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Combination of tacrolimus and mycophenolate mofetil induces oxidative stress and genotoxicity in spleen and bone marrow of Wistar rats

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ABSTRACT

Tacrolimus (TAC) and mycophenolate mofetil (MMF) are common immunosuppressive drugs used to avoid immunological rejection of transplanted organs. The risk of developing cancer is the most critical complication in organ transplant recipients undergoing immunosuppressive therapy. This study aims to explore the cytotoxic and genotoxic effects of TAC and MMF alone or combined orally administrated on spleen and bone marrow of Wistar rats. Our results showed that TAC (2.4; 24 and 60 mg/kg) and MMF (5; 50 and 125 mg/kg) induced a genotoxic effect on rat bone marrow. Moreover, the co-treatment with the TAC/MMF (2.4/5 mg/kg b.w.; 2.4/50 mg/kg b.w. and 60/50 mg/kg b.w.) produce a genotoxicity as measured by micronuclei (MN) frequencies, chromosomal aberrations (CA) rates and DNA damage levels. Furthermore, the TAC and MMF-treated animals developed oxidative stress in spleen, indicated by a significant increase of malondialdehyde (MDA), protein oxidation and decrease of anti-oxidant enzymes levels such as catalase (CAT) and superoxide dismutase (SOD). This damage was associated with an increase of DNA fragmentation. Co-treatment with TAC/MMF synergistically induced markers of oxidative stress in rat splenic tissue. In conclusion, TAC/MMF associated induction in oxidative stress plays a role in the splenic and bone marrow toxicity and enhances the different endpoints of genotoxicity, suggesting its mutagenic action *in vivo*.

1. Introduction

Tacrolimus (TAC) is an immunosuppressive drug used to avoid organ rejection in recipients of solid organ transplants [1,2]. TAC inhibit T-cells activation by repressing calcineurin (CN), it binds to the immunophilin protein FKBP-12 [3]. TAC-FKBP-12 complex inhibits CN phosphatase activity, preventing nuclear translocation of nuclear factors of T-cells [4]. TAC also inhibits gene transcription of cytokines, including interleukins and IFNγ in human T-cells [5,6].

Mycophenolate mofetil (MMF) is an anti-metabolite produced by the fungus *Penicillium brevicompactum* [7,8]. MMF acts as an immunosuppressive drug, and is clinically approved for the prevention of allograft rejection after organ transplantation or hematopoietic stem cell transplantation [9,10]. MMF is a specific noncompetitive inhibitor of inosine monophosphate dehydrogenase and suppresses T and B lymphocytes proliferation by blocking *de novo* pathway of guanosine nucleotide synthesis [11].

MMF and TAC were included in the majority of immunosuppressive combinations regimen for treatment of organ transplantation recipients. Immunosuppressive regimen with TAC and MMF combination predispose to over-immunosuppression, and hence reduces the rate of acute allograft rejection. Moreover, the use of MMF and TAC was associated with a reduced mortality among renal transplantation recipients [12].

The risk of developing cancer is considered one of the most severe complications in patients undertaking immunosuppressive

Abbreviations: TAC, tacrolimus; MMF, mycophenolate mofetil; LD_{50}, lethal dose 50%; ROS, reactive oxygen species; MDA, malondialdehyde; PC, protein carbonyl; SOD, superoxide dismutase; CAT, catalase; CA, chromosomal aberration; Micronuclei, MN; PCE, polychromatic erythrocyte; NCE, normochromatic erythrocyte.

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Table 1
Animal groups and treatments.

Groups	Doses (mg/kg b.w.)
Group 1	Water
Group 2	2.4 mg/kg of TAC (1% of the LD_{50})
Group 3	24 mg/kg of TAC (10% of the LD_{50})
Group 4	60 mg/kg of TAC (25% of the LD_{50})
Group 5	5 mg/kg of MMF (1% of the LD_{50})
Group 6	50 mg/kg of MMF (10% of the LD_{50})
Group 7	125 mg/kg of MMF (25% of the LD_{50})
Group 8	2.4 mg (TAC)/kg + 5 (MMF)mg/kg
Group 9	2.4 mg(TAC)/kg + 50 (MMF) mg/kg
Group 10	60 mg(TAC)/kg + 50 (MMF) mg/kg

treatment. Post-transplant patients have a higher rates of malignancy [13,14], with a higher incidence of certain types of cancer [15,16] and higher rates of morbidity and mortality (50%) after 30 years of treatment [17,18].

However, there is no clear understanding of the mechanism through which immunosuppressive agent combination induces the risk to develop cancer. So far, we did not found sufficient definite data on the genotoxic effects of TAC and MMF combination on primary and secondary lymphoid organs. In the present study, we examined the possible genotoxic effects of TAC and MMF combination according to the different endpoints of genotoxicity; chromosome aberrations, MN and DNA damage in bone marrow. Moreover, we evaluated the effects of TAC on reactive oxygen species (ROS) generation and genotoxicity in rat spleen.

2. Materials and methods

2.1. Chemicals

TAC and MMF were purchased from Sigma-Aldrich (Saint Quentin Fallavier, France). All other chemicals used in the study were of analytical grade.

2.2. Animals treatment

The study was performed on male Wistar rats (weighing 120–150 g). Animals were obtained from the Central Pharmacy (SIPHAT, Tunis, Tunisia). All animals were maintained under controlled ambient temperature (22 ± 3 °C) and a 12/12 h light/dark cycle for two weeks prior to commencement of the experiment. Animals received a nutritionally standard diet (SICO, Sfax, Tunisia) and tap water ad libitum. The experimental procedures were performed according to the National Institute of Health Guidelines for Animal Care and approved by the local Ethics Committee.

Rats were treated with approximately 1%,10% and 25% of previously determined acute lethal dose (LD_{50}) of TAC and MMF respectively are 240 mg/kg b.w. and 500 mg/kg b.w. [19]. Rats were randomly divided into twenty groups of six each, including ten groups are used to study of the biochemical analysis of spleen, micronucleus assay and DNA fragmentation of spleen and bone marrow. The remaining ten groups are used for the study of chromosomal aberrations. Rats received oral doses of TAC and MMF alone and their combination as presented in Table 1.

2.3. Preparation of spleen homogenates

Spleen was homogenized with an Ultra Turrax homogenizer in Tris-HCl (10 mM, pH 7.4) at 4 °C. The homogenate was centrifuged at 4000 rpm for 30 min at 4 °C. The supernatant was collected, aliquoted and stored at −80 °C until use for analysis. Protein concentrations were quantified using the BioRad protein assay kit according to the method of Bardford (BioRad, Marnes-la-Coquette, France).

2.4. Catalase (CAT) activity

CAT activity was quantified in the spleen extracts as described previously [20]. Briefly, spleen extracts were mixed with phosphate buffer (0.5 M, pH 7.4) and H_2O_2 (0.5 M). CAT activity was calculated using the molar extinction coefficient of H_2O_2 at 240 nm (0.04 mM^{-1} cm^{-1}). The results were expressed as μmol of H_2O_2/min/mg of protein.

2.5. Superoxide dismutase (SOD) activity

SOD activity was assayed as described previously [21]. Enzyme activity was measured by mixing spleen extracts with phosphate buffer (50 mM, pH 7.8) containing 50 μl of the sample, EDTA (0.1 mM), L-methionine (13 mM), nitroblue tetrazolium (NBT) (75 μM) followed by the addition of riboflavin for reaction initiation (2 μM). The control was simultaneously run without tissue homogenate. The absorbance was measured at 560 nm. Units of SOD activity were expressed as the amount of enzyme required to inhibit the reduction of NBT by 50%.

2.6. Determination of lipid peroxidation products

Lipid peroxidation was measured indirectly by quantifying the production of malondialdehyde (MDA) in spleen extracts as described previously [22]. Briefly, spleen extracts were mixed with tris-buffered saline (TBS) and trichloroacetic acid- butylated hydroxytoluene (TCA–BHT). After centrifugation at 1500 rpm, the supernatant were mixed with 0.6 N HCl and Tris-thiobarbituric acid (TBA). The absorbance was measured at 530 nm. The concentration of MDA (nmol/mg of protein) was calculated using the molar extinction coefficient of MDA (1.56×10^5 M^{-1} cm^{-1}).

2.7. Protein carbonyl (PC) assay

PC content was measured in spleen homogenates according to the previously described method [23]. The reactivity of carbonyl groups with 2,4-dinitrophenylhydrazine (2,4-DNPH) was measured by mixing spleen extracts with 2,4-DNPH (10 mM), followed by incubation for 1 h at ambient temperature. Samples were kept in ice after adding 1 ml of 20% TCA and then centrifuged for 5 min at 4000 rpm. The protein precipitates were dissolved in 6 M guanidine hydrochloride. The absorbance for PC was measured at 370 nm. PC concentration (nmoles/mg of protein) was determined using its molar extinction coefficient of 22 mM^{-1} cm^{-1}.

2.8. DNA fragmentation using comet assay

The alkaline comet assay was performed as previously described [24]. Spleen were placed in cold phosphate-buffered saline (PBS) and minced finely with scissors and forceps to obtain a splenic cellular suspension. For isolation of bone marrow, both femurs were placed in Petri dish. The epiphysis of femurs was removed and bone marrow cavity was flushed out using a 24-gauge needle and collected in a micro-centrifuge tube. Bone marrow cell suspension was mixed with PBS containing 20 mM EDTA and 1% DMSO. Spleen and bone marrow cell suspensions were embedded in1% low-melting-point agarose (LMA) and spread on glass slide. The slides were cooled down and then immersed in lysis solution and subjected to electrophoresis as previously described [25]. Each comet class was given a value of 0, 1, 2, 3, or 4 (from undamaged, 0, to maxi-