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Trans-anethole, the major constituent of the essential oil of anise, improves the antiarthritic effects of methotrexate in rats

O trans-anetol, principal componente do óleo essencial de anis, melhora os efeitos antiartríticos do metotrexato em ratos

El trans-anetol, componente principal del aceite esencial de anís, mejora los efectos antiartríticos del metotrexato en ratas

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Abstract

The aim of this study was to evaluate the effect of trans-anethole in combination with methotrexate, compared to monotherapy with trans-anethole or methotrexate, on inflammatory parameters and oxidative stress of arthritic rats. The experimental model of arthritis induced by Freund's complete adjuvant (AIA) in rats was used. Treatment with trans-anethole and methotrexate, in combination or monotherapy, was started on the day of AIA induction and continued for 21 days. The association of anethole (62.5 mg/Kg) to methotrexate (6 mg/Kg) therapy was able to further reduce both the oxidative stress induced by arthritis and the inflammatory events that characterize the disease, as demonstrated by the following indicators: 1) decrease in the edema of the hind legs, in the score of secondary lesions, in the recruitment of leukocytes to the articular cavity and in the plasma myeloperoxidase activity; 2) increase in plasma content of reduced thiols and total antioxidant capacity; 3) diminution of protein carbonylation in plasma, liver and kidneys; 4) decrease in lipid peroxidation in the liver; 5) reduction in the hepatic ROS content; 6) enhancement of the activity of the antioxidant enzymes and GSH/GSSG ratio. In general, the combination anethole + methotrexate presented a substantially higher effect than the monotherapy with anethole or methotrexate (at the same

doses). The data obtained in this work allow us to conclude that the use of trans-anethole in combination with methotrexate suppressed arthritic progression in rats, with the main advantage of reduction of methotrexate dose, attenuating adverse effects caused by high dose therapy.
Keywords: Natural product; Inflammation; Arthritis; Oxidative stress.

Resumo

O objetivo deste estudo foi avaliar o efeito do trans-anetol em combinação com metotrexato, comparado à monoterapia com trans-anetol ou metotrexato, sobre parâmetros inflamatórios e de estresse oxidativo de ratos artríticos. Foi utilizado o modelo experimental de artrite induzida pelo adjuvante completo de Freund (AIA) em ratos. O tratamento com trans-anetol e metotrexato, em combinação ou monoterapia, foi iniciado no dia da indução da AIA e continuou por 21 dias. A associação do anetol (62,5 mg/Kg) à terapia com metotrexato (6 mg/Kg) foi capaz de reduzir o estresse oxidativo induzido pela artrite e os eventos inflamatórios que caracterizam a doença, conforme demonstrado pelos seguintes indicadores: 1) diminuição no edema dos membros posteriores, no escore de lesões secundárias, no recrutamento de leucócitos para a cavidade articular e na atividade da mieloperoxidase plasmática; 2) aumento do conteúdo plasmático de tióis reduzidos e capacidade antioxidante total; 3) diminuição da carbonilação de proteínas no plasma, fígado e rins; 4) diminuição da peroxidação lipídica no fígado; 5) redução do conteúdo hepático de EROs; 6) aumento da atividade das enzimas antioxidantes e da relação GSH/GSSG. Em geral, a combinação anetol + metotrexato apresentou efeito substancialmente maior do que a monoterapia com anetol ou metotrexato (nas mesmas doses). Os dados obtidos neste trabalho permitem concluir que o uso do trans-anetol em combinação com o metotrexato suprimiu a progressão artrítica em ratos, tendo como principal vantagem a redução da dose de metotrexato, atenuando os efeitos adversos causados pela terapia com altas doses.
Palavras-chave: Produto natural; Inflamação; Artrite; Estresse oxidativo.

Resumen

El objetivo de este estudio fue evaluar el efecto del trans-anetol en combinación con metotrexato, en comparación con la monoterapia con trans-anetol o metotrexato, sobre los parámetros inflamatorios y el estrés oxidativo de ratas artríticas. Se utilizó el modelo experimental de artritis inducida por adyuvante completo de Freund (AIA) en ratas. El tratamiento con trans-anetol y metotrexato, en combinación o monoterapia, se inició el día de la inducción de AIA y se continuó durante 21 días. La asociación de anetol (62,5 mg/Kg) a la terapia con metotrexato (6 mg/Kg) logró reducir el estrés oxidativo y eventos inflamatorios inducido por la artritis, como lo demuestran los siguientes indicadores: 1) disminución en el edema de las patas traseras, en la puntuación de lesiones secundarias, en el reclutamiento de leucocitos a la cavidad articular y en la actividad mieloperoxidasa plasmática; 2) aumento del contenido plasmático de tioles reducidos y capacidad antioxidante total; 3) disminución de la carbonilación de proteínas en plasma, hígado y riñones; 4) disminución de la peroxidación de lípidos en el hígado; 5) reducción del contenido hepático de ROS; 6) mejora de la actividad de las enzimas antioxidantes y la relación GSH/GSSG. En general, la combinación anetol + metotrexato presentó un efecto sustancialmente mayor que la monoterapia con anetol o metotrexato (a las mismas dosis). Los datos obtenidos en este trabajo nos permiten concluir que el uso de trans-anetol en combinación con metotrexato suprimió la progresión artrítica en ratas, con la principal ventaja de la reducción de la dosis de metotrexato, atenuando los efectos adversos causados por la terapia a altas dosis.
Palabras clave: Producto natural; Inflamación; Artritis; Estrés oxidativo.

1. Introduction

Rheumatoid arthritis (RA) is a chronic inflammatory disease, autoimmune, of unknown etiology. Its main characteristic is a persistent inflammatory synovitis, which involves all synovial articulations, the peripheral ones preferably, in a symmetric form of distribution and with variable deformation potential (Firestein, 2003). Estimates reveal that the worldwide prevalence of RA is about 1%, women being more affected than men (Alam et al., 2017). Treatment of arthritis generally involves corticosteroids, non-steroidal anti-inflammatories, disease modifying antirheumatic drugs and biological agents (Batlouni, 2010; Blumenthal et al., 2018). The whole therapeutic set, however, does not always modify in a satisfactory way the pathologic process that is responsible for chronic inflammation and is often associated with high financial costs (McCarberg & Tenzer, 2013).

Methotrexate (MTX) can be considered one of the first-choice medicines for rheumatoid arthritis treatment (Alam et al., 2017). Nevertheless, its main limitation is the appearance of adverse effects, which include hepatotoxicity, nephrotoxicity, anemia, leukopenia, thrombocytopenia, and gastrointestinal and mucocutaneous disturbances (Gaies et al., 2012). Association of pharmacological agents with complementary mechanisms of action can be useful for preventing the adverse effects of MTX

and for, additionally, potentiate its antiarthritic actions (Bauerova et al., 2010; Tawfik, 2015). Considering that, the search for new and perhaps more efficient combinations is highly recommendable, especially among natural products. One such natural product could be trans-anethole (AN), a compound that can be found in the essential oils of anise species, such as Illicium verum, Pimpinella anisum and Foeniculum vulvare (Yang et al., 2010; Fitsiou et al., 2016). This compound has evoked great scientific interest due to its numerous biological activities, among which it is worth to mention the antimicrobial, anticancer, antioxidant, antinociceptive, hepatoprotective and anti-inflammatory properties (Yang et al., 2010; Ritter et al., 2014; Moradi et al., 2014).

Taking into account, thus, that the association of pharmacological agents can eventually become an effective therapeutic alternative for potentiating beneficial activities and diminishing adverse consequences, the purpose of the present study was to evaluate the effect of MTX in combination with AN on inflammatory parameters and oxidative stress in the Freund's complete adjuvant-induced arthritis in rats, an experimental immunopathology that presents many similarities to human rheumatoid arthritis.

2. Methodology

2.1 Animals

Male Holtzman rats (180-200 g), were housed under controlled temperature (22ºC) and a 12 h/12 h light/dark cycle with food and water available *ad libitum*. The experimental protocol was approved by the Ethics Committee of Animal Experimentation of the State University of Maringá (protocol # 9937300419).

2.2 Arthritis induction and animal treatment

Arthritis was induced by a subcutaneous injection of 0.1 mL of a complete Freund's adjuvant (CFA) suspension into the plantar surface of the left hind paw. The adjuvant consists in heat inactivated *Mycobacterium tuberculosis*, suspended in mineral oil (Nujol®, Schering-Plough, São Paulo, Brazil) at a concentration of 0.5%. Treatments with AN (62.5 and 250 mg/Kg; Sigma-Aldrich, USA), MTX (6, 12 or 24 mg/Kg; Libbs Pharmaceutics, Brazil) and the combination AN (62.5 mg/Kg) + MTX (6 mg/Kg) were initiated at the day of arthritis induction and evaluation was done at the 21th day. AN was dissolved in water and administered orally daily (gavage). MTX was administered once a week by intraperitoneal injection. The doses were stipulated based on literature data (Silva et al., 2005; Wisniewski-Rebecca et al., 2015). Control groups of healthy and non-treated arthritic rats were also carried out.

2.3 Evaluation of the inflammatory response induced by Freund's adjuvant

The volume of both hind paws, up to the tibiotarsal joint, was measured by plethysmography as previously described elsewhere (Winder et al., 1957). Secondary lesions were evaluated through the system of numerical grading proposed by Rosenthale (1970). Points were attributed to the following events: nodes in the tail, +1; nodes in one or both ears, +1 or +2; and increase in the volume of one or both hind paws, +1 or +2. The severity of the lesions was graded from 0 to 5, zero indicating absence of lesions.

For leukocyte counting in the articular cavity, animals were anesthetized with sodium thiopental (50 mg/kg) and euthanized 21 days after the injection of CFA. Thereafter, the femorotibial articulations were exposed by sectionning the patellar tendon. Before collecting the articular exsudate, intravascular washings were done with saline solution containing ethylene-diamino tetra-acetic acid (EDTA). Total leukocytes recruited into joint cavities were quantified in a Neubauer chamber and polymorphonucleated and mononucleated leukocytes were counted in microscope slides after May-Grünwald-Giemsa staining.

2.4 Analytical assays in plasma

Plasma activities of alanine aminotransferase (ALT), aspartate aminotransferase (AST) alkaline phosphatase (ALP), albumin, urea and creatinine were measured by means of commercial kits (Gold Analisa®, Brazil). Antioxidant capacity in plasma was estimated using the capacity of sequestering the $ABTS^{\bullet+}$ (2,2′-azino-bis (3-ethylbenzo-thiazoline-6-sulphonic acid) free radicals, as described by Erel (2004). A standard curve was constructed with Trolox (6-hydroxy-2,5,7,8-tetramethyl-chloraman-2-carboxylic acid) and the activity was expressed as mmol Trolox equivalents (TE) per mL plasma. Reduced thiol groups were measured spectrophotometrically using DTNB (5,5′-dithiobis 2-nitrobenzoic acid) (Sedlak & Lindsay, 1968). Protein carbonyls were quantified spectrophotometrically using 2,4-dinitrophenylhydrazine (DPNH) (ε_{370} = 22 x 10^3 M^{-1} cm^{-1}). The results were expressed as nmol per mg protein (Levine et al., 1990). The myeoloperoxidase activity (MPO) was determined by the o-dianisine-H_2O_2 assay (Bradley et al., 1982). The change in absorbance at 460 nm was followed for 1 minute. The activity was calculated using the molar extinction coefficient (ε = 1.13 ×10^4 M^{-1} cm^{-1}) and expressed as nmol.min^{-1}.(mg protein)$^{-1}$. Finally, for erythrocyte counting, blood was first diluted with an isotonic solution (Hayen solution) and the number of cells determined using a Neubauer chamber.

2.5 Liver and kidney homogenates preparation

Liver and kidneys were utilized for determining oxidative stress parameters because these organs are particularly affected by both arthritis (Daoussis et al., 2010; Comar et al., 2013) and treatment with MTX (Gaies et al., 2012). Liver and kidneys were immediately clamped in liquid nitrogen and stored at 80º C. For preparing the homogenate, approximately 2 g of each freezed tissue were weighed and homogenized in a Dounce homogenizer with 10 volumes of 0.1 M phosphate buffer (pH 7.4) at 4º C.

2.6 Glutathione determination

The tissue contents of GSH (reduced form) were measured spectrofluorimetrically (excitation at 350 nm and emission at 420 nm) after reaction with o-phthalaldehyde, as described in the literature (Hissin & Hilf, 1976). Standard curves were constructed with GSH or GSSG and the results were expressed as nmol (mg protein)$^{-1}$.

2.7 Lipid peroxidation and protein carbonylation

The levels of lipid peroxides in the liver and kidneys homogenate were measured using the thiobarbituric acid reactive substances (TBARS) assay (Buege & Aust, 1978). The level of carbonylated proteins was measured spectrophotometrically at 370 nm after reaction with 2,4-dinitrophenyl-hydrazine (DNPH) (ε_{370} = 22 x 10^3 M^{-1} cm^{-1}) (Levine et al., 1990). The results were expressed as nmol (mg protein)$^{-1}$.

2.8 Determination of reactive oxygen species (ROS)

Assay of the total contents of ROS in tissue samples was done using the probe 2'-7'-dichlorfluorescein-diacetate (DCFH-DA) as described by Siqueira et al. (2005). The formation of oxidized dichlorofluorescein (DCF) was measured spectrofluorimetrically (excitation at 504 and emission at 529 nm). A standard curve was constructed with oxidized dichlorofluorescein and the resuls were expressed as nmol. (mg protein)-^{1}.

2.9 Activity of antioxidant enzymes

The activity of catalase (CAT) was assayed by measuring the transformation of H_2O_2 at 240 nm and expressed as nmol.min^{-1}. (mg protein)$^{-1}$. The activity of superoxide dismutase (SOD) was estimated by its capacity of inhibiting the auto-

oxidation of pyrogallol in alkaline medium. This phenomenon was monitored spectrophotometrically at 420 nm (Marklund & Marklund, 1974). One superoxide dismutase unit (U) is defined as the amount of enzyme capable of causing 50% inhibition of pyrogallol auto-oxidation and the results were expressed as U. (mg protein)$^{-1}$.

2.10 Statistical analysis

The results were expressed as means ± standard errors of the means and submitted to variance analysis (ANOVA) followed by Tukey's post-hoc testing. The significance criterium was $p \leq 0.05$.

3. Results

3.1 Effects of treatments on paw volumes and secondary lesions

Figure 1 shows the time course in the evolution of the volumes of the left paw (which received CFA, panel A) and the right one (not injected, panel C) of healthy, non-treated arthritic (AIA), and treated arthritic rats. The final paw edema (at the 21th day) can be seen in Figures 1B and 1D. Treatment with the combination AN (62.5 mg/kg) + MTX (6 mg/kg) diminished significantly the inflammatory process in the left hind paw starting at the 13th day and continuing until de 21th day after the adjuvant injection. The reduction at this day, compared to the non-treated arthritic rats, was equal to 48%. With respect to the non-injected hind paw, the treatment with this combination almost suppressed the evolution of the inflammatory process during the whole experimental period, with a final 92% reduction in the paw volume relative to the non-treated condition. These effects were superior to the ones of monotherapy with AN (62.5 mg/Kg) or MTX (6 mg/Kg). Monotherapy with AN affected solely the inflammation of the left paw at a 4-fold higher dose (250 mg/kg), reaching 21% inhibition at the 21th day after adjuvant injection. However, even at this higher dose AN did not diminish the edema of the non-injected paw. MTX, at the dose of 6 mg/kg, delayed the development of the inflammatory response in the left paw, but at the 21th day the edema had advanced to a stage similar to that one found in non-treated arthritic rats. At the dose of 12 mg/kg, MTX reduced the volume of the left paw from the 9th day on and reached 48% reduction at the 21th day. The highest MTX dose used in this study, 24 mg/kg, had a pronounced anti-inflammatory action during the whole experimental period. It must be remarked, however, that this highest MTX dose caused intense diarrhea, an adverse effect that was not observed in the other groups.

The appearance of secondary lesions in the control arthritic rats occurred from the 10th day on after arthritis induction (Figure 2). Treatment with the combination AN + MTX delayed the appearance of the lesions as well as diminished their intensity at the 21th day. This effect was significantly superior to that one of AN in monotherapy at the doses of 62.5 and 250 mg/kg and to that of MTX in monotherapy (6 mg/kg). The results obtained with the combination were similar to those found with MTX 12 mg/kg.

3.2 Effects of treatments on the number of leukocytes in the articular cavity

Treatment with the combination AN + MTX reduced the number of total leukocytes in the left and right articular cavities when compared to the non-treated arthritic group (Figure 3). The number of both cell types (mononucleate and polymorphonucleate) was diminished. The effects of the combination were superior to the effects of AN alone at the lowest dose (62.5 mg/kg), but they were not significantly different from those of the AN monotherapy at the highest dose (250 mg/kg) or from those of the MTX monotherapy in general.

3.3 Plasma biochemistry and red cell counts

The results of all plasma biochemical indicators are summarized in Table 1. Arthritis induction did not change the activities of AST, ALT and ALP in plasma nor did it affect the creatinine concentration. These parameters were also not

significantly modified by the treatments. The urea concentration, however, was 35% increased by the 24 mg/kg MTX treatment, but not by the combination AN + MTX. On the other hand, arthritis diminished the circulating albumin concentration by 32%, an effect that was not prevented by any of the treatments used in this study.

Arthritis diminished the number of red blood cells. Treatment with MTX in monotherapy, with the doses of 12 and 24 mg/kg, diminished further the number of red blood cells. Nonetheless, the combination AN + MTX (62.5 + 6 mg/kg, respectively) almost prevented the drop in the number of red blood cells, which under this condition was closer to normal.

Figure 1. Evolution of the inflammatory response in the hind paws of Holtzman rats after induction of arthritis with complete Freund's adjuvant and the influence of treatments. The results were expressed as the increase in volume of the injected (left) and non-injected (right) paw after adjuvant injection. The treatments with anethole (AN; 62.5 and 250 mg/Kg), methotrexate (MTX; 6, 12 or 24 mg/Kg) and AN (62.5 mg/Kg) + MTX (6 mg/Kg) combination were initiated at the day of arthritis induction and evaluation was done at the 21th day. Panel A: Evolution of left (injected) paw edema; Panel B: Injected paw edema at the 21st day; Panel C: Evolution of right (non-injected) paw edema; Panel D: Non-injected paw edema at the 21st day. Each datum point represents the mean ± mean standard error of 8-10 animals. One-way ANOVA followed by Tukey's post-hoc testing was performed. *p ≤ 0.05 compared to the arthritic group (AIA); **p≤0.05 compared to the AN+MTX group; # p≤ 0.05 compared to the healthy (control) group.

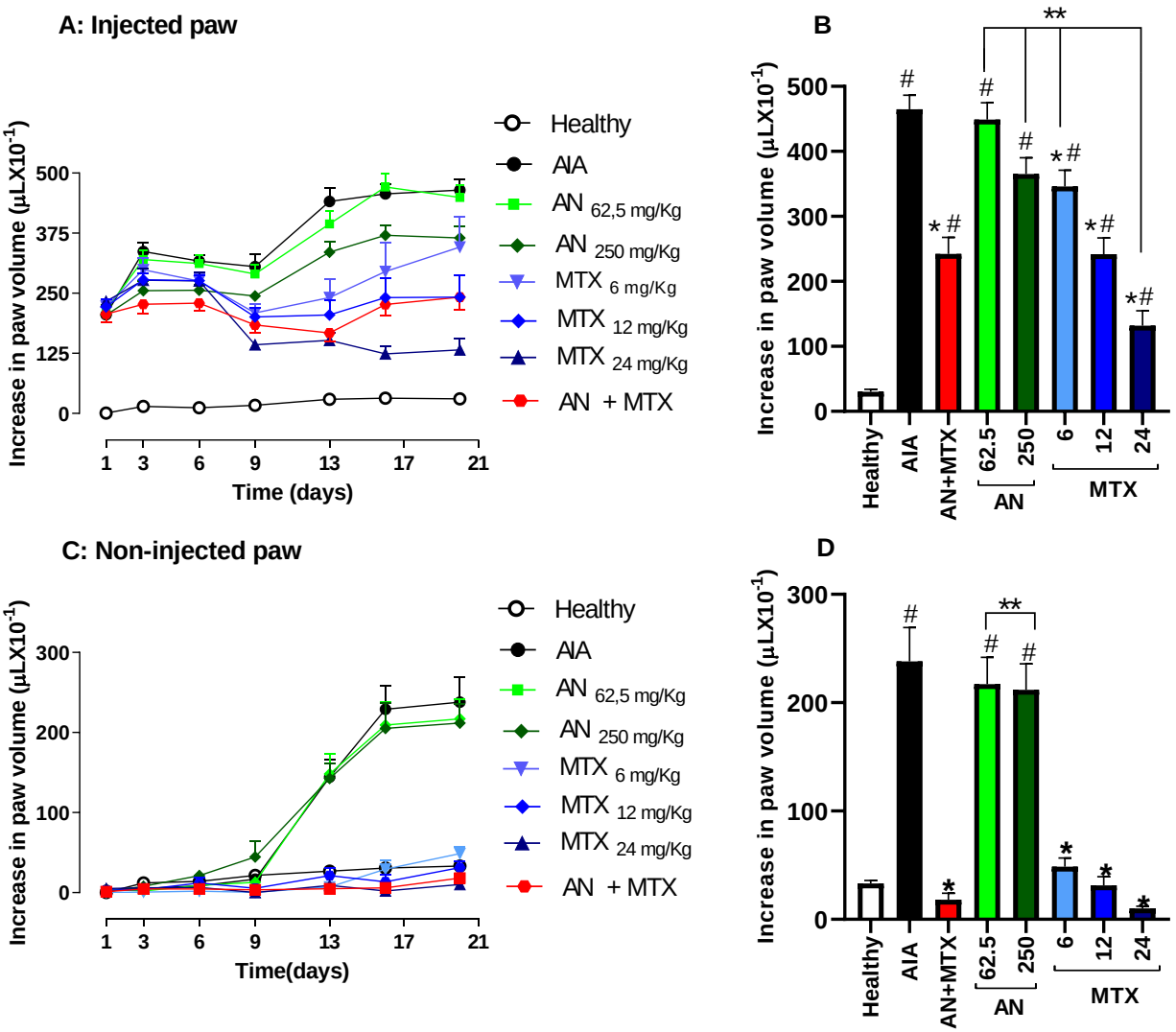

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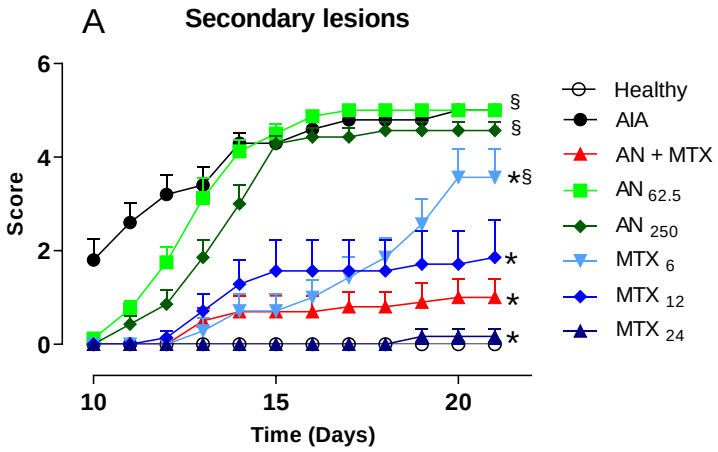
Figure 2 Development of the secondary lesions of healthy and arthritic rats and the influence of treatments. The treatments with anethole (AN; 62.5 and 250 mg/Kg), methotrexate (MTX; 6, 12 or 24 mg/Kg) and AN (62.5 mg/Kg) + MTX (6 mg/Kg) combination were initiated at the day of arthritis induction and evaluation was done at the 21th day. Data are the mean ± mean standard errors of 6 to 12 animals (n). #Indicate significant differences between healthy and AIA groups and *indicate significant differences of the various experimental groups (treatments) and AIA group, as inferred from one-way ANOVA followed by Tuckey's test ($p \leq 0.05$).



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3.4 Plasma oxidative stress parameters

Figure 4 illustrates the influence of arthritis and of the various treatments on several oxidative stress parameters in plasma. Confirming previous observations (Correa et al., 2019), the levels of protein carbonyls and plasma activity of myeloperoxidase were increased by arthritis. On the other hand, plasma content of thiol groups and plasma total antioxidant capacity (TAC - $ABTS^{\bullet+}$ scavenging capacity) were decreased. The AN + MTX combination totally prevented the increased protein carbonylation induced by arthritis, in a similar way than monotherapy with MTX 24 mg/Kg. For MPO activity, AN + MTX combination was effective in preventing its increase, while AN in monotherapy was significantly effective solely at the 250 mg/kg dose and MTX monotherapy was effective in all doses. Treatment with AN + MTX in combination maintained thiols levels 72% above those found in non-treated arthritic rats. Treatments with AN and MTX in monotherapy, however, did not prevent thiols content diminution. Plasma TAC diminution in non-treated arthritic rats is partially associated to the reduced circulating concentration of albumin as well as with the reduced concentration of protein thiols. None of the treatments in monotherapy prevented the diminution of the plasma antioxidant capacity. However, the AN + MTX partially prevented the $ABTS^{\bullet+}$ scavenging capacity which was 47% above that one found in non-treated arthritic rats.

3.5 Oxidative stress in liver and kidney

Results of our evaluation of the oxidative stress indicators in liver and kidney, namely protein carbonyls, reactive oxygen species (ROS) and lipid peroxidation (TBARS), are shown in Figure 5. Adjuvant–induced arthritis increased all parameters, as can be expected from previous reports (Sá-Nakanishi et al., 2018). Treatment with the AN (62.5 g mg/kg) + MTX (6 mg/kg) combination attenuated all the modifications caused by arthritis. In general terms the effects were more pronounced in the liver when compared to the kidney.

The effects of AN + MTX tended to be superior to monotherapy with the lower doses of both AN and MTX. The 62.5 mg/kg dose of AN alone did not affect at all the oxidative stress parameters that were measured in liver or kidney.

Figure 3. Number of leukocytes recruited into the articular cavities upon arthritis induction and the influence of treatments. The treatments with anethole (AN; 62.5 and 250 mg/Kg), methotrexate (MTX; 6, 12 or 24 mg/Kg) and AN (62.5 mg/Kg) + MTX (6 mg/Kg) combination were initiated at the day of arthritis induction and evaluation was done at the 21th day. Leukocytes in both left (panel A) and right (panel B) knees were counted and discriminated. The types of leukocytes are indicated on each panel and the type of treatment, including doses, is given under the bars. The bars represent means ± SEM of 8-10 animals per group. #p≤0.05 between healthy and AIA groups; *p≤0.05 compared to AIA group, as inferred from one-way ANOVA followed by Tuckey´s test.

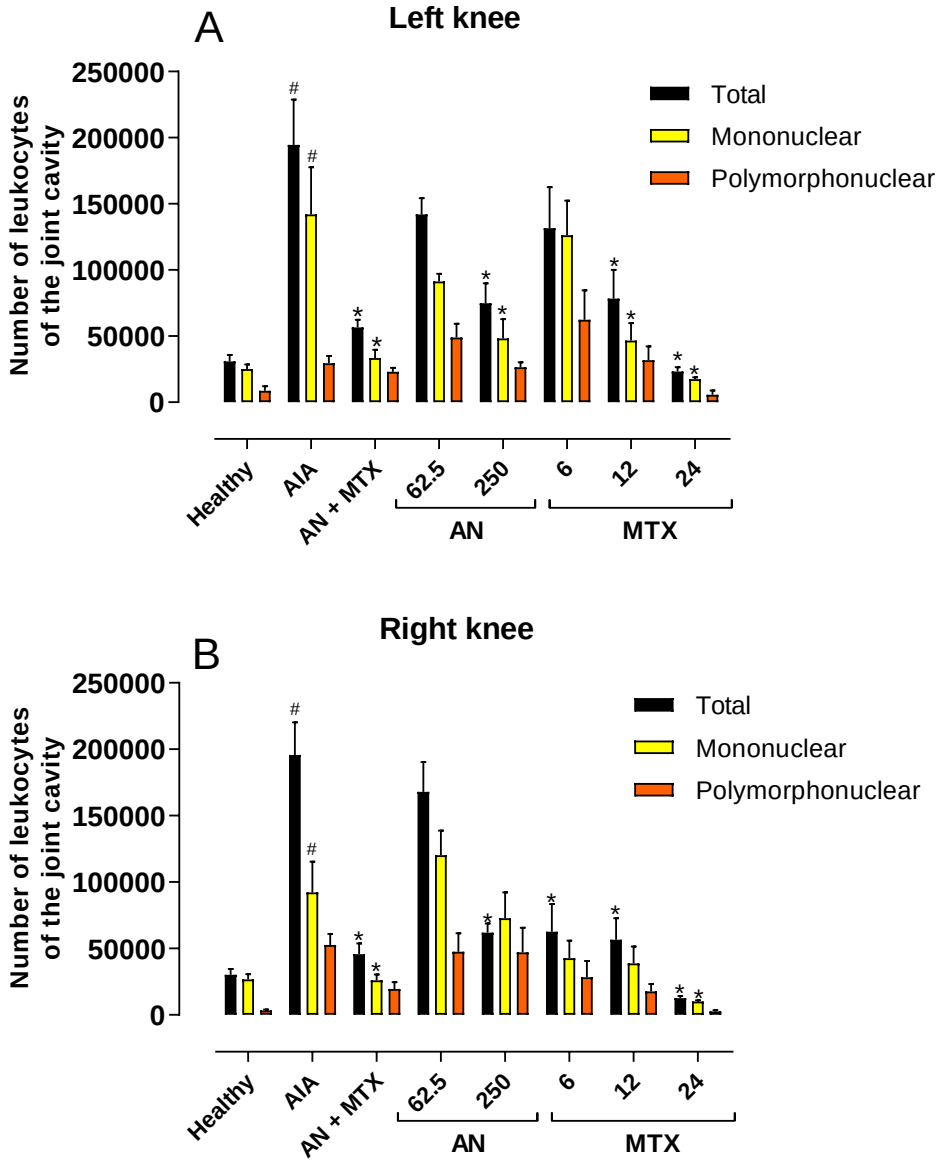

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Table 1. Biochemical parameters and red cell counts in healthy and arthritic rats and influence of the treatments. Evaluations were done at the 21th day after Freund's adjuvant inoculation.

Parameters	Healthy	Arthritic						
		No treatment AIA	Anethole (62.5 mg/kg)	Anethole (250 mg/kg)	Methotrexate (6 mg/kg)	Methotrexate (12 mg/kg)	Methotrexate (24 mg/kg)	AN (62.5 mg/kg) + MTX (6 mg/kg)
Albumin ($g \cdot dL^{-1}$)	3.26 ± 0.06^{a}	2.20 ± 0.09^{b}	2.33 ± 0.19^{b}	2.42 ± 0.21^{b}	2.18 ± 0.12^{b}	2.17 ± 0.16^{b}	2.63 ± 0.18^{b}	2.50 ± 0.09^{b}
ALP ($U \cdot L^{-1}$)	195.8 ± 6.20^{a}	221.5 ± 4.5^{a}	234.3 ± 15.3^{a}	223.4 ± 17.3^{a}	219.8 ± 27.7^{a}	215.2 ± 29.40^{a}	218.5 ± 15.07^{a}	238 ± 4.77^{a}
AST ($U \cdot L^{-1}$)	46.00 ± 1.91^{a}	46.8 ± 3.56^{a}	46.45 ± 4.60^{a}	48.75 ± 3.15^{a}	43.2 ± 1.78^{a}	46.5 ± 2.85^{a}	45.17 ± 3.78^{a}	44.33 ± 1.90^{a}
ALT ($U \cdot L^{-1}$)	59.65 ± 3.57^{a}	57.4 ± 4.35^{a}	63.48 ± 5.08^{a}	65.92 ± 6.57^{a}	54.41 ± 3.33^{a}	61.77 ± 3.29^{a}	61.55 ± 2.81^{a}	55.30 ± 5.62^{a}
Urea ($mg \cdot dL^{-1}$)	46.08 ± 2.20^{a}	52 ± 2.73^{ab}	46.56 ± 2.94^{a}	45.45 ± 2.72^{a}	60.3 ± 5.56^{ab}	59.1 ± 3.82^{ab}	62.42 ± 3.80^{b}	50.44 ± 4.19^{ab}
Creatinine ($mg \cdot dL^{-1}$)	0.39 ± 0.02^{a}	0.38 ± 0.02^{a}	0.39 ± 0.02^{a}	0.39 ± 0.02^{a}	0.41 ± 0.05^{a}	0.42 ± 0.03^{a}	0.41 ± 0.05^{a}	0.42 ± 0.04^{a}
Red blood cells ($cells \cdot mm^{-3} \cdot 10^{-6}$)	6.83 ± 0.37^{a}	5.72 ± 0.34^{cde}	5.55 ± 0.15^{bde}	5.59 ± 0.18^{bde}	5.16 ± 0.20^{bde}	4.64 ± 0.18^{b}	5.05 ± 0.16^{bd}	6.05 ± 0.11^{ace}

Data are the mean ± mean standard errors of 8 to 12 animals. Differently superscripted letters indicate significant differences between the various experimental groups within a given line, as inferred from one-way ANOVA followed by Tuckey's test ($p \leq 0.05$). Source: Authors

Figure 4. Plasma oxidative stress indicators in heathy and arthritic rats and the influence of treatments. The treatments with anethole (AN; 62.5 and 250 mg/Kg), methotrexate (MTX; 6, 12 or 24 mg/Kg) and AN (62.5 mg/Kg) + MTX (6 mg/Kg) combination were initiated at the day of arthritis induction and evaluation was done at the 21th day. The parameters that were measured are indicated on each panel and the type of treatment, including doses, is given under the bars. The bars represent means ± mean standard errors of 8-10 animals per group. #p≤0.05 between healthy and AIA groups; *p≤0.05 compared to AIA group, as inferred from one-way ANOVA followed by Tuckey's test (p ≤ 0.05).

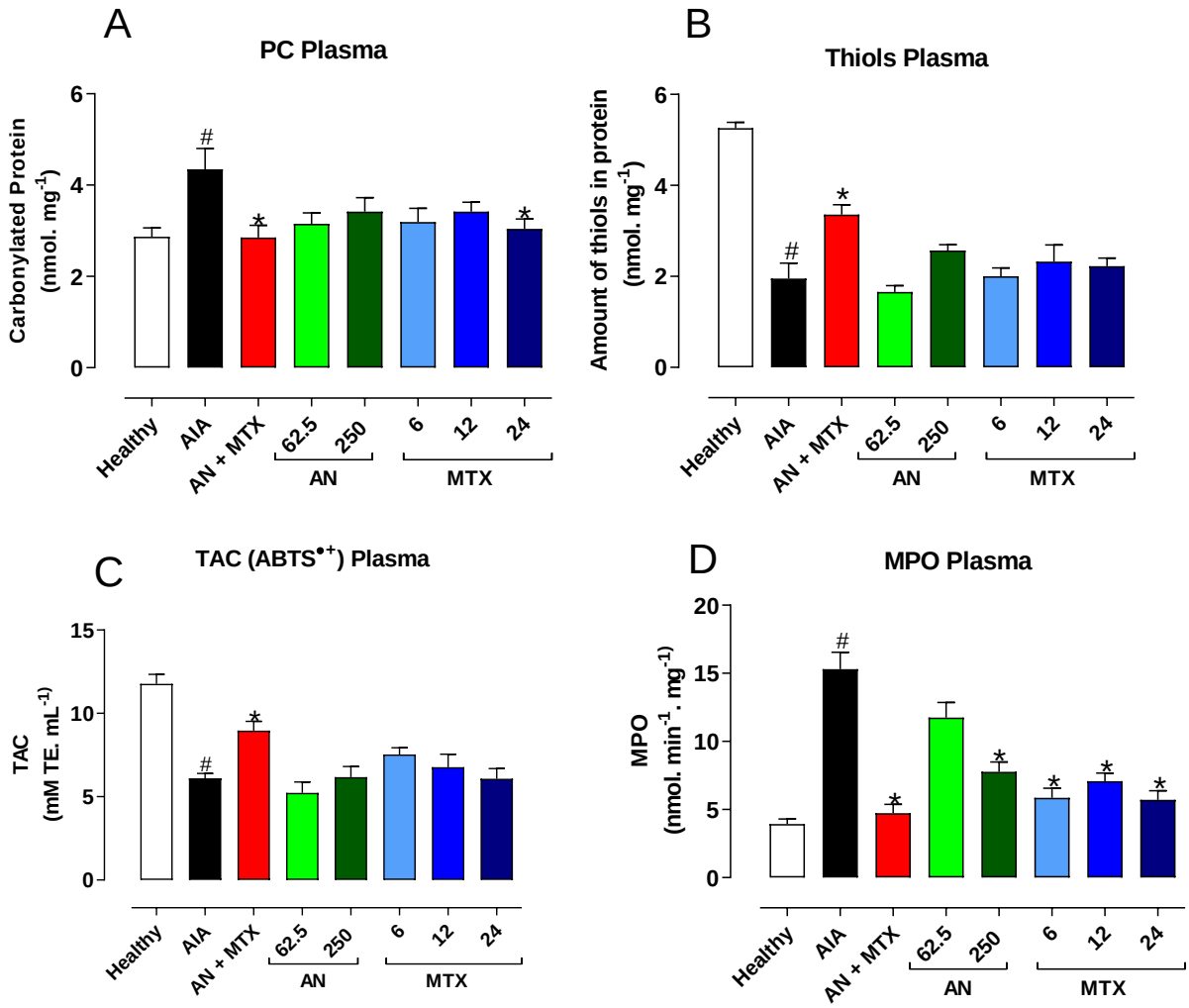

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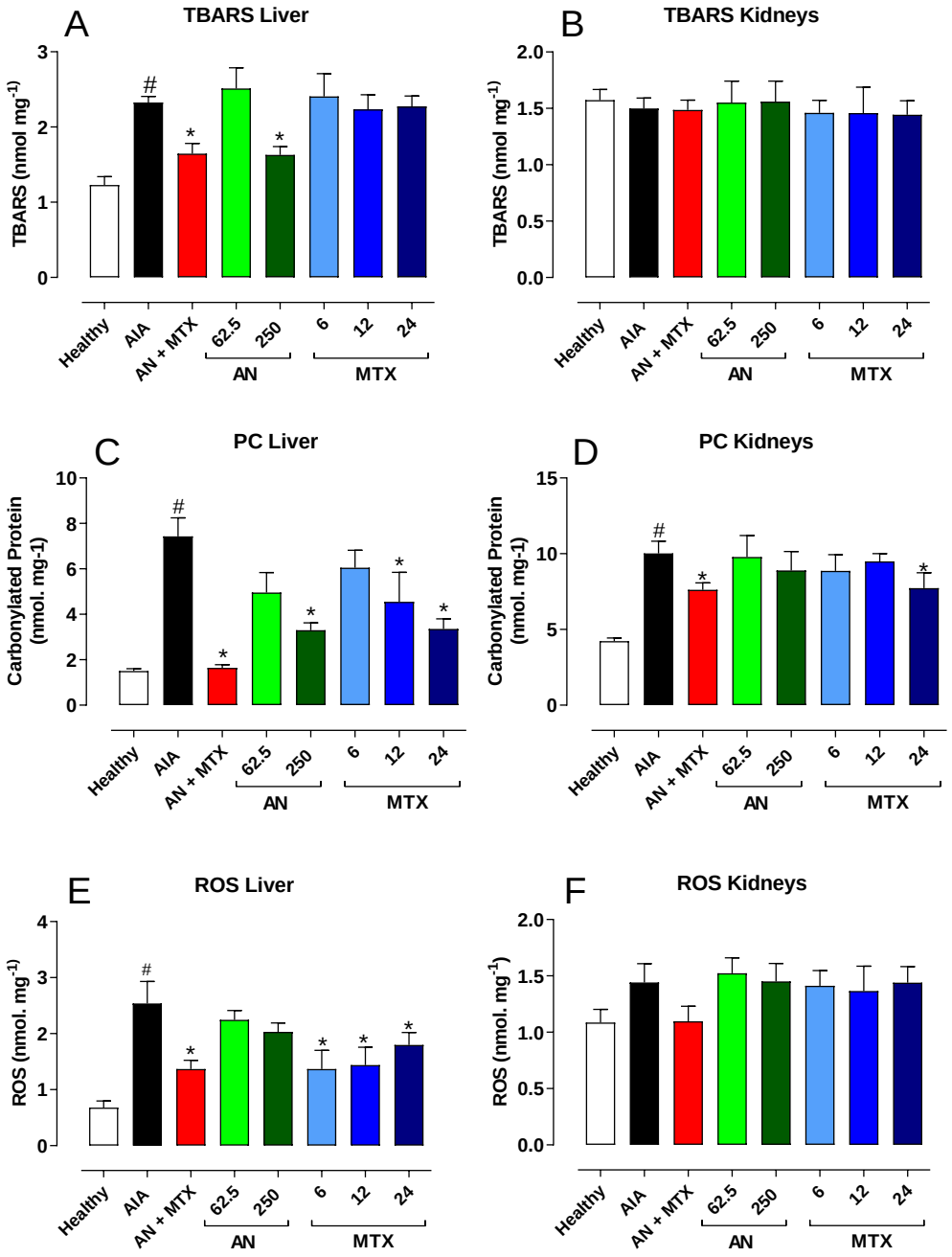
Figure 5. Liver and kidney oxidative stress indicators in heathy and arthritic rats and the influence of treatments. Treatments with anethole (AN; 62.5 and 250 mg/Kg), methotrexate (MTX; 6, 12 or 24 mg/Kg) and AN (62.5 mg/Kg) + MTX (6 mg/Kg) combination were initiated at the day of arthritis induction and evaluation was done at the 21th day. The parameters that were measured and the tissue that was analyzed are indicated on each panel. The type of treatment, including doses, is given under the bars. The bars represent means ± mean standard errors of 8-10 animals per group. Different letters on the bars identify statistically significant differences between #p≤0.05 between healthy and AIA groups; *p≤0.05 compared to AIA group, as inferred from one-way ANOVA followed by Tuckey's test.



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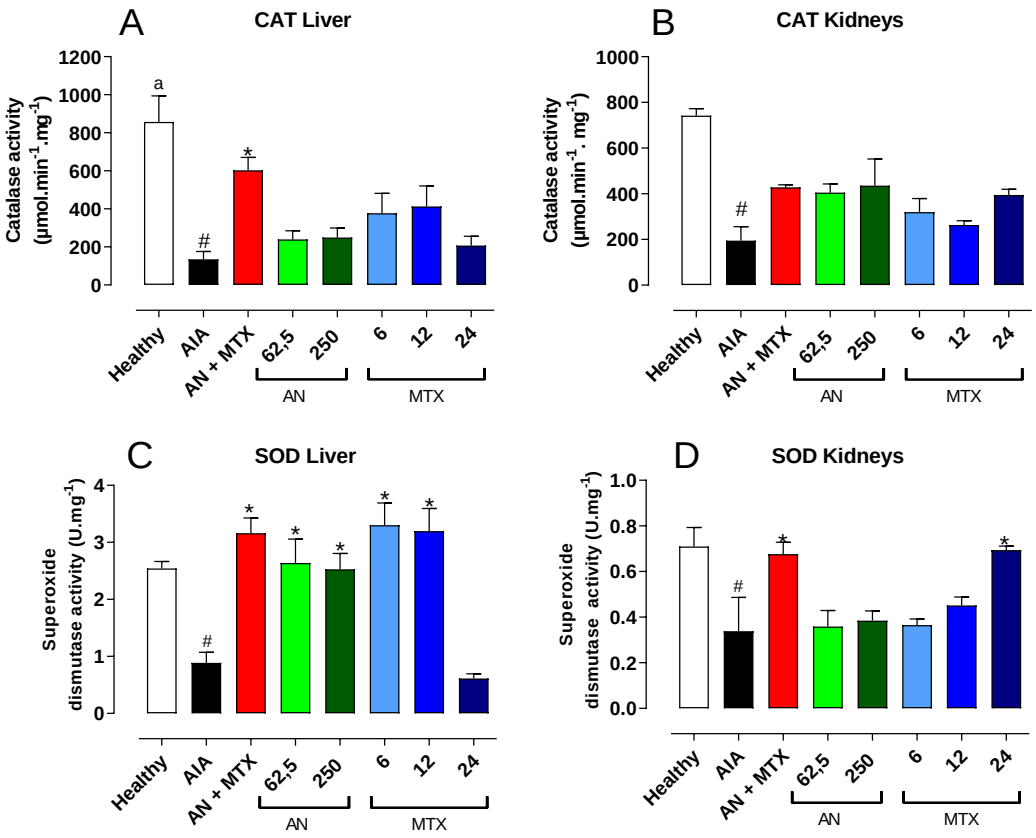
Methotrexate alone at the 6 mg/kg dose was unable to prevent the increased protein carbonylation and lipid peroxidation in the liver, but it influenced the levels of reactive oxygen species which was comparable to that one when it was associated to AN. In the kidney the 6 mg/kg dose of MTX was inactive.

Monotherapy with the highest MTX doses of 12 and 24 mg/kg, on the other hand, prevented the increased protein carbonylation and ROS levels in the liver, but not those of TBARS. In the kidney only the 24 mg/kg dose had some effect on the carbonylated protein levels, but not on the ROS levels. AN at the dose of 250 mg/kg kept the levels of carbonylated proteins and TBARS in the liver of arthritic rats at low levels but acted poorly on the ROS concentrations. In the kidney this AN dose was inactive on the parameters that were measured.

Figure 6 Liver and kidney antioxidant enzyme activities in heathy and arthritic rats and the influence of treatments. The treatments with anethole (AN; 62.5 and 250 mg/Kg), methotrexate (MTX; 6, 12 or 24 mg/Kg) and AN (62.5 mg/Kg) + MTX (6 mg/Kg) combination were initiated at the day of arthritis induction and evaluation was done at the 21th day. The parameters that were measured and the tissue that was analyzed are indicated on each panel. The type of treatment, including doses, is given under the bars. The bars represent means ± mean standard errors of 8-10 animals per group. #p≤0.05 between healthy and AIA groups; *p≤0.05 compared to AIA group, as inferred from one-way ANOVA followed by Tuckey's test.



Source: Authors

3.6 Antioxidant enzymes in liver and kidney

Figure 6 shows the results of the measurements of the CAT and SOD activities in the liver and kidney. As expected, based on previous reports, arthritis greatly depressed the activities of both enzymes (Sá-Nakanishi, 2018; Correa et al., 2019). AN + MTX treatment prevented the decrease in SOD activities in both liver and kidney. Under the same treatment, CAT activity in the liver also remained fairly close to that of healthy rats, but in the kidney preservation was partial. Monotherapy with AN (62.5 and 250 mg/kg) and MTX (6 and 12 mg/kg) did not prevent the diminution of the CAT activities in both liver and kidney, the same being true for the SOD activity in the kidney. These treatments, however, preserved almost entirely SOD activity in the liver. MTX at the highest dose, 24 mg/kg, on the other hand, had no effect at all on the arthritis-induced low CAT and SOD activities in the liver. In the kidney, on the contrary, there was total preservation of SOD activity and partial preservation of the CAT activity.

3.7 Reduced and oxidized glutathione in liver and kidney

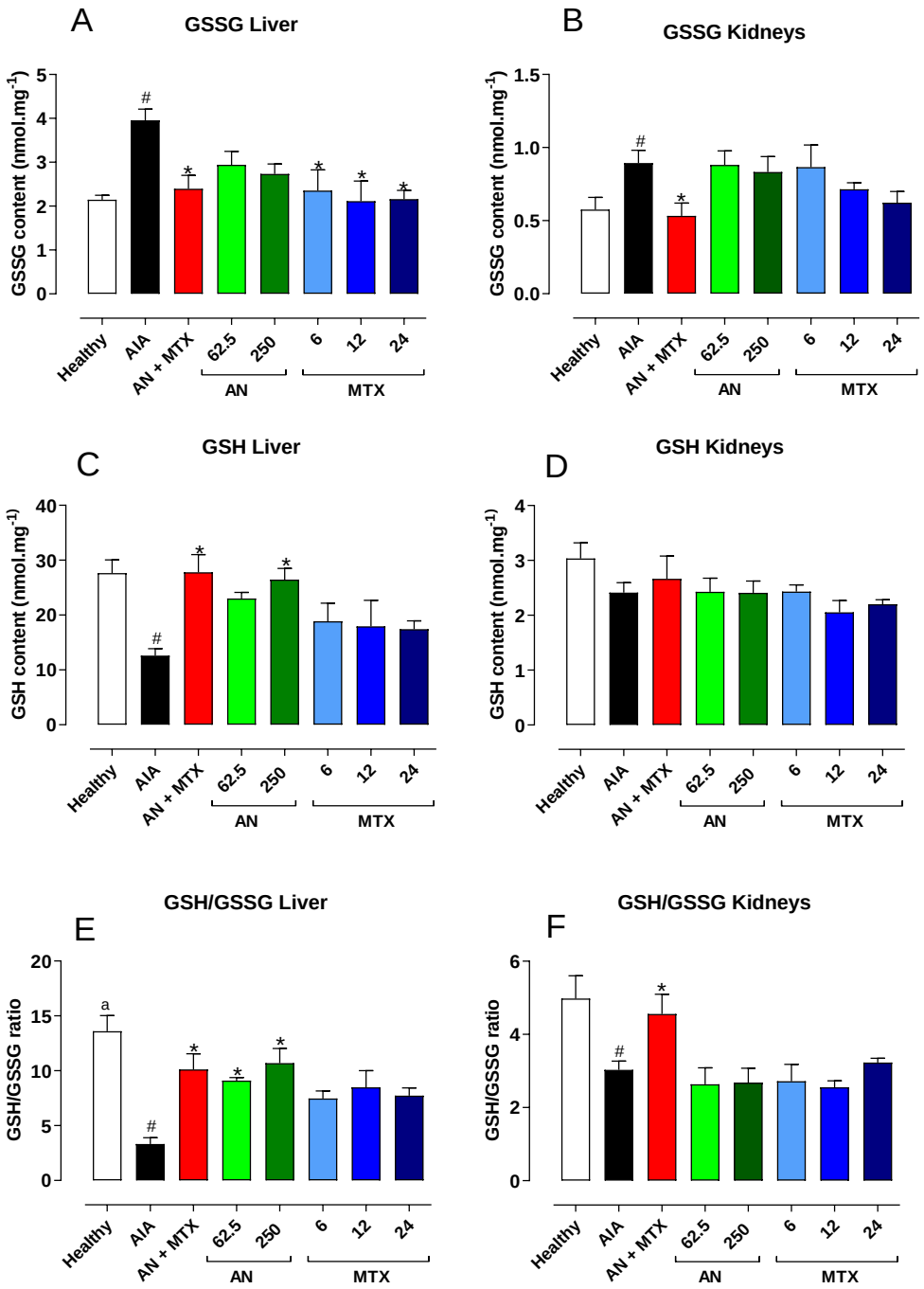
The concentrations of the two important components of the cellular oxidative homeostasis, GSH and GSSG, were measured and the results are shown in Figure 7. Our measurements confirm the early notion that arthritis diminishes the GSH levels and increases the GSSG levels, leading to a diminution in the GSH/GSSG ratio (Comar et al., 2013). These alterations were more pronounced in the liver when compared to the kidney. In the liver the combination AN + MTX clearly prevented the diminution in the GSH concentration and the increase in the GSSH concentration; this implies also in the maintenance of the GSH/GSSG ratio. In the kidney, the GSH/GSSG ratio was also maintained at values that were close to those found in healthy rats. The general trend of monotherapy with either AN or MTX in the liver was in the direction of partially preserving the GSH and GSSG levels as well as the GSH/GSSG ratio. In the kidney, however, monotherapy was inefficient.

4. Discussion

Rheumatoid arthritis is characterized by a systemic inflammatory state, in which immune cells and soluble mediators, such as cytokines, play a crucial role (Gomes et al., 2017). Oxidative stress is also tightly associated with arthritis development. Reactive oxygen and nitrogen species (ROS and RNS) are systemically increased and can effectively contribute to cartilage damage and also to the severity and chronicity of the disease (Tak et al., 2000). In this regard, the results obtained in this work demonstrate that treating adjuvant-induced arthritic rats with AN (62.5 mg/kg) and a relatively low dose of MTX (6 mg/kg) attenuates in a significant and often pronounced way several inflammatory parameters and oxidative stress indicators in plasma, liver and kidneys. A notable characteristic of this series of antiarthritic effects is the possibility of reducing the therapeutic dose of methotrexate. For many indicators examined in this work the effects of the combination of 6 mg/kg MTX with 62.5 mg/kg AN were fairly comparable to the effects of methotrexate in monotherapy with the dose of 12 mg/kg. The observation gains in importance if one takes into account that the latter compound is often considered the first choice therapeutic agent in the clinical treatment of rheumatoid arthritis and other autoimmune diseases in humans (Zimmerman et al., 2017). Furthermore, the effects of the combined treatment were, in several instances, superior to the effects of 250 mg/kg AN in monotherapy, a dose that exceeds by a factor of four the dose of the compound in the associated therapy.

The antiarthritic effects of MTX observed in this work plainly confirm its prominent therapeutic position (Alam et al., 2017). Even in monotherapy (especially at the doses of 12 and 24 mg/kg), MTX was capable of preventing the paw edema, the appearance of secondary lesions and the migration of leukocytes into the articulations. Most of the actions of MTX are proba-

Figure 7 Liver and kidney reduced and oxidized glutathione contents in heathy and arthritic rats and the influence of treatments. The treatments with anethole (AN; 62.5 and 250 mg/Kg), methotrexate (MTX; 6, 12 or 24 mg/Kg) and AN (62.5 mg/Kg) + MTX (6 mg/Kg) combination were initiated at the day of arthritis induction and evaluation was done at the 21th day. Parameters and tissue are specified on each panel. The type of treatment, including doses, is given under the bars. The bars represent means ± mean standard errors of 8-10 animals per group. #p≤0.05 between healthy and AIA groups; *p≤0.05 compared to AIA group, as inferred from one-way ANOVA followed by Tuckey's test.



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bly consequence of its already described anti-inflammatory and antioxidant effects. With respect to its antioxidant effects, Zimmerman et al. (2017) have shown that MTX acts in a direct way, being able to scavenge the superoxide anion in vitro. The mechanism of the anti-inflammatory action of MTX is not fully understood, but it could be largely due to the impairment of the synthesis of several inflammatory cytokines, such as interleukin 1β (IL-1β), IL-4, IL-6, IL-13, TNF-α, interferon gamma (IFN-γ) and the granulocyte-macrophage colony-stimulating factor (GM-CSF) (Wessels et al., 2008). Inhibition of the synthesis of all these cytokines is likely to be consequence of the inhibition of purine and pyrimidine synthesis (Segal et al., 1990).

Complementary to this main mode of action, the anti-inflammatory effects of MTX could also be reflecting its interference with the formation of polyamines such as spermine and spermidine (Chan & Cronstein, 2013). It is known that polyamines accumulate in the synovial fluid and in the urine of patients with rheumatoid arthritis (Stamp et al., 2010). Finally, an additional mechanistic possibility is an extra generation of adenosine (Wessels et al., 2008), a compound that possesses endogenous anti-inflammatory activity (Chan & Cronstein, 2013).

Monotherapy with MTX, however, caused some adverse effects in the arthritic rats: diarrhea (which was intense in animals treated with the 24 mg/kg dose), increased plasma concentrations of urea (renal toxicity) and diminution in the number of red blood cells (anemia). These effects were not observed with the lowest dose used in this work (6 mg/kg), although, as discussed above, this dose only partially affected the inflammation and oxidative stress. In this regard, AN, co-administered at the dose of 62.5 mg/kg, was able to potentiate many of the antiarthritic effects of the 6 mg/kg dose of MTX. This is notably beneficial because it avoids the adverse effects of the higher MTX doses.

One can consider AN as having an action mechanism that is largely complementary to that of MTX. The antiarthritic effects of AN had already been demonstrated by previous work (Ritter et al., 2017; Ames et al., 2020). These effects are related to a diminished production of the pro-inflammatory cytokines involved in the pathogenesis of adjuvant-induced arthritis, such as IL-17, IL-6 and TNF-α, but also to a diminished generation of nitric oxide (Ritter et al., 2017) and prostaglandins (Wisniewski-Rebecca et al., 2015). Recently it was demonstrated that AN is a potent inhibitor of the activation of the transcription factor NF-κB induced by TNF, inhibiting the phosphorylation and consequent degradation of IκBα (Chainy et al., 2000), and compromising, thus, the pro-inflammatory effects of NF-κB. There are few reports about the antioxidant effects of AN. The available data reveal that AN display a weak antioxidant activity *in vitro*, as deduced from DPPH assays and from its capacity in reducing ferric ions (Freire et al., 2005; Senatore et al., 2013). It is highly probable, thus, that the beneficial effects of AN on oxidative stress in arthritic animals, as observed in the present study, are much more the consequence of its anti-inflammatory action, as the connection between both phenomena is almost indissociable (McGarry et al., 2018).

5. Conclusion

As a conclusion, AN improved the antiarthiritic action of MTX, creating conditions that diminish both inflammation and oxidative stress. The main advantage in using AN is the reduction of the MTX dose, what eliminates or attenuates adverse effects caused by high dose therapy. Consequently, the combination of AN with MTX therapy may represent a new and efficient strategy for treating rheumatoid arthritis in humans if one takes into account the similarities between the pathological manifestations of the latter and those of the adjuvant-induced experimental arthritis.

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