Effect of anethole and anethole+itraconazole assiciantion on experimental arthritis induced by *Paracoccidioides brasiliensis*

Efeito do anetol e da associação anetol+itraconazol sobre a artrite experimental induzida por *Paracoccidioides brasiliensis*

Efecto del anetol y de la asociación anetol+itraconazol sobre la artritis experimental inducida por *Paracoccidioides brasiliensis*

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Abstract
In this study, the effect of treatment with anethole (AN) and the combination anethole+itraconazole (AN+IT) compared to IT, on infectious arthritis induced by *Paracoccidiodes brasiliensis*, was evaluated. The animals were treated for 14 days at doses of anethole (AN - 62.5, 125 and 250 mg/kg), itraconazole (IT - 12.5, 25 and 50 mg/kg) and the combination of anethole+itraconazole (AN+ IT - 62.5 and 12.5 mg/kg). The parameters evaluated were: the development of knee edema, the number of leukocytes recruited into the joint cavity, the body weight of the animals, the walking capacity, the plasmatic concentration of nitric oxide, the concentration of TNF in the knee joint exudate, the production of anti-Pb antibodies and the activity of plasma transaminases (AST and ALT). Histological changes in the right knee joint of the hind paw were evaluated using Hematoxylin-eosin and Grocott staining. The results showed that treatment with monotherapies and combinations reduced knee joint edema, the number of leukocytes recruited into the synovial cavity and improved the animals' gait. The concentration of plasma NO, tissue TNF and anti-Pb were reduced by treatment with IT at all doses tested and with the AN+IT combination. Treatment with AN only reduced the plasma concentration of NO and tissue TNF at a high dose. Altogether, the data showed that treatments with IT monotherapy and the combination of AN + IT showed a similar inhibitory effect on the development of arthritis.
Keywords: Anethole; Itraconazole; Infectious arthritis; Antifungal.

Resumo
Neste estudo foi avaliado o efeito do tratamento com o anetol (AN) e a associação anetol+itraconazol (AN+IT) comparativamente ao IT, sobre a artrite séptica induzida pelo *Paracoccidiodes brasiliensis*. Os animais foram tratados

por 14 dias nas doses de anetol (AN - 62,5, 125 e 250 mg/kg), itraconazol (IT - 12,5, 25 e 50 mg/kg) e associação de anetol+itraconazol (AN+IT - 62,5 e 12,5 mg/kg). Os parâmetros avaliados foram: o desenvolvimento do edema de joelho, o número de leucócitos recrutados na cavidade articular, o peso corporal dos animais, a capacidade de marcha, a concentração plasmática de óxido nítrico, a concentração de TNF no exsudato articular do joelho, a produção de anticorpos anti-Pb e a atividade de transaminases plasmáticas (AST e ALT). Alterações histológicas na articulação do joelho direito da pata posterior foram avaliadas utilizando coloração Hematoxilina-eosina e Grocott. Os resultados mostraram que o tratamento comas monoterapias e com a associação, reduziram o edema da articulação do joelho, o número de leucócitos recrutados na cavidade sinovial e melhoraram a marcha dos animais. A concentração de NO plasmática, de TNF tecidual e de anti-Pb foram reduzidos pelo tratamento com o IT em todas as doses testadas e com a associação AN+IT. O tratamento com AN somente reduziu a concentração plasmática de NO e de TNF tecidual em dose alta. No conjunto, os dados mostraram que os tratamentos com o IT em monoterapia e com a associação AN + IT, mostraram um efeito inibitório similar sobre o desenvolvimento da artrite.
Palavras-chave: Anetol; Itraconazol; Artrite infecciosa; Antifúngico.

Resumen
En este estudio se evaluó el efecto del tratamiento con anetol (AN) y la combinación anetol+itraconazol (AN+IT) en comparación con IT, sobre la artritis séptica inducida por Paracoccidioides brasiliensis. Los animales fueron tratados durante 14 días con dosis de anetol (AN - 62,5, 125 y 250 mg/kg), itraconazol (IT - 12,5, 25 y 50 mg/kg) y la combinación de anetol+itraconazol (AN+ IT - 62,5 y 12,5 mg/kg). Los parámetros evaluados fueron: el desarrollo del edema de rodilla, el número de leucocitos reclutados en la cavidad articular, el peso corporal de los animales, la capacidad para caminar, la concentración plasmática de óxido nítrico, la concentración de TNF en el exudado de la articulación de la rodilla, la producción de anticuerpos anti-Pb y la actividad de las transaminasas plasmáticas (AST y ALT). Los cambios histológicos en la articulación de la rodilla derecha de la pata trasera se evaluaron mediante tinción con hematoxilina-eosina y Grocott. Los resultados mostraron que el tratamiento con monoterapias y combinaciones redujo el edema de la articulación de la rodilla, el número de leucocitos reclutados en la cavidad sinovial y mejoró la marcha de los animales. La concentración de NO en plasma, TNF tisular y anti-Pb se redujo mediante el tratamiento con IT en todas las dosis probadas y con la combinación AN+IT. El tratamiento con AN sólo redujo la concentración plasmática de NO y TNF tisular en dosis altas. En conjunto, los datos mostraron que los tratamientos con monoterapia IT y la combinación de AN + IT mostraron un efecto inhibidor similar sobre el desarrollo de la artritis.
Palabras clave: Anetol; Itraconazol; Artritis infecciosa; Antifúngico.

1. Introduction

Paracoccidioidomycosis (PMC) is a systemic mycosis that affects more than ten million people in Latin America. It is considered the eighth cause of death among infectious and parasitic diseases (Restrepo et al. 2015; Wagner et al. 2021). The highest prevalence of cases occurs in Brazil, Venezuela, Argentina, Ecuador and Colombia, where the estimated incidence of the disease is 1 to 3 new cases per 100,000 inhabitants/year (Martinez, 2017). In Brazil, PMC is an important public health problem, and the states that concentrate the highest number of cases are those in the Southern and Central-western regions, highlighting high mortality rates in the states of São Paulo and Paraná (Shikanai-Yasuda et al. 2018; Loth et al. 2014; Ramos et al. 2005).

The fungus Paracoccidioides brasiliensis (P. brasiliensis), a eukaryotic microorganism belonging to the phylum Ascomycota, is the etiological agent of PMC (Bagagli et al. 2006). Man is its host, and when inhales conidia present in soil and, inside his organism, it gives rise to a spherical, yeast-like shape (Shikanai-Yasuda et al. 2018; Ricci et al. 2004). This characteristic of dimorphism, transition from mycelium to yeast form, represents the microorganism ability to colonize, invade, survive and cause disease in a host (Andrade et al. 2005; Andrade et al. 2006). Subsequently, granulomas occurs are produced in several organs, such as lungs, spleen and liver, which characterizes the systemic development of PMC disease (Restrepo et al. 2001).

Paracoccidioides brasiliensis (Pb) also causes damage to other tissues such as joints, leading to infectious arthritis. This treatment is usually carried out with itraconazole (IT) in monotherapy for a long term, until the etiological agent elimination is recorded. This treatment is already well established in the Brazilian Consensus on PMC (Shikanai-Yasuda et al.

2018), but, as it is a long process, it can cause adverse effects in patients, from mild reactions such as gastrointestinal discomfort to severe reactions such as liver damage (Brunton et al., 2012).

The use of natural products that allowed propitious therapeutic effects, but with fewer adverse effects, has been gaining more attention in the treatment of several diseases (Singh, 2007). Anethole (AN), a natural compound extracted from star anise (Illicium verum), has several biological activities, with its antimicrobial, anti-inflammatory and antinociceptive activities already well proven (Kosalec et al. 2005; Sabry et al. 2021; Yutani et al. 2011; Domiciano et al. 2013; Ritter et al. 2013; Wisniewski-Rebecca et al. 2015; Ritter et al. 2017). In vitro studies have shown that AN effect on yeast and filamentous fungi may be due to the induction of apoptosis and formation of reactive oxygen species. However, Fujita et al., (2014) reports that the AN antimicrobial activity is lower when compared to reference antimicrobials.

Other studies show that AN in monotherapy inhibits the acute and chronic inflammatory response in rats (Domiciano et al. 2013; Ritter et al. 2017), and that when associated with ibuprofen (traditional anti-inflammatory), it has greater anti-inflammatory activity at much lower doses when compared to monotherapy (Wisniewski-Rebecca et al. 2015). Such AN activity seems to be due to its inhibitory action on the production/release of some inflammatory mediators (NO, TNF) (Domiciano et al. 2013; Ritter et al. 2013; Wisniewski-Rebecca et al. 2015; Ritter et al. 2017; Chainy et al. 2000).

In recent years, drugs combination therapy has been highlighted as an important strategy to increase their therapeutic efficacy in several diseases (Barrett et al. 2012; Park et al. 2012). Rosato et al. (2007) and Rosato et al. (2008) investigated the association of antimicrobials with some essential oils, and showed a synergistic effect of these ones (Origanum vulgare, Pelargonium graveolens and Melaleuca alternifolia) when combined with norfloxacin and amphotericin B against strains of Bacillus cereus, Bacillus subtilis, Escherichia coli, Staphylococcus aureus and several strains of Candida. As well as on Dąbrowska et al. (2021) study, who evaluated the additive effects of eugenol and anethole in combination with miconazole against clinical isolates of Candida albicans.

This study aimed at evaluating the effectiveness of treatments with AN and the association anethole+itraconazole (AN+IT) compared to IT, to treat infectious arthritis induced by PCM.

2. Methodology

2.1 Animals

Male Wistar rats, from 45 to 60 days age (200-230g), were kept in standard vivarium conditions (temperature of 22ºC, controlled humidity, and a 12-hour light-dark cycle) and had free access to the standard diet and water). The experimental procedure was approved by the Ethics and Animal Experimentation Committee of the State University of Maringá (CEAE/UEM 7318020417). Rats were randomly divided into the following experimental groups (Figure 1).

Figure 1 - Scheme showing the experimental groups in the model of arthritis induced by *P. brasiliensis* (AIP). N = number of animals per group; AN = anethole; IT = itraconazole.
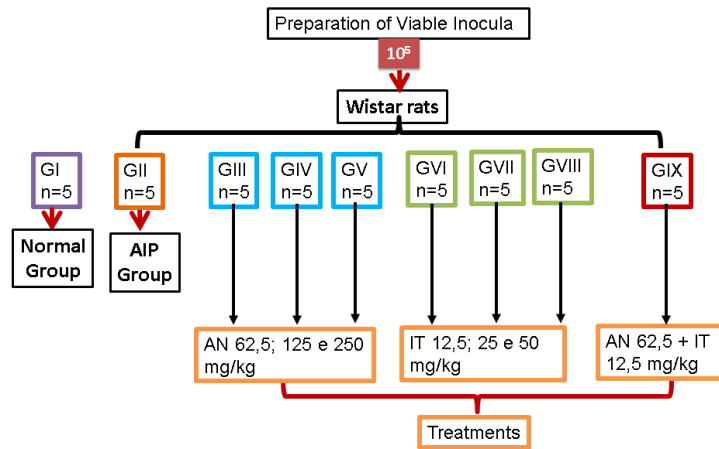
Source: Authors.

2.2 Induction of Infectious Arthritis by *P. brasiliensis*

Infectious arthritis was induced according to Loth et al. (2014). Fungi (*P. brasiliensis* - Pb18) were transmitted in phosphate buffered saline solution (PBS) at a concentration of 10^5 viable yeast cells. The animals, previously anesthetized with 5% isoflurane, received 100µl of an intra-articular suspension in the right knee of the hind paw. The animals from the normal group received 100 µl of PBS by the same via of administration.

Initially, experiments were carried out to establish the evolution pattern of infectious arthritis and in which period the clinical manifestations were more severe. This was done to define the treatment period for the animals. Disease progression was evaluated on the 3rd, 7th, 14th and 21st days after arthritis induction (AIP). The evaluated parameters were: knee edema development, number of leukocytes recruited in the joint cavity, animals' body weight, plasma concentration of nitric oxide, TNF concentration of in joint exudate of knee, walking capacity, production of anti-Pb antibodies and plasma transaminases activity (AST and ALT). Histological analyses were also carried out using hematoxylin-eosin and Grocott staining techniques. After the animals were euthanized, blood samples from the inferior vena cava and synovial fluid samples were collected, processed and stored at -80° for further analyses.

2.3 Edema determination and total score and leukocyte differential in synovial fluid

An edema was evaluated by measuring the diameter of the knee (side-to-side diameter/mm) using an analog caliper (Western). Results were calculated by the difference between the initial knee diameter measurement before and after arthritis induction.

The determination of number of leukocytes recruited in the joint cavity was carried out immediately before arthritis induction and at different periods established after disease induction. And exudate was collected after an intra-articular washing with 40 µl of PBS containing EDTA. The number of total leukocytes recruited in the joint was quantified in a Neubauer chamber and polymorphonuclear number (PMN) and mononuclear (MN) leukocytes were determined on slides stained by the May-Grunwald-Giensa method. Results were expressed as means ± standard error of mean (S.E.M.).

2.4 Animals' Care

The animals were daily treated, orally, with AN (Sigma Aldrich®) at doses of 62.5; 125; 250 mg/kg; IT (100 mg capsules prepared in a handling pharmacy in Cascavel-PR) at doses of 12.5; 25 and 50 mg/kg and AN+IT association at doses

of 62.5 and 12.5 mg/kg, diluted in 10% carboxymethylcellulose (CMC - vehicle). The animals of both normal and AIP groups received the vehicle daily, orally. The treatment of different experimental groups started on the day of infectious arthritis induction (day zero) and continued until the 14th day.

2.5 Determination of nitric oxide concentration in plasma

The Griess Method was carried out to determine the plasmatic concentration of nitric oxide (NO), which determines nitrite concentration in the samples. Thus, to quantify nitrite, 50 µL of each plasma sample was placed in a microplate of 96 cavities, in triplicate. Then, 50 µL of Griess solution (1% sulfanilamide in 5% phosphoric acid and 0.1% N-1-naphthylethylonediamine dihydrochloride in water) were added at room temperature. After 10 minutes, the reading was performed in an ELISA plate reader (550 nm). NO concentrations were calculated from a sodium nitrite standard curve and the results were expressed in µmol/mL.

2.6 Determination of TNF concentration in joint exudate

TNF concentration was determined in the knee joint exudate of the animals. After the rats' euthanasia, femur- joints were collected, shredded in a Homo Mix® homogenizer and the macerate was centrifuged at 1200 rpm, for 10 minutes at 4°C. The supernatant was separated to determine TNF concentration, using a commercial ELISA kit, following manufacturer's recommendations. TNF concentration was expressed in picograms (pg/ml)

2.7 Titration of anti-Pb antibodies

ELISA test (enzyme-linked immunosorbent assay) was carried out as described by Ramos et al. 2005. Flat-bottomed 96-well plates were sensitized with 100 µl in each well of the solution containing the protein extracted from Pb wall at a 2-ng/ml dilution, which were incubated for 18 hours at 4 °C. Plasma samples obtained from the animals' blood were distributed on the plate according to each group. After incubation and washing with PBS, 50 µl of secondary antibody (immunoglobulin G, IgG, with peroxidase, produced by SIGMA-ALDRICH) were added per well, at 1:1000 dilution in PBS, followed by incubation at 37 °C for one hour.

In order to get some development/exposure, 100 µl per well of orthophenilenediamine solution (Sigma) at 0.4 mg/ml and hydrogen peroxide at 5% in 1 molar sodium citrate buffer at pH 4.5 were added to the plates. The reaction was kept in a dark room for 15 minutes, then, it was stopped with 0.5-molar sulfuric acid for 10 minutes to obtain the sample reading by absorbance in a microplate reader at 492nm. It should be reinforced that all assays were carried out in triplicate.

2.8 Animals' evaluation in the open field test

The studied animals were evaluated in the open field test, which consists of a wooden box (45 cm × 45 cm) with a 30-cm high wall. Rats were placed individually in the center of the field to explore the box for 10 minutes, and the distance covered (in meters) by each animal was recorded.

2.9 Histological analyses of femorotibial joint

Just after the animals' euthanasia, the dissection product of the right knee joint of the hind paw of each animal was fixed in a 10% formalin solution for 48 hours at room temperature. Subsequently, samples were washed with distilled water and kept in a 5% trichloroacetic acid solution for seven to nine days at room temperature. After verifying the decalcification of the pieces, they were dehydrated in increasing series of alcohols until absolute and diaphanized in n-

butyl alcohol solution. Samples of translucent pieces were embedded in paraffin. Then, 5 µm-thick sections (longitudinal sections of joints) were made on an Olympus CUT 4055® microtome, stained with hematoxylin-eosin (HE) technique to analyze the general morphology of tissue and granulomas formation, while the presence of *P. brasiliensis* was demonstrated by Grocott technique (1955).

Morphological evaluations of the joints were carried out using a light microscope (Olympus®), while the visual fields of interest were photomicrographed using 20 and 40X magnification objectives. The morphological characteristics of articular cartilage of femur and tibia were: the surface shape of cartilage and the presence of flocculation, chondrocytes distribution in specific zones cell clones, presence of fissures in the extracellular matrix, *pannus* formation, as well as the subchondral bone. In synovial membrane, the morphological characteristics of synovial intima and subintima and the presence of inflammatory infiltrate in the joint cavity were analyzed. The results were presented in histological plates forms.

2.10 Determination of transaminase activity

Assays to evaluate transaminase activity (aspartate aminotransferase - AST and alanine aminotransferase - ALT) were carried out using plasma from the animals. Blood samples collected from the inferior vena cava were centrifuged (700g/15 min.) and analyzed using analytical kits (Gold Analisa Diagnostica Ltda - Belo Horizonte, MG, and Brazil). The results were expressed in units per liter (U/L).

2.11 Statistical analysis

Results are presented as mean ± S.E.M. The difference among groups was analyzed by ANOVA followed by Tukey test. The significance level was $P < 0.05$, and data were analyzed using Prism 4.0 software (GraphPad, San Diego, CA, USA).

3. Results

3.1 Development of arthritis induced by *P. brasiliensis* (edema evaluation and number of leukocytes recruited in joint)

Fungus inoculation,via intra-articularly, into the animal's knee, has triggered an intense inflammatory reaction with a progressive increase in the diameter of joint from the 3rd day to the 21st day of infection (Figure 2A). On the 14th day, the highest response peak occurred after arthritis induction, and the results are shown in Figure 2. The number of total leukocytes in synovial fluid of the right knee of arthritic animals increased significantly when compared to the one of normal animals (Table 1). Cell recruitment increased from the 3rd day onwards, with a very intense increase on the 14th day after arthritis induction. On the 21st day, the number of recruited leukocytes was reduced when compared to the AIP group on the 14th day, but it was still higher when compared to normal animals. The number of total leukocytes increased due to both the increase in the number of polymorphonuclear and mononuclear leukocytes.

As a whole, these data indicated that AIP manifestations were more intense on the 14th day after infection, and, therefore, this period was chosen to evaluate the animals in the treatment groups (14 days). The treatment of AIP animals with AN at doses of 62.5; 125 and 250 mg/kg reduced joint diameter by 32%, 44% and 64%, respectively, on the 14th day after arthritis induction. The treatment of animals with AN+IT association at doses of 62.5+12.5 mg/kg significantly reduced (71%) joint diameter. IT at doses of 12.5 and 25 significantly reduced knee edema (56% and 61%, respectively) while the 50 mg/kg dose caused a reduction of 45% on the 7th day and 81% on the 14th day. The results are shown in Figure 2B.

Figure 2 - Development of arthritis (AIP) in the animals' right back knee joint (A). Treatment with AN 62.5, 125, 250 mg/kg, IT 12.5, 25, 50 mg/kg and AN+IT 62.5+12.5 mg/kg was carried out orally (gavage), daily , in a single dose, and it started on the same day as the arthritis induction. The effect of the respective treatments was evaluated on the 7th and 14th day after AIP induction (B). Each value represents the mean ± SE of 5 animals per group. One-way ANOVA followed by the Tukey test. *P<0.05 compared to AIP group.
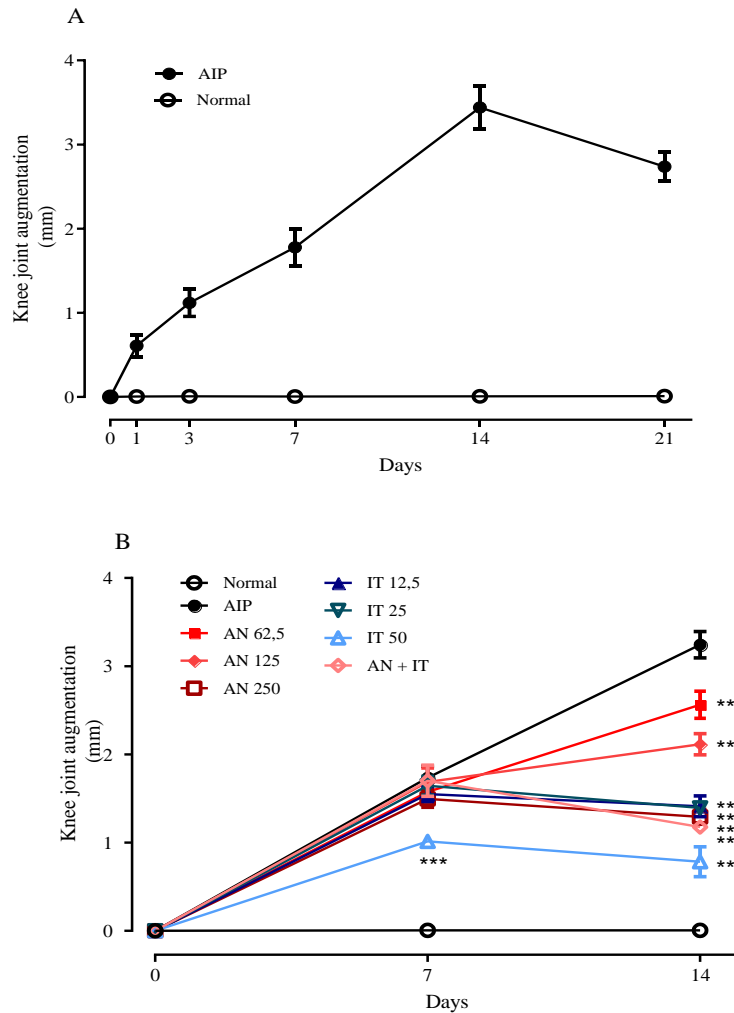
Source: Authors.

In the treatments presented, the reduction in edema produced by the inflammatory process with the combination of IT+AN was compared to the highest dose of IT monotherapy.

Treatment with AN at doses of 62.5 mg/kg, 125 mg/kg and 250 mg/kg significantly reduced the number of total leukocytes, as well as mononuclear and polymorphonuclear leukocytes when compared to the AIP group (Table 1). On the 14th experimental day, animals were treated with IT and the association AN+IT, at different doses, significantly reduced the number of total, mononuclear and polymorphonuclear leukocytes in synovial fluid of the right joint cavity. The analysis of treatments on cell recruitment in AIP model shows that IT in monotherapy at the highest dose and the association AN+IT at lower doses were able to significantly reduce the number of cells recruited to the infection area.

Table 1 - Number of total and differential leukocytes on knee joint inflammatory exudate of rats.

	Animals' group	N	TL/mm^3	PMN/mm^3	PMN (%)	MN/mm^3	MN(%)
	Normal	5	2200 ± 200	1476 ± 36	67	724 ± 164	33
AIP	3 days	5	13900 ± 1109	10994 ± 1103	79	2907 ± 171	21
	7 days	5	72300 ± 8870	55204 ± 8450	76	17096 ± 1364	24
	14 days	5	946989 ± 45673^a	780864 ± 36988^a	76	137536 ± 6878^a	24
	21 days	5	318000 ± 15369^a	261984 ± 11089^a	82	56016 ± 7872^a	18
AIP - 14 dias	AN 62.5 mg/Kg	5	266240 ± 36294^b	213241 ± 23597^b	80	71584 ± 17767^b	20
	AN 125 mg/Kg	5	259520 ± 10023^b	200022 ± 12006^b	77	66468 ± 5001^b	23
	AN 250 mg/Kg	5	243200 ± 15145^b	193715 ± 15098^b	80	49485 ± 6493^b	20
	IT 12.5 mg/Kg	5	178800 ± 23812^b	94444 ± 26296^b	53	40576 ± 11733^b	47
	IT 25 mg/Kg	5	36800 ± 1461^b	25486 ± 1439^b	69	17134 ± 3693^b	31
	IT 50 mg/Kg	5	8880 ± 933^b	6269 ± 960^b	71	1831 ± 302^b	29
	AN 62.5 mg/Kg + IT 12.5 mg/Kg	5	11824 ± 1153^b	16760 ± 1164^b	73	5960 ± 790^b	27

Each value represents the mean of leukocytes number $\pm$ SEM. in the joint cavity of the right knee of rats. AIP = *P. brasiliensis*-induced arthritis. N = Number of animals per group. TL = total leukocytes. PMN = polymorphonuclear leukocytes. MN = mononuclear leukocytes. One-way ANOVA followed by Tukey test. [a]$P<0.0001$ compared to normal group, [b]$p < 0.0001$ compared to 14 days group. Source: Authors.

Cell migration is one of the body's defenses against the infectious agent and IT and AN monotherapies and the IT+AN combination managed to reduce this inflammatory process.

3.2 Body weight of animals

The body weight evolution of the different groups of animals is shown in Table 2. The AIP induction and the treatments with AN, IT or AN+IT association did not change significantly the animals' body weight.

Table 2 - Variation in animals' body weight.

|	Animals' group	N	1 day	3 days	7 days	14 days
	Normals	5	4.00 ± 0.6	11.33 ± 2.3	28.00 ± 1.1	44.67 ± 3.7
AIP 14 dias	AIP	5	3.21 ± 0.5	8.57 ± 1.2	27.71 ± 2.4	58.21 ± 5.1
	AN 62.5 mg/Kg	5	3.40 ± 0.6	6.20 ± 1.1	28.70 ± 2.6	57.00 ± 4.3
	AN 125 mg/Kg	5	2.80 ± 0.7	9.00 ± 1.0	20.70 ± 3.0	52.00 ± 4.5
	AN 250 mg/Kg	5	3.70 ± 0.9	7.20 ± 1.0	20.30 ± 2.6	45.00 ± 4.9
	IT 12.5 mg/Kg	5	3.67 ± 0.7	5.00 ± 1.8	24.08 ± 3.9	52.42 ± 4.7
	IT 25 mg/Kg	5	3.90 ± 0.6	8.60 ± 1.6	32.00 ± 2.6	59.50 ± 5.8
	IT 50 mg/Kg	5	5.10 ± 1.1	8.80 ± 2.2	26.20 ± 2.1	59.90 ± 8.0
	AN 62.5 mg/Kg + IT 12.5 mg/Kg	5	4.00 ± 0.8	5.17 ± 1.1	28.00 ± 2.5	43.00 ± 3.8

Each value represents the mean variation in animals' body weight $\pm$ SEM. AIP = *P. brasiliensis*-induced arthritis. N = number of animals per group. AN = anethole. IT = itraconazole. AN+IT = anethole+itraconazole. One-way ANOVA followed by Tukey test. [a]$P < 0.0001$ compared to the normal group, [b]$p < 0.0001$ compared to 14 days group. Source: Authors.

Joint infection by *P. brasiliensis* promotes a local inflammatory process, without changing weight. Therefore, the treatments also did not vary.

3.3 Determination of plasma concentration of nitric oxide

Nitric oxide concentration in AIP plasma was significantly higher when compared to the one of normal animals on the 14th day after fungus injection (Figure 3A). However, NO concentration on the 21st day was approximately 25% lower than on the 14th day (Figure 3A). The animals' treatment with AN at 250 mg/kg dose reduced significantly NO concentration in the animals' plasma when compared to AIP, with 37% of an inhibition percentage (Figure 3B). However, the lowest doses of AN (62.5 or 125 mg/kg) did not cause a significant reduction (11% and 24%, respectively). Treatment with IT (12.5; 25 and 50 mg/kg), at all doses tested and with the AN+IT association (62.5 + 12.5 mg/kg) reduced NO concentration by 52%, 60%, 66% and 76%, respectively (Figure 3B).

Figure 3 - Nitric oxide (NO) concentration in animals' plasma after arthritis induction. Arthritic animals without treatments on days 3, 7, 14 and 21 compared to normal animals (A). Nitric oxide levels in groups of animals treated with AN, IT and AN+IT association (B). Each value represents the mean ± SE of 5 animals per group. One-way ANOVA followed by Tukey test. [a]P<0.05 compared to the normal group. [b]p<0.05 compared to the AIP group.
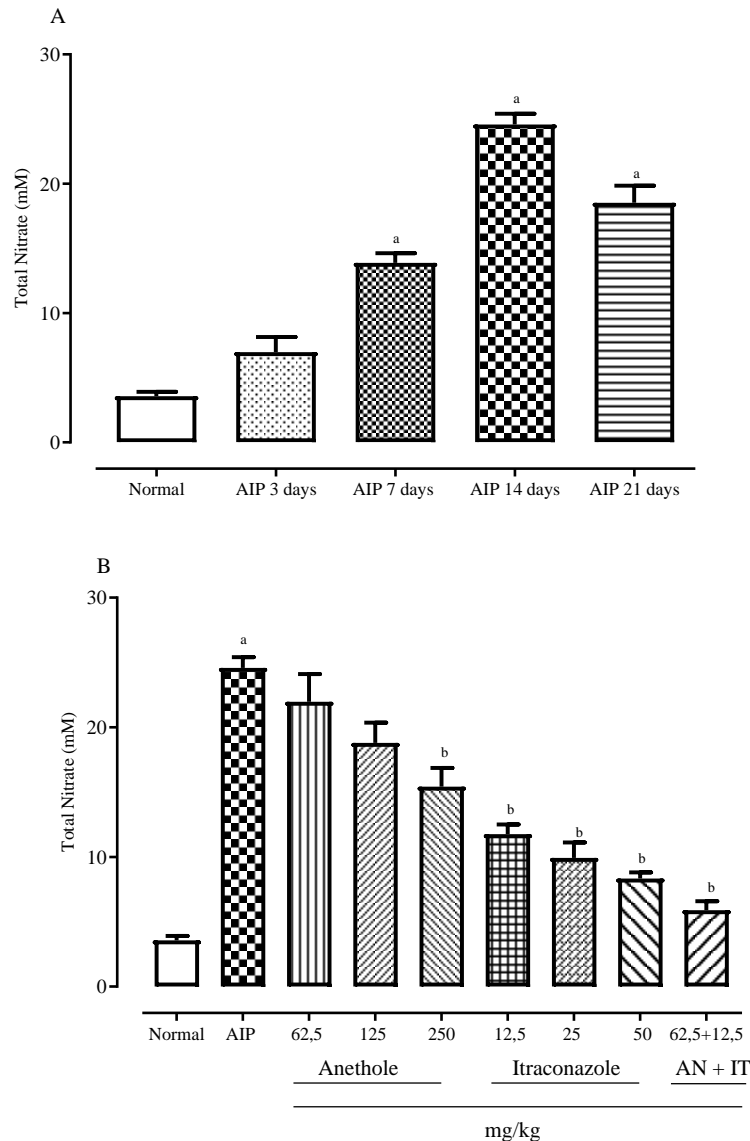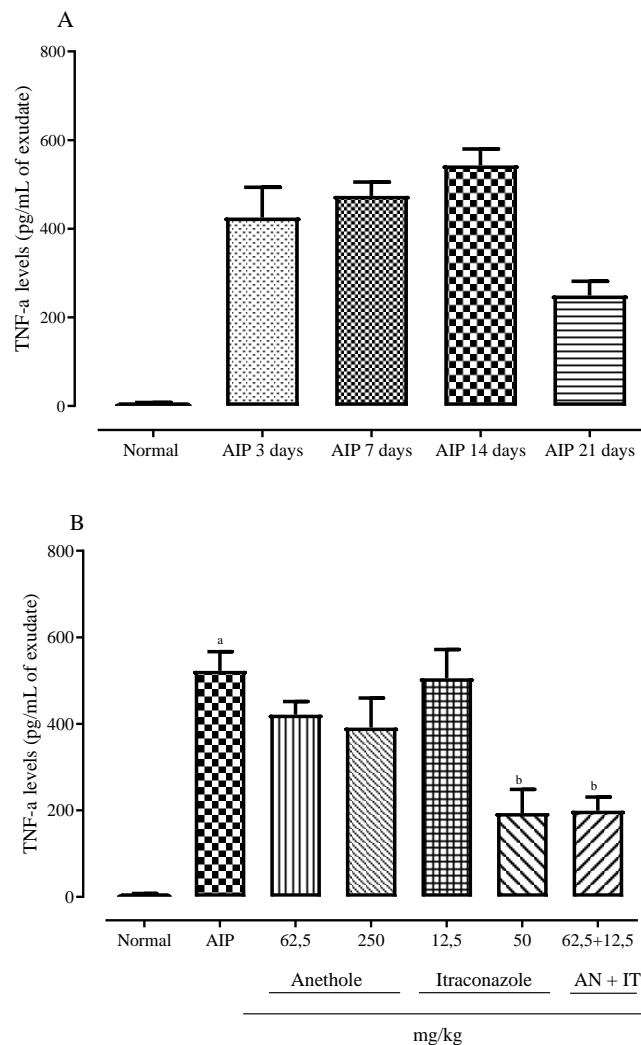

Source: Authors.

The 14th day of infection is the peak of plasma NO levels and treatments as a reference drug showed a significant reduction. However, AN monotherapy requires a high dose for a considerable effect, but the combination of IT+AN in low doses promoted the smallest reduction in plasma NO levels.

3.4 TNF concentration in knee joint exudate

The concentration of tumor necrosis factor (TNF) in knee joint exudate of arthritic animals increased significantly on the 3rd, 7th and 14th day after AIP induction, when compared to normal animals. The animals' treatment with AN (125 and 250 mg/kg), IT (12.5 and 50 mg/kg) and AN+IT association (62.5+12.5 mg/kg) reduced TNF concentration when compared to the animals of AIP group on the 14th day after induction, an inhibition percentage of 19% and 25% for AN, 3% and 63% for IT and 62% for the treatment with AN+IT association (Figure 4).

Figure 4 - TNF concentration in exudate of the animals' knee joint after 3, 7, 14 and 21 days of arthritis induction (A). Effect of AN, IT and AN+IT association on TNF concentration in exudate from animals' knee joint on the 14th day after arthritis induction (B). Each value represents the mean ± SE of 5 animals per group. One-way ANOVA followed by Tukey test. [a]P<0.05 compared to normal group, [b]p<0.05 compared to AIP group.
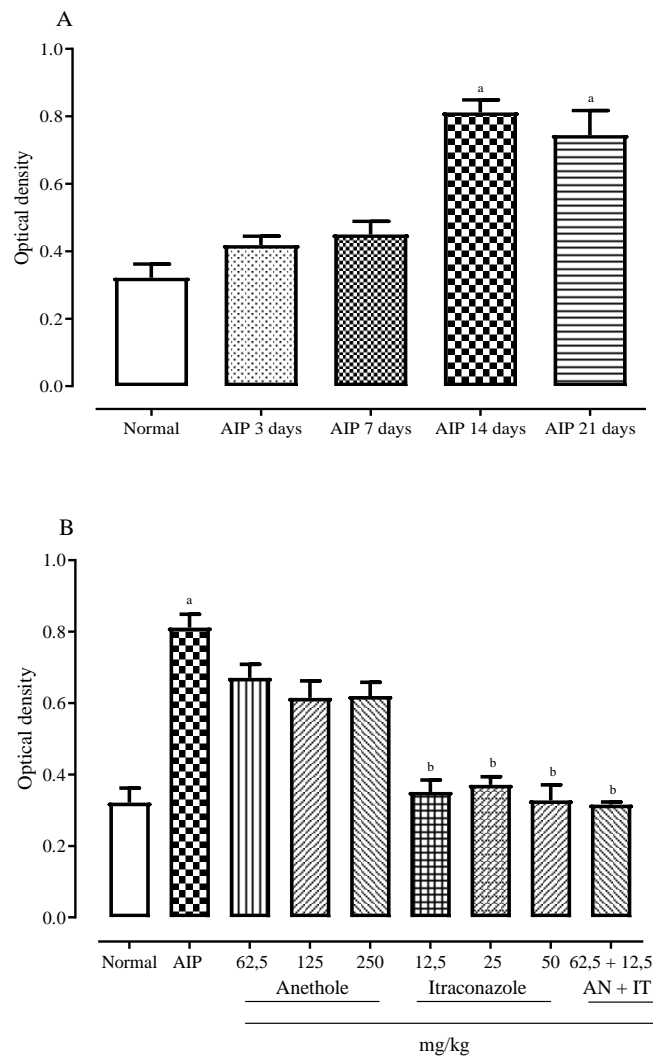

Source: Authors.

Monotherapy with the highest dose of IT promoted the greatest reduction of TNF in the inflammatory process, which was not observed with AN monotherapy. However, the combination of compounds showed a reduction consistent with the reference drug.

3.5 Titration of anti-Pb antibodies

The anti-Pb antibody titration assay showed higher production of specific antibodies on the 14th day of infection (Figure 5A). Treatment with AN, at all tested doses, did not significantly reduce anti-Pb levels, but IT reduced anti-Pb titers at all applied doses. Treatment with AN+IT association reduced significantly (61%) antibodies production (Figure 5B).

Figure 5 - Production of anti-Pb antibodies in animals' plasma 3, 7, 14 and 21 days after AIP induction (A). Effect of treatments with AN 62.5, 125 or 250 mg/kg, IT 12.5, 25 or 50 mg/kg and AN+IT 62.5+12.5 mg/kg on anti-Pb antibodies production in animals' plasma on the 14th day after AIP induction (B). The treatments were carried out orally, daily, in a single oral dose, starting on the same day as the arthritis induction. Each value represents the mean $\pm$ SE of 5 animals per group. One-way ANOVA followed by Tukey test. [a]$P<0.05$ compared to AIP group, [b]$p<0.05$ compared to AIP group.
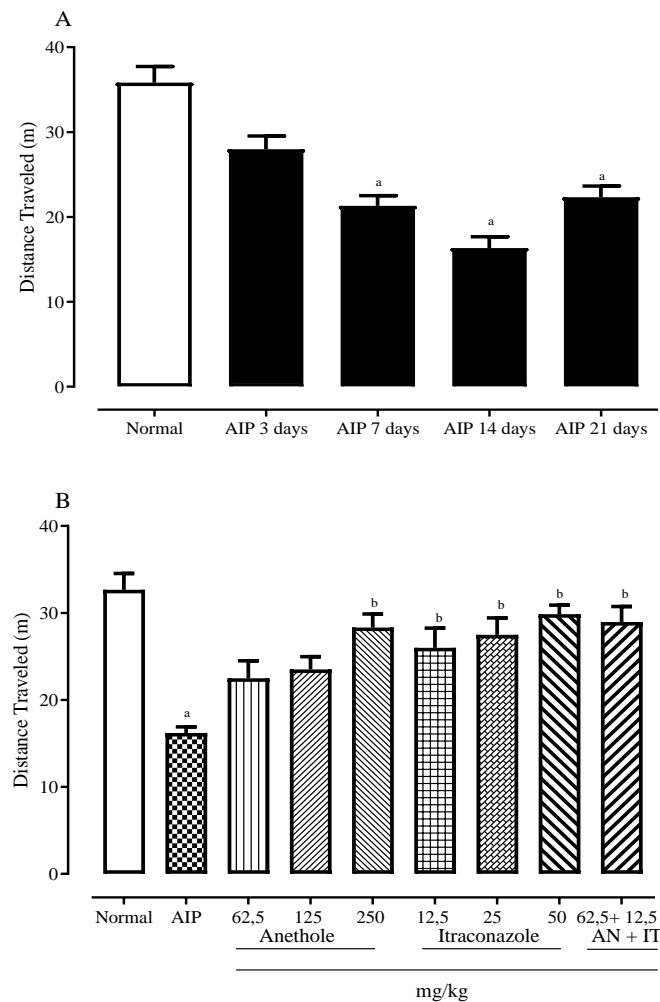

Source: Authors.

Adminstration of AN monotherapy did not reduce the production of anti-Pb antibodies. It was only possible to observe when associated with IT, as well as IT monotherapies.

3.6 Open Field Test

In the open field test, the distance covered by normal animals was 32.8 ± 1.9 meters during the 10-evaluated minutes (Figure 6). With AIP induction, animals' mobility was reduced (Figure 6A), consequently, there was a reduction of 22% in the distance covered by the animals on the 3rd day and of 55% on the 14th day.

While the treatment with AN increased the distance covered by the animals in the open field test only at a 250-mg/Kg dose (28.3 ± 1.6). Treatment with doses associated with AN+IT (62.5+12.5 mg/kg) increased the distance covered in relation to the AIP group, which caused an improvement in animals' mobility.

Figure 6 - Mean distance covered by the animals in the open field test on days 3, 7, 14 and 21 after AIP induction (A). Effect of treatments with AN 62.5, 125 or 250 mg/kg, IT 12.5, 25 or 50 mg/kg and AN+IT 62.5+12.5 mg/kg on the average distance covered by the animals in the field test opened on the 14th day after AIP induction (B). The treatments were carried out daily, and orally, in a single dose. It started on the same day as the arthritis induction. Each value represents the mean $\pm$ SE of five animals per group. One-way ANOVA followed by Tukey's test. aP<0.05 compared to normal group, bp<0.05 compared to AIP group.

Source: Authors.

The inflammatory process decreased the animals' mobility and the treatments reduced activity and increased the distance covered. The treatment that produced the most important improvement was AN monotherapy.

3.7 Histological analyses of femoral and tibial joint

Along the morphological joints analysis of normal animals stained with hematoxylin-eosin (HE), normal characteristics of femur and tibia cartilage were observed, with a smooth articular surface and cellular organization (Figure 7A). With chondrocytes arrangement in layers or zones, they were arranged side by side with a flattened appearance in the superficial zone. In the transition zone, cells have a rounded shape, and arranged singly or in isogenous groups. While in the deep zone, chondrocytes were arranged in a gap and below it, the calcified zone.

The synovial membrane also showed normal morphological characteristics, with two to three cell layers (Type A and Type B synoviocytes) in intima and subintima layers, with a predominance of adipocytes and blood vessels presence (Figure 7B). At the beginning of the infection (3rd day), there were no morphological changes in the cartilage. On the other hand, the presence of fungus *P. brasiliensis* in the joint caused significant changes at the beginning of the experiment, from the 3rd day of infection, in the synovial membrane (Figure 7C), which was with inflammatory characteristics, and a large number of leukocytes in both layers, disorganization of intima and subintima ones (Figure 7D), as well as a significant reduction of adipocytes, neovascularization and inflammatory infiltrate in the joint cavity.

Figures 7E and 7F show some results on joints on the 7th day of infection. As we can observe, although there were no morphological changes in cartilage, there is a large inflammatory infiltrate and a reduction of adipocytes in the synovial membrane when compared to normal animals.

Figure 7 - Photomicrographs of Wistar rats knee in longitudinal sections, stained with hematoxylin and eosin (H.E). A: Normal animal femur cartilage. B: Normal animal synovial membrane. C: AIP animal femur cartilage 3rd day. D: AIP animal synovial membrane 3rd day. E: AIP animal femur cartilage 7th day. F: AIP animal synovial membrane 7th day. Articular cartilage (AC); Synovial membrane (SM); Intima layer (IC); Subintimate layer (CSI); Adipocytes (AD); Polymorphonuclear leukocytes (PMN).
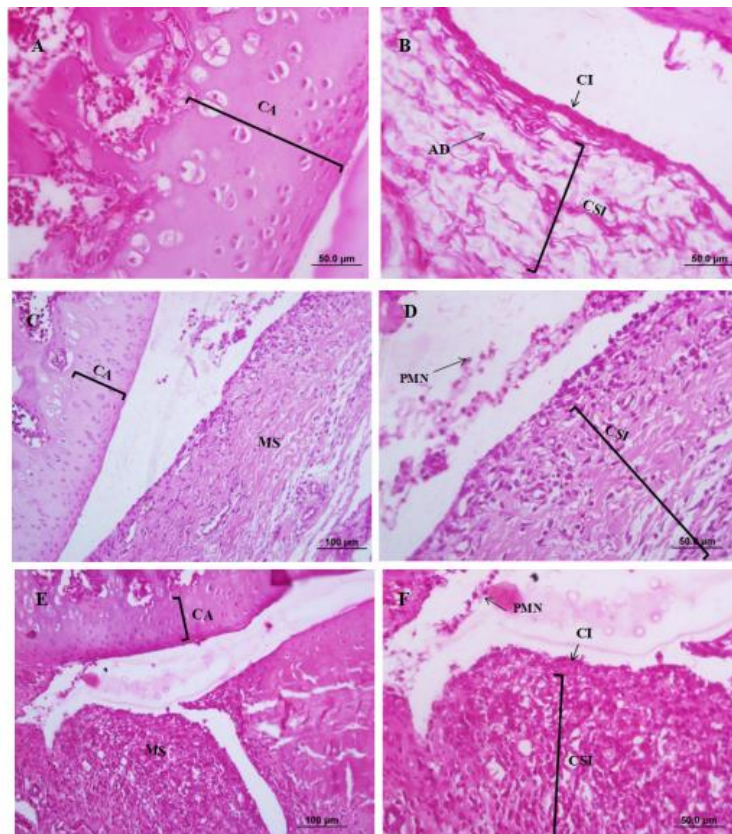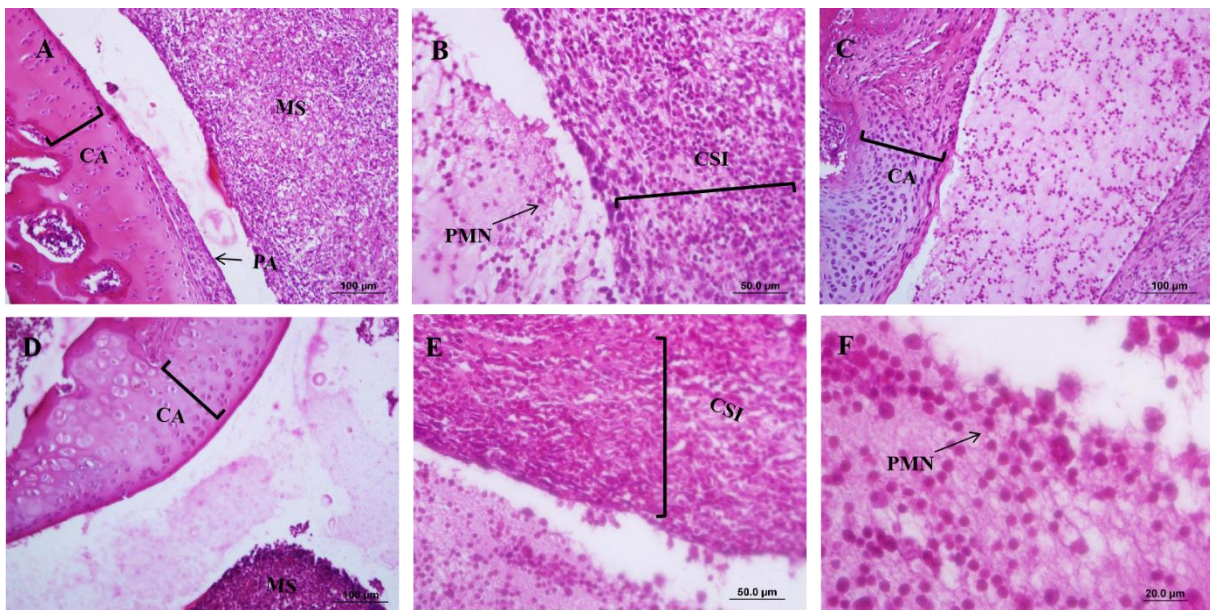

Source: Authors.

In the first week after P. brasiliensis inoculation, the inflammatory process caused changes in the synovial membrane due to the recruitment of leukocytes into the joint cavity.

On the 14th and 21st days of infection, changes were observed in the characteristic morphology of the knee joint (Figure 8). It was identified a degradation on cartilage and a subchondral bone invasion in the calcified area, as well as intense inflammatory infiltrate in joint cavity and the presence of pannus. The intima layer of synovial membrane have some cell spacing and the subintima shows intense reduction of adipocytes and vascularization.

Figure 8 - Photomicrographs of femoral and tibial joints of AIP rats on the 14th day (A), synovial membrane (B) and joint degradation (C). femoral and tibial joints of AIP rats on the 21st day (D), synovial membrane (E) and inflammatory infiltrate (F). Synovial membrane (SM); articular cartilage (AC); subintimal layer (CSI); Pannus formation (PA). The slides were stained using the hematoxylin and eosin (H.E) technique and the images were captured at 20X and 40X magnification.
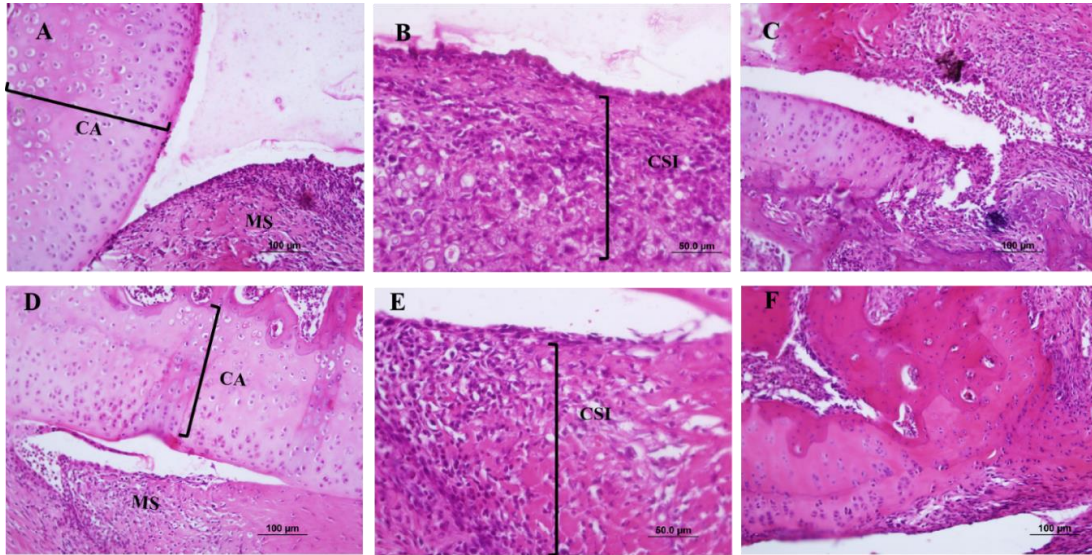

Source: Authors.

With the advancement of the inflammatory process caused by infectious arthritis, joint damage intensifies from the 14th day onwards, with significant changes in the cartilage and joint cavity.

Figure 9 shows joints morphology of animals in the treatment groups. Invasion of the subchondral bone in the calcified zone, presence of pannus, cells spacing in intimal layer were observed in animals treated with 62.5 mg/kg AN. And at 250 mg/kg of AN dose, it was possible to observe a small inflammatory infiltrate, some reduction of adipocytes in subintima layer and changes in animals' cartilage.

Figure 9 - Photomicrographs of femur-tibial joints of AIP rats treated during 14 days with AN at 62.5-mg/kg doses (A), synovial membrane (B) and joint degradation (C). femur-tibial joints of AIP rats treated during 14 days with AN at 250-mg/kg doses (D), synovial membrane (E) and joint degradation (F). Synovial membrane (SM); articular cartilage (AC); subintimate layer (CSI). The slides were stained using hematoxylin and eosin (H.E) technique and images were captured at 20X and 40X magnification.
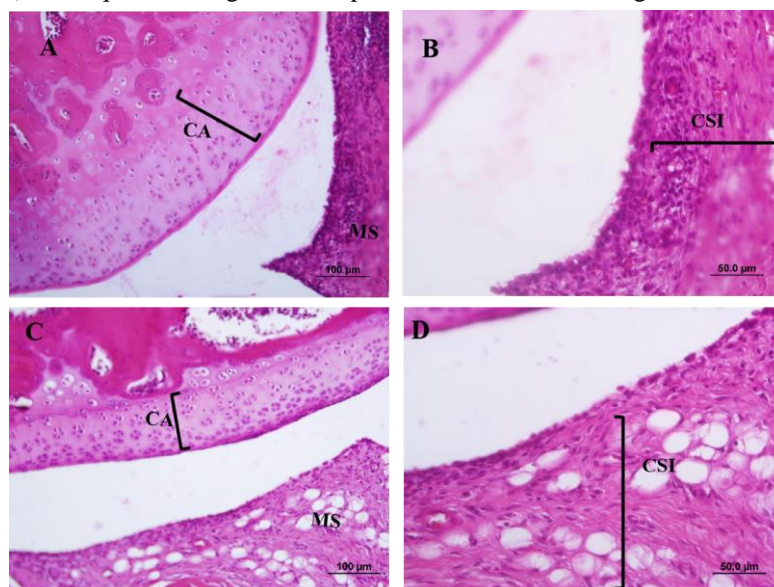

Source: Authors.

In monotherapy, AN did not show significant improvement in the joints of animals with infectious arthritis caused by *P. brasiliensis*.

Treatment with IT at 12.5 and 50-mg/kg doses showed a reduction in inflammatory infiltrate, with a slight change in subintima layer with a reduction in adipocytes and vascularization (Figure 10).

Figure 10 - Photomicrographs of femur-tibial joints of AIP rats treated during 14 days with IT at 12.5 mg/kg dose (A) and synovial membrane (B). Femur-tibial joints of AIP rats treated during 14 days with IT at 50-mg/kg dose (C) and synovial membrane (D). Synovial membrane (SM); articular cartilage (AC); subintimal layer (CSI). The slides were stained using hematoxylin and eosin (H.E) technique and images were captured at 20X and 40X magnification.
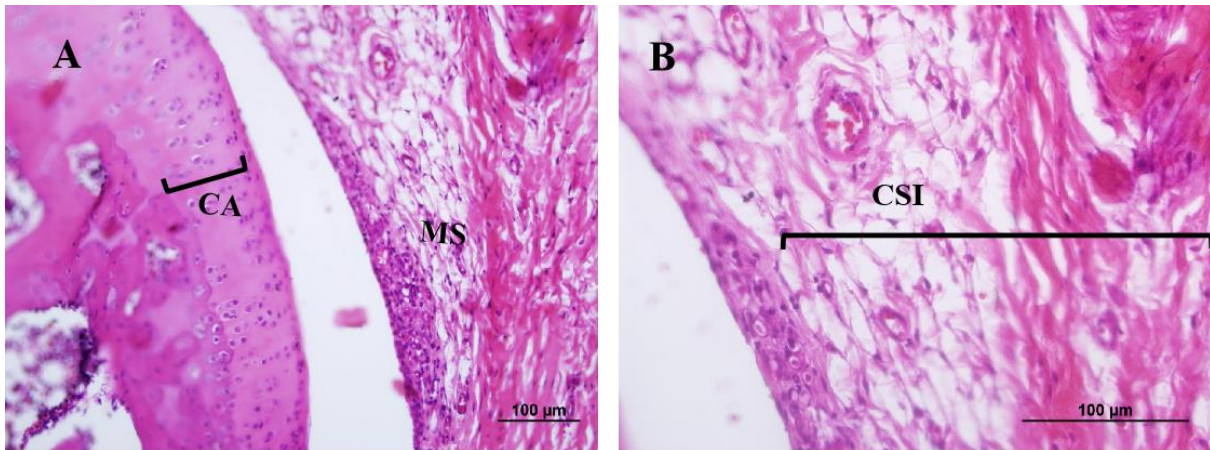

Source: Authors.

Monotherapy with IT, a reference drug for the treatment of infectious arthritis, controlled the inflammatory process and reduced signs of joint damage.

The animals' join cartilage from the group treated with AN + IT association did not show a modified morphology (Figure 11). However, the joint cavity showed an inflammatory infiltrate with a slight change in subintimal layer, with a reduction in both adipocytes and vascularization.

Figure 11 - Photomicrographs of femur-tibial joints of AIP rats treated during 14 days with AN+IT association at 62.5+12.5-mg/kg dose (A) and synovial membrane (B) Synovial membrane (MS); articular cartilage (AC); subintimal layer (CSI). The slides were stained using the hematoxylin and eosin (H.E) technique and images were captured at 20X and 40X magnification.
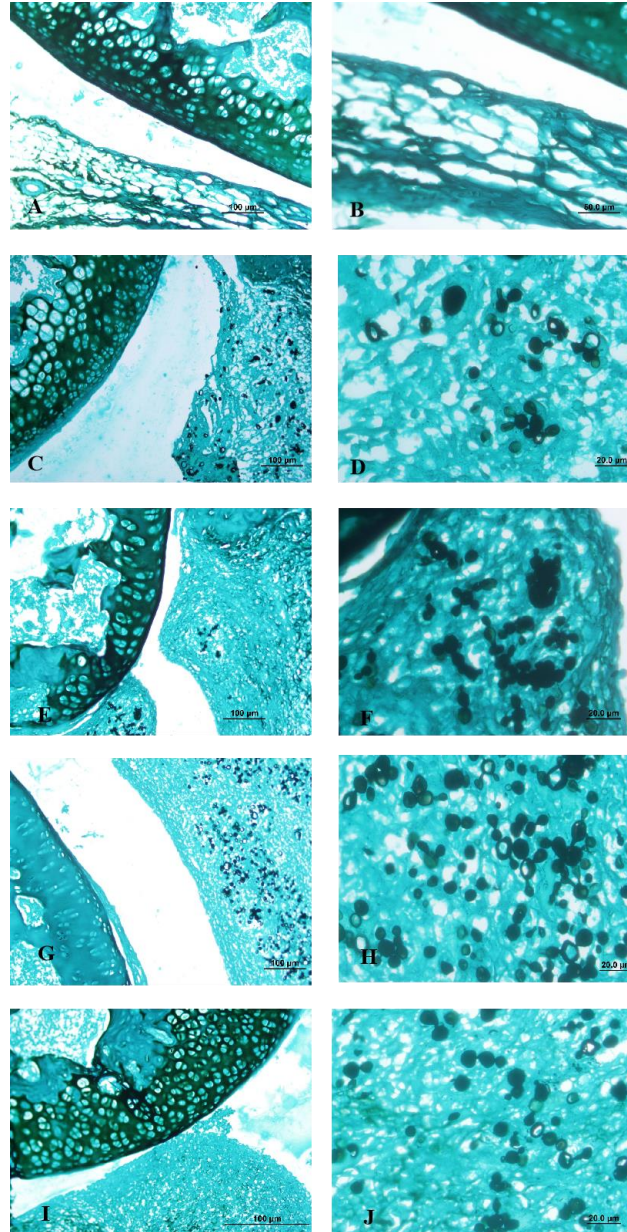

Source: Authors.

Treatment with lower doses of IT+AN associated histologically showed a minor inflammatory process in the joint cavity and absence of cartilage damage.

The presence of *P. brasiliensis* in the synovial membrane of the femur-tibial joint of the infected animals, in the 3rd, 7th, 14th and 21st days after arthritis induction, was confirmed by the Grocott staining technique (Figure 12), in order to visualize the budding fungal cells stained brown. In the treatment groups (Figure 13), it was also possible to observe the budding yeast form of fungus in the synovial membrane of joints of those treated animals. According to a qualitative analysis, it was observed a reduction of fungal cells in treatments with IT and with AN+IT association.

16

Figure 12 - Photomicrographs of femur-tibial joints of normal rats (A), AIP 3rd day (C), AIP 7th day (E), AIP 14th day (G), AIP 21st day (I) and synovial membrane of normal rats (B), AIP 3rd day (D), AIP 7th day (F), AIP 14th day (H), AIP 21st day (J), showing the presence of fungus *P. brasiliensis* in budding. Slides were stained using the Grocott technique and images were captured at 20X and 40X magnification.

Source: Authors.

The experimental model of arthritis induced by *P. brasiliensis* was evidenced histologically by the presence of the fungus in the animals' joints during the 21 days of the inflammatory process.

Figure 13 - Photomicrographs of femur-tibial joints of AIP rats treated during 14 days with AN 62.5 mg/kg (A), AN 250 mg/kg (C), IT 12.5 mg/kg (E), IT 50 mg /kg (G), AN 62.5 mg/kg + IT 50 mg/kg (I) association and synovial membrane of AIP rats treated during 14 days with AN 62.5 mg/kg (B), AN 250 mg/kg (D), IT 12.5 mg/kg (F), IT 50 mg/kg (H), association AN 62.5 mg/kg + IT 50 mg/kg (J), showing the presence of fungus *P. brasiliensis* in budding. The images were captured at 20X and 40X magnification and stained by Grocott.
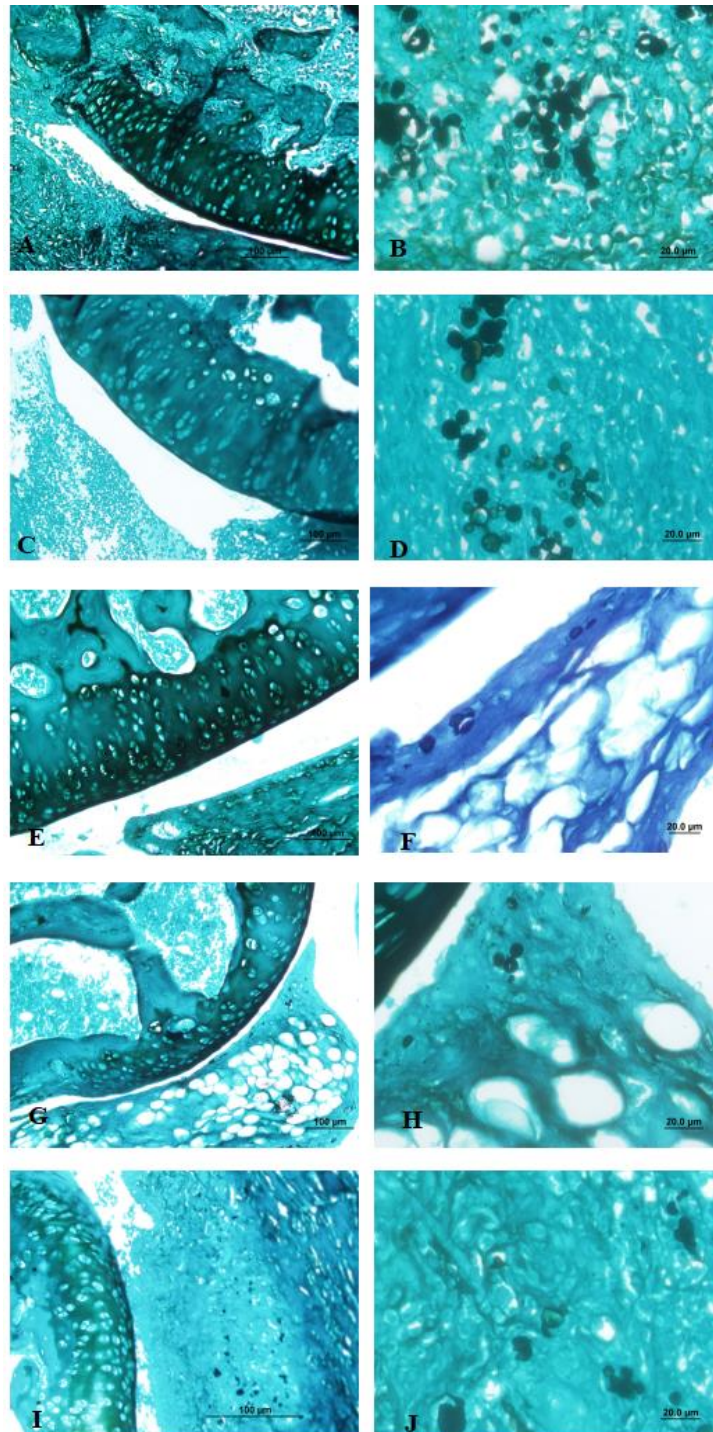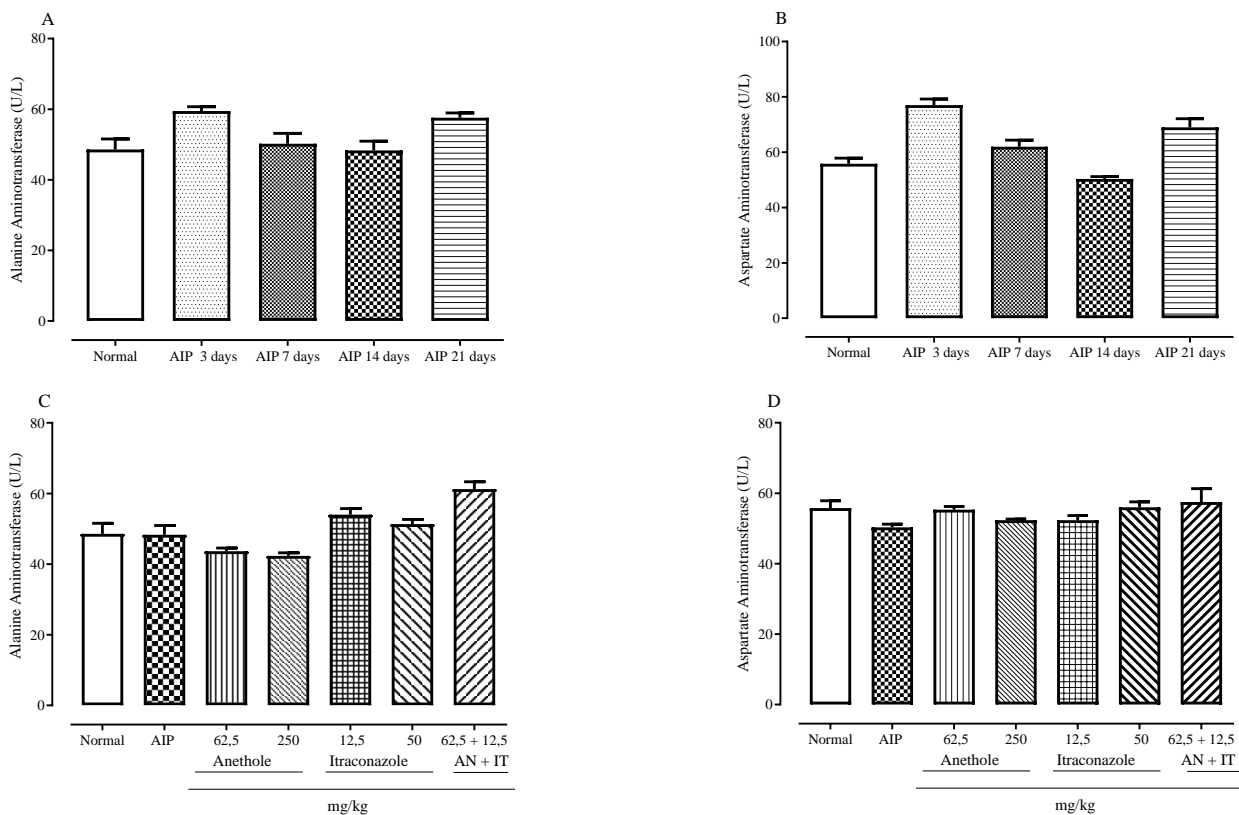

Source: Authors.

All monotherapy or combined doses showed a qualitative reduction in the fungal load, but were unable to completely eliminate the infectious agent within 14 days of treatment.

3.8 Effect of treatments on plasma transaminase activity

The activities of aspartate aminotransferase (AST) and alanine aminotransferase (ALT) in rats' plasma from AIP group, on the 3rd, 7th, 14th and 21st day after disease induction, were not modified when compared to normal animals (Figures 14A and 14B). Treatments with AN, IT and AN+IT association at different tested doses did not change ALT and AST activities on the 14th day when compared to normal animals and AIP (Figures 14C and 14D).

Figure 14 - Activity of alanine aminotransferase (A) and aspartate aminotransferase (B) in rats' plasma on days 3, 7, 14 and 21 after AIP induction. Effect of treatments with AN 62.5, 125 or 250 mg/kg, IT 12.5, 25 or 50 mg/kg and AN+IT (62.5+12.5 mg/kg), orally given, daily, in a single dose on alanine aminotransferase (C) and aspartate aminotransferase (D) activity on the 14th day after AIP induction. Treatment started on the same day as the arthritis induction. Each value represents the mean ± SEM of 10 animals per group. One-way ANOVA followed by Tukey's test.

Source: Authors.

The analysis of liver transaminases helps to ensure the safety of long-term treatments for chronic infections. However, none of the doses administered to the study animals showed changes during the 14 days of treatment.

4. Discussion

The experimental model of infectious arthritis induced by *P. brasiliensis* is characterized by the development of an intense infectious response, plus an inflammatory response, represented by the formation of joint edema and an increase in leukocytes number, recruited at the lesion area, corroborating with Loth et al. (2012) research. This response reached the highest peak on the 14th day after induction, and it remained stable on the 21st day.

In this scenario, a high concentration of plasma nitric oxide (NO), a pro-inflammatory cytokine (TNF) in the joint exudate and a decrease in the animal's mobility were observed. Morphological analyses using the HE staining technique showed changes in the synovial membrane and degeneration of the joint cartilage. While, when the Grocott technique was applied, the presence of *P. brasiliensis* was evident in the synovial membrane of knee joint of infected animals. All these parameters were significantly modified on the 14th day after infection induction.

P. brasiliensis has put forward an edema formation at the infection area by progressively increasing vascular permeability until the 15th day of induction, and there was some stabilization of the joint diameter after this period (Loth et al. 2012). Loth et al. (2014) deployed the model of infectious arthritis induced by *P. brasiliensis* in rats and verified, by radiological analysis, an increase in joint destruction. It started on the 7th day after the infection induction and persisted until the 30th day. After this period, it was observed some infection chronicity. Rocha et al. (2007) identified similar manifestations, such as edema formation, in the knee of patients infected with *P. brasiliensis*, and this suggests that there is a development of infectious arthritis.

In this study, it was shown that the presence of this fungus in animals' joint has caused an intense inflammatory process in the area, with the participation of inflammatory mediators such as NO and TNF, and an increase in the number of leukocytes recruited in the joint, predominantly polymorphonuclear leukocytes (neutrophils - 70%). The presence of neutrophils is important, since these cells constitute the first line of defense of the innate response of adaptive immunity against the pathogen (Nathan, 2006; Mócsai, 2013). These findings have corroborated with previous studies by Gonzáles et al. (2001) and Gonzáles et al. (2003), who showed the role of neutrophils to fight infection cause by *P. brasiliensis*, especially in the first hours of the infectious process (24-96 hours), that is, in the acute phase.

In this context, some studies show that the cellular infiltrate helps to eliminate this fungus, mainly by exercising a defense mechanism such as phagocytosis (Behnsen et al. 2007), release of chemical substances such as proteases and peroxidases (Gunzer, 2014; Nauseef, 2007) and production of mediators such as cytokines, chemokines and growth factors (IFN-γ, TNF, IL-1, IL-17, G-CSF and GM-CSF) (Tsuda et al. 2004; Sergejeva et al. 2009; Kumar et al. 2010), which play an important role to fight and destroy pathogens (Gunzer, 2014; Köhler et al. 2011).

It was also observed that the infectious process evolved with a simultaneous increase in the number of neutrophils and NO concentration at fungus inoculation area, in relation to time. And this has revealed the importance of cell and NO recruitment to control the microorganism dissemination. Pino-Tamayo et al. (2016) reported the importance of neutrophils regarding infection prognosis. The authors also recorded a significant increase in fungal burden and mortality in animals depleted of neutrophils. Studies that were carried out by Bernardino et al. (2013) and Parente et al. (2015) showed that the inhibition of iNOS decreases NO production and consequently the proliferation of fungal cells of *P. brasiliensis*.

As it has been already reported above, our results showed an increase in the number of neutrophils and in NO concentration regarding the joint of arthritic animals, on the 14th day after fungus inoculation. These evidences may indicate the modulation of an inflammatory response that this fungus causes during an infectious process, as already demonstrated by Nishikaku et al. (2009) and Bernardino et al. (2013). These authors highlighted that the high level of NO reduces fungal dissemination, by formation of granulomas, and that the same infection in knockout mice (for NO production) causes fungal dissemination by reducing the formation of granulomas.

An interesting finding was the increase in tissue TNF concentration already found out at the beginning of infection (3rd day), whose peak was on the 14th day. *In vitro* studies show that Pb strains are able of increasing TNF-α production in human monocytes (Romagnolo et al. 2018), and that increasing the concentration of this mediator can increase the phagocytic and microbicidal ability of phagocytic cells (Ricci-Azevedo et al. 2018).

Treatment with IT at all tested doses, with AN at a high dose and with AN+IT association at low doses reduced TNF concentration. Studies using a pulmonary PMC model in rats have shown that IT reduces the production of inflammatory cytokines such as TNF and consequently fungal burden (Naranjo et al. 1990; Puerta-Arias et al. 2016). Thus, it can be suggested that the inhibitory activity caused by treatments on infectious arthritis may be, at least partially, related to a reduction in TNF concentration, thus, it controls the infection.

The gp43 glycoprotein that is present in cell wall of fungus *P. brasiliensis* is a virulence factor that promotes an immunomodulatory effect with TNF production (Torres et al. 2013). Thus, the anti-Pb antibody can be used for the diagnosis and prognosis of acute and chronic PMC.

There was an increase in anti-Pb production with the infectious arthritis induction by *P. brasiliensis*, and simultaneously with TNF concentration until the 14th day post-infection. Treatment with IT and AN+IT association decreased anti-Pb levels, thus, it has shown the favorable prognosis of the treatment. On the other hand, treatment with AN in monotherapy, even at a high dose, showed no effect on anti-Pb production. These data suggest that the reduction in anti-Pb levels is related to IT antifungal activity and AN modulator, that is, the lower the fungal load, the lower NO and TNF concentrations as well as anti-Pb levels. Therefore, the treatment is more favorable to the cure prognosis. It is also important to emphasize that the recruitment of leukocytes at the infection area is the body defense in an attempt to eliminate the microorganism, however, when they are very high, leukocytes can cause tissue damage by releasing lysosomal enzymes, lipoproteins and reactive oxygen species and nitrogen (Eyles et al. 2006). Thus, the use of compounds that control migration and recruitment of leukocytes to the lesion area do not modulate negatively the immune response of the infected organism, but it can contribute to reduce tissue damage.

Previous studies have shown that AN inhibits the production/release of several cytokines involved in cell recruitment and migration to lesion area, such as tumor necrosis factor alpha (TNF-α), interleukin 1 beta (IL-1β) and interleukin 17 (IL-17) (Ritter et al. 2013; Chainy et al. 2000; Ponte et al. 2012). AN treatment has also been shown to decrease the expression of certain intercellular adhesion molecules, including intercellular adhesion molecule 1 (ICAM-1) (Sung et al. 2013) and it increased interleukin concentration 10 (IL-10) (Kim et al. 2013). Therefore, we believe that the combination of these biological activities may be, in some way, responsible for the AN modulator effect suggested in this research.

According to the II Brazilian Consensus on Paracoccidioidomycosis (Shikanai-Yasuda et al. 2018), the first drug to be chosen to treat PMC is the antifungal IT. This drug is a triazole derivative that has a fungistatic action. It acts out by competition in the ergosterol biosynthesis pathway in the plasmatic membrane of the fungus (Ponte et al. 2012). However, IT treatment is only effective after prolonged use, and therefore may exhibit a potential for hepatotoxicity that may be worsened in patients with hepatic impairment (Sung et al. 2013).

The standardization of the AIP experimental model allowed the investigation of the pharmacological efficacy of compounds both in monotherapy (IT and AN) and in combination (AN+IT). As observed, the treatment with IT was quite effective at all tested doses (12.5, 25 or 50 mg/kg), since it reduced all evaluated parameters, consequently, caused a satisfactory antifungal effect and improved the other modified parameters. However, it is important to mention that the fact that we did not find changes in transaminase activity (ALT and AST) in animals treated with IT may be due to the relatively short treatment period (14 days).

The AN, at higher doses (125 and 250 mg/kg), promoted a reduction in edema and in the number of recruited leukocytes. The concentrations of both nitric oxide and TNF were only reduced with the highest dose of AN (250 mg/kg). These data suggest that AN, in monotherapy, has an effect only on the inflammatory and nociceptive response, which resulted from an infectious process, since it reduced the inflammatory mediators involved in such response.

Some studies, using other experimental models, have shown the inhibitory activity of AN on inflammatory and nociceptive response. So, there is a reduction on the production/release of inflammatory mediators such as TNF, IL-1β, prostaglandin and NO (Domiciano et al. 2013; Ritter et al. 2013). Other studies show a fungicidal action of the essential oil of *Illicium verum* (star anise), which has more than 90% of its composition of AN (Sung et al. 2013; Kim et al. 2013; Visbal et al. 2005; Bellmann & Smuszkiewicz, 2017). The antifungal activity of AN, as an isolated compound, was demonstrated against *Candida spp., Microsporum canis, Mucor mucedo* and *Aspergillus fumigatus*. Such activity seems to be due to the AN action on fungal cell wall metabolism, as it changes the biosynthesis of chitin and chitosan and fosters greater fragility of the cell wall, and/or an action that depends on oxidative stress that causes cell death, similar to apoptosis (Shukla & Tripathi, 1987; Yutani et al. 2011; Fujita et al. 2014). However, according to our knowledge, there is no study in literature regarding AN action on infectious arthritis, induced by *P. brasiliensis*.

The treatment with AN+IT association showed similar efficacy to treatment with IT, but at lower doses (62.5 + 12.5 mg/kg). Fujita et al. (2017) and Ueda et al. (2021) reported a synergism of AN+dodecanol association and AN+naglinactone E, respectively, changing the expression of efflux pumps in yeast, and potentiating antifungals effects. As it was already mentioned above, the Grocott staining technique showed fungus presence in the joint of an infected animal, and with the histological analysis using the HE staining technique, it was observed that this fungus causes an inflammatory response with intense cell recruitment and change in synovial membrane and cartilage degradation, as demonstrated by Loth et al. (2014).

Treatment with IT and AN+IT association showed an inhibitory activity on infectious arthritis induced by *P. brasiliensis*, which preserved the synovial membrane morphology and prevented cartilage degradation. Cartilage preservation can be explained by the improved mobility of the animals. Kunz et al. (2014) demonstrated that the animal's mobility improvement can cause a reorganization of the joint and synovial membrane when compared to immobilized animals, which presented degeneration of joint cavity and changes in synovial membrane.

According to all these observations, the effect of AN + IT association can be explained by a complementary action of AN and IT on some mediators involved in the response, such as the concentrations of NO and TNF, as evidenced in this study. IT caused fungal cell death and improved infectious process, while, AN, by modulating activity, reduced inflammation and nociception. However, we cannot rule out the possibility of a more satisfactory antifungal action of AN+IT association on fungal cells.

This study showed important and unpublished data, although there is some restriction because it was not possible to determine the concentrations of other cytokines, such as anti-inflammatory cytokines (for example - IL-10) and interferon gamma (INF-γ), which could explain in a safer way the mechanism of action of AN+IT association in this experimental model.

5. Conclusion

Infectious arthritis, induced by inoculation of fungus *P. brasilensis* in animal's knee joint, came up as a very intense local infectious response, followed by an inflammatory response, with severe clinical manifestations on the 14th day after induction. Treatments with IT in monotherapy, at doses of 12.5, 25, 50 mg/kg, and with NA + IT association at much lower doses (62.5+12.5 mg/kg), showed a similar inhibitory effect on the development of arthritis. The AN in monotherapy, even at high dose (250 mg/kg), did not show the same pharmacological efficacy when compared to IT and AN + IT association, in this experimental design.

Thus, based on all these observations, it can be reinforced that the effect of AN + IT association can be explained by a supplemental action of AN and IT on some mediators involved in the response, such as NO and TNF levels, as evidenced in this study. IT causes fungal cell death and improves the infectious process, while AN reduces inflammation, and nociception

favorably modulates the response. These actions in combination may be responsible for joint edema reduction and may improve animals' mobility. This may have contributed to the preservation and cellular organization of the animals' cartilage and synovial membrane.

Based on these results, this research leads to a more accurate investigation of the mechanism of pharmacological action of the IT+AN association, such as inflammatory cytokines (IL-10 and INF-γ) and the possible side effects in long-term treatment. As well as the search for the association of other compounds with IT in the inflammatory process caused by *P. brasiliensis*, discovering treatment options with more efficacy and safety.

Acknowledgments

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