart was placed in 0.5 mL of cold phosphate-buffered saline (PBS) and finely minced in order to obtain a cellular suspension. Hearts cells suspensions (60 μL) were embedded in 60 μL of 1 % low melting point agarose and spread on slide precoated with a layer of 1 % (w/v) normal melting point agarose prepared in PBS. The agarose was allowed to set at 4°C for 5–10 min and the slides immersed in lysis solution (2.5 M NaCl, 100 mM EDTA, 10 mM Tris, NaOH to pH 10.0) with freshly added 1% Triton X-100 and 10 % DMSO at 4°C over night to remove cellular proteins and membranes. Slides were then placed in a horizontal electrophoresis tank containing 0.3 M NaOH and 1 mM EDTA for 20 min before electrophoresis at 25 V for 15 min at an ambient temperature. The slides were then washed three times for 5 min each with 0.4 M Tris–HCl, pH 7.5 before staining with 50 μL ethidium bromide (20 μg/mL). The experiment was repeated three times for each sample. Ethidium bromide stained slides were examined with a Nikon Eclipse TE 300 fluorescence microscope (Nikon, Tokyo, Japan). A total of 100 comets on each slide were visually scored according to the relative intensity of fluorescence in the tail and classified as belonging to one of five classes. Each comet class was given a value of 0, 1, 2, 3, or 4 (from undamaged, 0, to maximally damaged, 4) as described previously by Collins *et al.*[29] The total score was calculated by the following equation: (percentage of cells in class 0×0) + (percentage of cells in class 1×1) + (percentage of cells in class 2×2) + (percentage of cells in class 3×3) + (percentage of cells in class 4×4). Consequently, the total score was ranging from 0 to 400.

Evaluation of lipid peroxidation status

Lipid peroxidation was determined indirectly by measuring the production of MDA in the heart extracts following the method of Aust.[30] Briefly, 200 μL of heart extracts were

mixed with 150 μL of TBS (Tris 50 mM and NaCl 150 mM, pH 7.4) and 250 μL trichloroacetic acid-butylated hydroxytoluene (TCA–BHT) (20% TCA and BHT 1%). The mixture was vigorously vortexed and centrifuged at 1500 g for 10 min. About 400 μL of the supernatant were added with HCl 0.6 N and 320 μl Tris–thiobarbituric acid (TBA) (Tris 26 mM and TBA 120 mM), the content was mixed and incubated for 10 min at 80°C. The absorbance was measured at 530 nm. The optic density corresponding to the complex formed with the TBA–MDA is proportional to the concentration of MDA and to the lipid peroxide. The concentration of MDA (nmol/mg of proteins) was calculated from the absorbance at 530 nm using the molar extinction coefficient of MDA 1.56×10^5 M/cm.

PC assay

PC content was determined as described by Mercier *et al.*[31] in heart homogenates by measuring the reactivity of carbonyl groups with 2,4-dinitrophenylhydrazine (2,4-DNPH). About 200 μL of supernatant of heart extracts were placed in two glass tubes. About 800 μL of 10 mM DNPH in 2.5 M HCl were added. Tubes were left for 1 h of incubation at room temperature in the dark. Samples were vortexed every 15 min. Then, 1 mL of 20% TCA was added to samples, and the tubes were left on ice for 10 min and centrifuged for 5 min at 4000 rpm to collect the protein precipitates. Next, another wash was performed using 1 mL of 10 % TCA, and protein pellets were broken mechanically with the aid of a glass rod. Finally, the pellets were washed with 1 mL of ethanol–ethyl acetate (1:1, v/v) to remove the free DNPH. The final precipitates were dissolved in 500 μL of guanidine hydrochloride 6 M and were left for 10 min at 37°C with general vortex mixing. Any insoluble materials were removed by additional centrifugation (10 min at 4000 rcf). PC concentration was determined from the absorbance at 370 nm, applying the molar extinction coefficient of 22.0 mM/cm. A range of nmoles of carbonyl per mL was usually obtained for most proteins and was related to the protein content in the pellets.

Determination of CAT activity

CAT activity was measured in heart extracts at 240 nm, 25°C according to Clairbone.[32] Briefly, 20 μL of the extract were added to a quartz cuvette contain 780 μL phosphate buffer and 200 μL of H_2O_2 0.5 M. The activity of CAT was calculated using the molar extinction coefficient (0.04 mM/cm). The results were expressed as μmol of H_2O_2/min/mg of proteins.

Determination of SOD activity

SOD activity was according to a modified method of Beyer and Fridovich.[33] The supernatant (50 μL) was added to a reaction mixture containing potassium phosphate buffer (pH = 7.8), 0.1 mM EDTA, 12 mM L-methionine, 75 μM nitroblue tetrazolium (NBT), and 2 μM riboflavin to a total volume of 3 mL. The reaction mixture was kept under a fluorescent light for 15 min at 25°C. One SOD unit was described as the amount of enzyme needed to reduce the NBT ratio to 50%. NBT reduction ratios were measured with a spectrophotometer at 550 nm. The activity was expressed as units/mg of proteins.

Statistical analysis

All data were expressed as means ± SD. Statistical significance of differences among different groups was evaluated by one-way analysis of variance (ANOVA) followed by Fisher multiple-comparisons test as a *post hoc* test. A $P < 0.05$ was considered to correspond with statistical significance.

Results

Determination of the median lethal dose (LD_{50}) of TAC and MMF in male rats

Based on our results, the oral LD_{50} value for TAC and MMF were respectively 240 mg/kg b.w. and 500 mg/kg b.w (Figures 1 and 2).

Effect of MF on TAC-induced DNA damage

The antigenotoxic effect of MMF was assessed through the alkaline comet assay. Results of the visual scoring of total basic DNA damage are illustrated in Figure 3. We observed a significant increase of the total DNA damage in hearts of rats treated with TAC alone in a dose dependant manner. MMF induced a significant DNA damage only at the highest tested dose (250 mg/kg b.w.), but at lower doses (50 and 125 mg/kg b.w.), no specific DNA fragmentation was detected. The co-administration of MMF at 50 mg/kg b.w. with TAC at 60 mg/kg b.w. reduced DNA fragmentation caused by TAC alone. The amount of DNA damage decreased about 55, 80%.

Induction of lipid peroxidation

To evaluate lipid peroxidation status, MDA level was measured and the results are shown in Figure 4. MDA is the end product of the major reactions leading to significant oxidation of polyunsaturated fatty acids in cellular membranes and thus, serves as a reliable marker of oxidative stress. When compared to control group, MDA level in the heart was significantly higher ($P < 0.05$) in groups treated with TAC alone at 24 and 60 mg/kg b.w. Therefore, the MDA level increased from a basal level of 0.1616 ± 0.05 nmol/mg of protein to 0.554 ± 0.2 and 0.7823 ± 0.213 nmol/mg of protein. On the other hand, MMF administration at 50 mg/kg b.w. simultaneously with TAC at 60 mg/kg b.w. was associated with a significant fall in the MDA levels. MDA level was significantly decreased from 0.7823 ± 0.213 in heart extracts of rats treated with TAC at 60 mg/kg b.w. to 0.4826 ± 0.118 nmol/mg of protein in group treated with TAC 60 mg/kg b.w. + MMF 50 mg/kg b.w. This reduction was estimated about 56.4%. It is of note that MMF alone, at the highest tested dose (250 mg/kg b.w.) induced a significant enhancement of MDA level. The MMF dose used in combination with TAC did not induce any increase in MDA level.

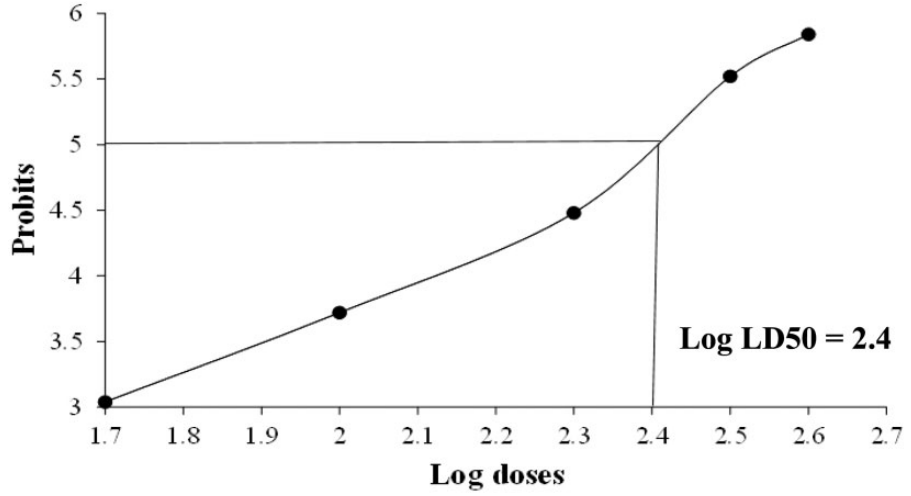

Figure 1 Determination of the median lethal dose (LD_{50}) of TAC in male rats. Mortality rates were recorded at 24 h following TAC administration. The plot shows the predicted mortality rates at different doses of TAC. The LD_{50} was 240 mg/kg

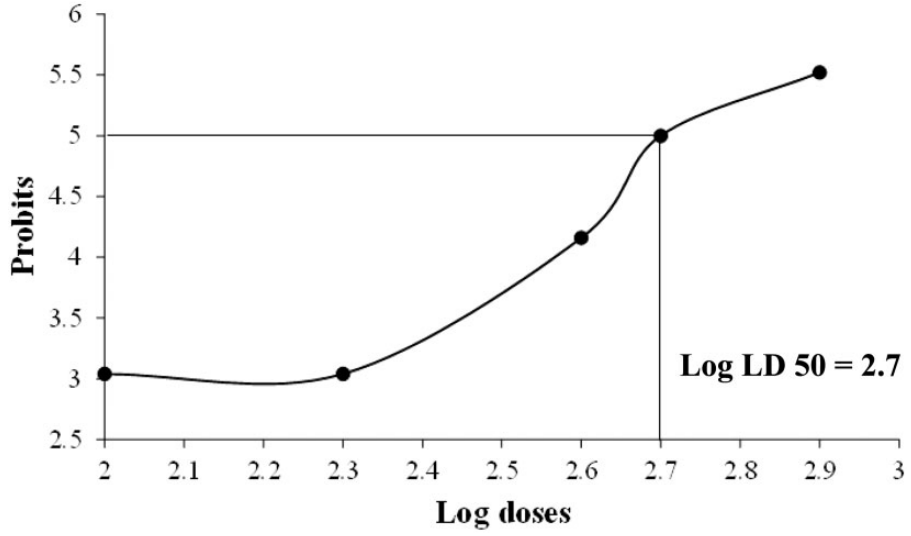

Figure 2 Determination of the median lethal dose (LD_{50}) of MMF in male rats. Mortality rates were recorded at 24 h following MMF administration. The plot shows the predicted mortality rates at different doses of MMF. The LD_{50} was 500 mg/kg

PC assay

The formation of PCs, the most widely used marker of severe protein oxidation was assayed in heart homogenates and results are illustrated in Figure 5. We showed that TAC alone generated a significant increase of PCs formation in heart as compared to control group. The PCs level increased from the basal value of 0.325 ± 0.22 nmol/mg of protein in control group to 0.67 ± 0.14 and 1.4 ± 0.5 nmol/mg of protein in groups treated with TAC at 24 and 60 mg/kg b.w. MMF (at 50 mg/kg b.w.) administration simultaneously with TAC (at 60 mg/kg b.w) decreased significantly the PCs formation induced by TAC alone (60 mg/kg b.w.). Thus, PCs level decreased from the value of 1.4 ± 0.5 nmol/mg of protein in TAC group to 0.75 ± 0.246 nmol/mg of protein in group treated with MMF (50 mg/kg b.w.) + TAC (60 mg/kg b.w.).

CAT activity

CAT is an endogenous antioxidant enzyme that protects cell from detrimental effects of reactive oxygen species. The levels of CAT can indicate the magnitude of oxidative stress that occurs during several injuries. The effect of TAC and MMF on CAT activity was illustrated in Figure 6. Our results showed that TAC alone induced a marked decrease in CAT activity in heart extracts. CAT activity decreased from the basal value of 220.5 ± 20 nmol/min/mg proteins in control group to 117.5 ± 23.63 and 62 ± 31 nmol/min/mg in TAC-treated groups, at respectively, 24 and 60 mg/kg b.w. MMF administration (at 50 mg/kg b.w.) simultaneously with TAC (at 60 mg/kg b.w) promoted a striking increase of this activity. CAT activity increased from 117.5 ± 12.5 nmol/min/mg in TAC

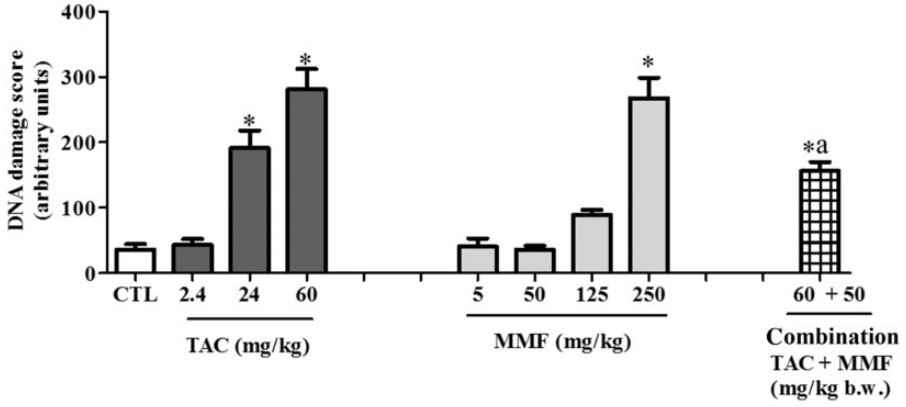

Figure 3 Total DNA fragmentation was measured by the alkaline Comet assay in isolated rat heart cells. Values are expressed as means ± SD ($n = 6$). Superscript characters indicate a significant difference at $p < 0.05$. (*) Significantly different from the control group (CTL). [a]$P < 0.05$ Significant difference from TAC-treated rats

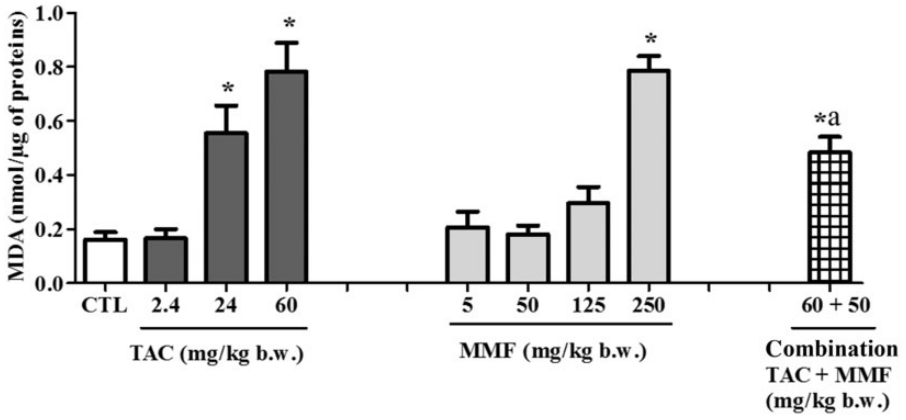

Figure 4 Lipid peroxidation as determined by MDA level in Wistar rat heart. The MDA level was estimated after exposure of TAC and MMF alone and combined after 24 h. Values are expressed as mean + SD ($n = 6$). *$P < 0.05$ significant difference from control (CTL). [a]$P < 0.05$ Significant difference from TAC-treated rats

group to 141.3 ± 23.94 nmol/min/mg in group treated with TAC and MMF.

SOD activity

A second activity of an antioxidant enzyme, SOD, was measured in heart extracts of treated rats. As shown in Figure 7, TAC alone markedly decreased SOD activity when compared to control group. This activity passed from 6.15 ± 0.8 in control group to 3.4 ± 1.4 and 1.86 ± 0.79 in groups treated with TAC at, respectively, 24 and 60 mg/kg b.w. MMF alone did not induce any change in SOD activity unless at the highest dose (250 mg/kg b.w) which decrease significantly SOD activity. MMF (50 mg/kg b.w.) co-treatment with TAC (60 mg/kg b.w.) restored significantly SOD activity in heart extracts. Indeed, SOD activity increased from 1.86 ± 0.79 nmol/min/mg in TAC group to 3.95 ± 1.22 nmol/min/mg in group treated with TAC and MMF.

Discussion

Oxidative stress has been implicated in the pathophysiology of several types of cardiovascular disease (CVD), including ischemic stroke, myocardial ischemia, myocardial stunning, ischemia-reperfusion injury, hypertension and atherosclerosis. Previous studies have demonstrated that the majority of patients with CVD are likely to have chronic oxidative stress.[34,35] The increase of oxidative stress formation by immunosuppressive drugs such as TAC altered profoundly the endothelial function.[8,36] For this reason, our major interest was directed toward the development or discovery of antioxidant compounds that reduce the cardiovascular dysfunction caused by TAC, at the same time, increase TAC efficacy. In this study, we explored the protective effect of MMF against cardiotoxicity induced by TAC and we assessed the involvement of oxidative stress in this eventual protective effect.

In a first set of experiments, we realized the comet assay, one of the standard methods for assessing DNA damages including single- and double-strand DNA breaks.[37] Our results showed that TAC alone caused a significant increase in DNA fragmentation. MMF treatment simultaneously with TAC induced a noticeable decrease in DNA fragmentation in heart of rats.

In a second set of experiments, we evaluated the oxidative damage. Thus, we have measured lipid peroxidation

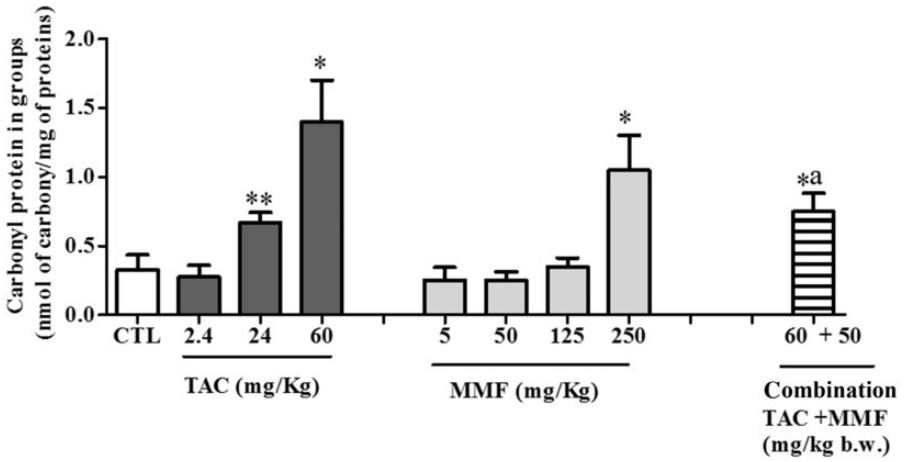

Figure 5 Effect of TAC and MMF treatment alone and their combination on protein carbonyl content in the heart tissues of rats after 24 h. Values represent mean ± SD of six animals in each group. *$P < 0.05$ from control (CTL). **$P < 0.01$ vs. control (CTL). [a]$P < 0.05$ Significant difference from TAC-treated rats

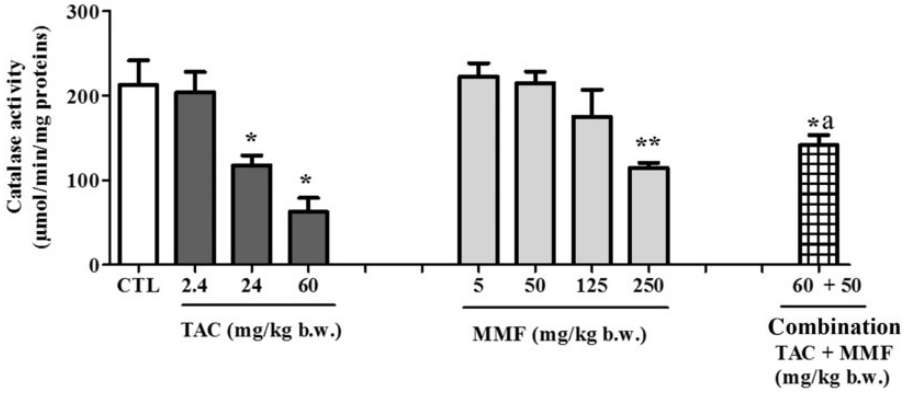

Figure 6 Effect of TAC and MMF alone and combined on catalase enzyme activity in rat heart after 24 h. Values are expressed as mean + SD ($n = 6$). *Significantly different from control group at $P < 0.05$. **Significantly different from control group (CTL) at $P < 0.01$. [a]$P < 0.05$ Significant difference from TAC-treated rats

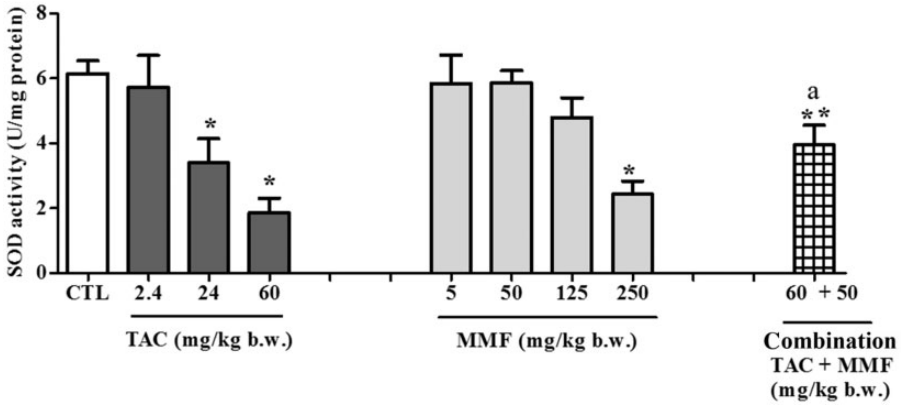

Figure 7 Effect TAC and MMF individually and combined treatment after 24 h in Superoxide dismutase (SOD) activity in rat Heart. The data are expressed as mean + SD ($n = 6$). **$P < 0.01$ vs. control group (CTL). *$P < 0.05$ vs. control group. [a]$P < 0.05$ Significant difference from TAC-treated rats

and PC content as two biomarkers of oxidative stress as well as CAT and SOD activities which are considered as two biomarkers of antioxidant defense. Results of the present study showed that TAC alone increased the MDA and PC levels. It decreased also CAT and SOD activities. These findings were in accordance with other reports showing that TAC induced oxidative stress in rat kidney and mesanglial cells.[38–41] TAC induced also vascular ROS generation

by increasing endothelial NAD(P)H oxidase activity.[42] Interestingly, the induction of oxidative stress by TAC can explain its implication in cardiovascular problems. In fact, many studies showed that the accumulation of proteins modified by the reactive oxygen and nitrogen species was associated with various cardiovascular disorders, such as hypertension, atherosclerosis, cardiac hypertrophy, heart failure, ischemia-reperfusion injury.[43–45] In the current investigation, the MMF co-treatment with TAC reduced significantly MDA and PC levels induced by TAC alone. Thus, MMF could protect heart cells from lipid peroxidation and protein oxidation caused by TAC. Furthermore, MMF increased CAT and SOD activities declined with TAC. Our results were in agreement with other studies which showed that MMF reduced endothelial ROS formation by blockade of the constitutively active endothelial NAD(P)H oxidase activity and superoxide formation which could contribute to the reduction in oxidative stress and improvement in hypertension.[39,42] Moreover, it was reported that MMF (40 mg/kg b.w.) can prevent the cardiovascular risk related to the administration of other CNI such as cyclosporine A (CsA) after organ transplantation by reduction of nitric oxide (NO).[46] Other studies showed that MMF treatment of psoriasis and rheumatoid arthritis and against others xenobiotics indicated a significant reduction in hypertention that parallels a decline in the oxidative stress.[47,48] In fact, MMF administration represents a drug with interesting antigenotoxic property in combination with TAC. This property was associated with the reduction of oxidative stress induced by TAC.

Conclusion

Results of the current study indicated the high efficacy of MMF at low dose against TAC-induced heart injuries. We concluded that the cardiotoxicity induced by TAC is in relationship with oxidative stress. In fact, MMF exerted an antigenotoxic effect against TAC-induced genotoxicity in hearts of treated rats via an antioxidant activity. Thus, it is important to support MMF and TAC treatment for transplants patients to minimize heart injury.

Author contributions: H.F. designed the study, conducted the study, and wrote the manuscript. R.T., I.A. helped in the statistical analysis. I.B.-A. supervised the study. S.A. revised the manuscript. The authors are thankful to H.B. and A.A. for their laboratory supports and for their constructive suggestions during the course of study. All authors have read and approved the final manuscript.

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DECLARATION OF CONFLICTING INTERESTS

The author(s) confirm that this article content has no conflict of interest.

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