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RESEARCH ARTICLE

Study of anti-inflammatory and analgesic properties of 3-benzoyl-propionic acid

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Summary Inflammation is an attempt by the body to remove noxious stimuli and initiate thus a cascade of responses in order to promote healing. There are a variety of inflammatory mechanisms involved in infections, chronic diseases and other tissue damage. Understanding these mechanisms and the search for new anti-inflammatory drugs with greater specificity and fewer side effects, underlying the development and improvement of new protocols and standardization of experimental inflammatory models to understand better these issues. The aim of this study was to evaluate the anti-inflammatory and analgesic activity of 3-benzoyl-propionic acid (3BPA) and its potential toxicological effect. To test the 3BPA as new anti-inflammatory and analgesic drug, the use carrageenan air pouch model 1% by *in vitro* model of cell culture to test genocytotoxicity. In the *in vitro* model the 3BPA presented low level of genotoxic and low cytotoxicity risk, shown by comet assay and no damage to the plasma membrane by hemolytic test erythrocytes. In the study of anti-inflammatory activity *in vivo* by the air pouch method were conducted nitrite dose trials, PGE_2 levels and cell migration. To verify analgesic effects of 3BPA drug *in vivo* tests of abdominal contortions induced by acetic acid and formalin were performed. Regard to the anti-inflammatory activity, 3BPA showed intense activity shown in marked reduction of cell migration and levels of NO, with large populations of neutrophils and reduction of PGE_2 values at a dose of 0.5mg/kg. In studies of antinociceptive activity, 3BPA reduced the number of writhes and the time lick the neurogenic and inflammatory phases of the formalin test. The results of this study also advanced substantially with respect to anti-inflammatory and analgesic properties of 3BPA by providing evidence of their likely mechanism of action, through the evaluation of antinociceptive activity, as well as the anti-inflammatory activity *in vitro* and *in vivo*, where the 3BPA showed no genotoxic effect.

Keywords: inflammation; analgesia; nonsteroidal anti-inflammatories (NSAIDs); 3-benzoyl-propionic acid.

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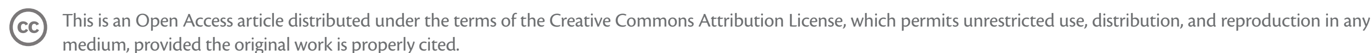

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Introduction

Clinically, inflammation is characterized by presenting five cardinal signs: redness, swelling, heat, pain and loss of function[1]. However, this process can be defined as an inflammatory cascade, in which occurs the activation of mediators and cells that aims the repair of injured tissue[2].

The use of chemicals substances to soften and control the various mechanisms involved in inflammatory infections, chronic diseases and other tissue injuries is a need of humanity. These mechanisms and the search for new anti-inflammatory and analgesic drugs with greater specificity and less side effects justify the development of new protocols and standardization of experimental inflammatory models to better understand these issues[3].

The pain is constantly fought by taking nonsteroidal anti-inflammatory drugs (NSAIDs) as the more prescribed medication for this purpose. The action of these drugs is twofold: first, to interfere with the prostaglandin system. Secondly, most of these drugs reduces inflammation, swelling and irritation that often come along with a wound and increases the pain due to high pressure on the local[4]. Given this, it is possible to identify their broad applicability in the treatment of pain, fever and inflammation[5].

The NSAIDs, due to differences in chemical structures are subdivided into groups; among them are those derivatives of 2-aryl-propionic acid, which constitute an important group of NSAIDs[6], making part of it the 3-benzoyl-propionic acid (3BPA), purpose of this research study on the development of anti-inflammatory and analgesic safer and more reliable drugs.

Methods

Study of anti-inflammatory activity in vitro

Materials

The reagents used were Penicillin-Streptomycin (P4333), Fetal Bovine Serum (F2442), Benzoyl-propionic Acid 3 (B13802), Lipopolysaccharide of Escherichia coli 0111: B4 (LPS, L4391), PMA (P1585), Phosphoric Acid (V000145), Sulfanilamide (S33626), N-(1-naphthyl) Dihydrochloride of Dtilenodiamina® (E222488), Blue of Thiazolyl Tetrazolium (MTT, m2128), Sodium Resazurin Salt (R7017), Agarose (A9539), NaCL (S7653), Ethylenediaminetetraacetic Acid (EDTA, E9884), Tris (T1503), Triton X-100 (X100), Dimethyl Sulfoxide (DMSO, d2650), N-lauroylsarcosinate (L9150), NaOH (S8045), Doxorubicin Hydrochloride (D1515), Ethidium Bromide (E7637) and MRC-5 PD 19 (05,072,101) all purchased from Sigma Chemical Co. (St. Louis, MO, USA).

Animals

This study was conducted in accordance with the guidelines of the Research Ethics Committee with Experimental Animals (CEPAE) of the Federal University of Para in the protocol of number 228-14 UFPA-CEPAE and 111-13 UFPA-CEPAE. They made every effort to minimize the number of animals used as well as their suffering. *Mus musculus* swiss male mice (40-45g) were purchased from the Animal Center of the Federal University of Pará (Belém, Pará, Brazil) and placed in experimental animal unit of Neuroinflammation Laboratory. The animals were housed under controlled temperature (22 ± 1°C) in cycle 12h day/night and water *ad libitum*.

Cell culture of the J774 lineage

This macrophages cell lineage from mice were cultured in DMEM supplemented with 10% fetal bovine serum, 1% penicillin and streptomycin, kept in an incubator at 37°C with 5% CO_2. Subcultures were performed when the culture reached 80% of confluence. For treatment, the cells were plated at a concentration of $0,6x10^6$ cells/ml in 24 wells plates.

Cell culture of peritoneal macrophages

Peritoneal macrophages were obtained from *Swiss* mice. The animals were sacrificed by cervical dislocation for the collection of cells, made by peritoneal lavage with sterile DMEM culture without serum, with a sterile syringe and needle. The cleaned material was centrifuged at 1500rpm for 5 minutes at room

temperature. The cells were re-suspended in serum-free culture in Neubauer Counting Chamber and the number of cells was adjusted to $2x10^6$ cells/ml. The macrophages were plated in 24 wells plate in medium DMEM and maintained in oven at 37°C with 5% CO_2 and 95% of air, during two hours for adhesion.

Peritoneal macrophage celular treatment

After 2h incubation for cell adhesion, the culture was washed with PBS and the adherent macrophages were treated with different concentrations of 3BPA. The macrophages were exposed for 1 hour to 3BPA, washed and incubated with LPS 1µg/ml for 24 or 48 hours in CO_2 incubator.

Analysis of cell viability by the MTT method

For analysis of cell viability, the method of Mosmann[7] was followed, which consists of the cleavage of the tetrazolium salt (MTT), performed only by mitochondria of viable cells, having as a product the formazan compound. The amount thereof is measured by colorimetric analysis being proportional to the number of viable cells. After treatment with 3BPA at concentrations of 1, 10, 25, 50 and 100µM for 24 and 48 hours, the cells had been incubated in 0.5mg/ml MTT diluted in medium culture for 2 hours. After the incubation period, the medium was removed and the deposited salt was solubilized in 500µl DMSO. The reading was performed in 96-wells plates in the Enzyme-linked immunosorbent assay reader (ELISA) at a wavelength of 570nm.

Dosage nitrite

The concentration of NO was indirectly determined from the measuring nitrite concentration in the cell supernatant by the Griess reaction[8].The macrophages were treated as described above, and the supernatant collected and centrifuged at 3500rpm for 5 minutes at room temperature. 100µl of sample was added to 100µl of Griess reagent (0.1% nafitil and 1% sulfanilamide in 5% phosphoric acid) in a 96 wells plate. Absorbance values were measured at OD 570nm and compared to a standard curve (sodium nitrite solution), to determine the concentration of NO_2^- and NO_3^-.

DNA damage assessment by the comet assay

To perform the comet, slides were previously prepared covered with normal melting point of the 1.5% agarose solution. This layer was used to promote adhesion of the second layer of agarose of low melting point. MRC-5 PD 19 cells were seeded in 6 well plates at a density of $2.5x10^5$ cells/well and maintained for 24 hours in the oven with atmosphere of 5% CO_2 at 37°C. After these 24 hours, the treatment was performed with 3BPA concentrations 31µM, 62µM and 124µM for 3 hours.

Then, the slides were arranged horizontally in electrophoresis chamber and filled with electrophoresis buffer (1mM EDTA, 300mM NaOH; pH ≥ 13).The slides were kept at rest for 20 minutes before starting the electrophoresis in order to allow DNA deployment, relaxing their connections and the exposure of alkali labile sites. After this process, electrophoresis was conducted in low light for 20 minutes, 34V and a current of 300mA.

The analysis was performed according to standard scores predetermined by the size and intensity of comet tail[9]. Comets 100/blade were counted and classified according to the percentage of DNA in the comet tail, to indicate the degree of break DNA. The doxorubicin 1µM was used as positive control of cytotoxicity and corresponds to the negative control untreated. After counting cells and assigning scores of the two blades of each sample, was established damage index (DI) made by summing the score of the product as a corresponding number of damage at each level, and therefore, obtained a final average for each sample.

Damage assessment to the plasma membrane - erythrocytes hemolytic test

This method allows us to evaluate the potential of the substance test to cause damage to the cell membrane, either by formation of pores or the total rupture or the presence of hemoglobin in solution[10].

When the sample tested cause hemolysis into 1h incubation, the cytotoxic mechanism of action is considered nonspecific to cause direct damage to the membrane. In this test the cytotoxic activity of 3BPA at a concentration of 250µg/ml was evaluated. For hemolysis test *Mus musculus Swiss* mice venous blood

was used, which was taken by cardiac puncture and placed in saline, and centrifuged at 1500rpm for 5 minutes to prepare erythrocyte 2% solution. This solution was used 100 µL in 96 wells plates and more 100 µL of each sample (concentration of 250µg/mL), Triton X-100 (positive control) and DMSO (negative control) and 100µL of saline (white) in triplicate. The plate was incubated at room temperature for 1 hour and centrifuged at 1500rpm for 5 minutes. The reading of the supernatant was performed in plate reader at 541nm.

Study of the anti-inflammatory activity in vivo

Materials

The reagents used were K-carrageenan (C22048), Celecoxib (PZ 0008), Acetylsalicylic Acid (A5376), 3-Benzoyl-Propionic Acid (B13802), Phosphoric Acid (V000145) Sulfanilamide (S33626), N- (1- naphthyl) DihydrochlorideEtilenodiamina® (E222488), Ethylenediaminetetraacetic Acid (EDTA, E9884), all purchased from Sigma Chemical Co. (St. Louis, MO, USA).

Animals

Wistar male rats (120-150g) and *Mus musculus swiss* male mice (40-45g) were purchased from the Animal Center of the Federal University of Pará (Belém, Pará, Brazil) and placed in experimental animal unit of Neuroinflammation Laboratory. The animals were housed under controlled temperature (22 ± 1°C) in cycle 12h day/night and water *ad libitum.*

Air pouch preparation and induction of the inflammatory process

The air pouch were carried out as described by Bastos et al.[11]. The sterile air was obtained by capturing in a laminar flow hood to prevent contamination. The pouch was inflated with 20ml of air injected into the intra-scapular area, and then re-inflated with 10ml of air for 2 days. After the preparation of air pouch, inflammation was induced by different concentrations of carrageenan and different pattern anti-inflammatory drugs.

Sample collection

Samples were obtained 16 h after administration of the phlogistic agent. A small incision was made in the wall of the pouch and its content were carefully removed with a sterile Pasteur pipette. To increase the total volume of exudate and thus improving the accuracy of the measurement, 1mL of PBS (137mM NaCl, 10mM Phosphate, 2.7 mM KCI, and a pH of 7.4) was previously injected into the air pouch. Thereafter, the animals were euthanized where were evaluated for the microvasculature of nitrergic activity, cell migration, leukocyte cell counts and levels of prostaglandins (PGE_2).

Nitrergic activity evaluation

The samples were initially diluted 1:20 in PBS and then 500µL of each diluted sample were mixed with an equal volume of Griess reagent (0.1% nafitileno and 1% sulfanilamide in 5% phosphoric acid)[8]. Absorbance values were measured at OD 570nm and compared to a standard curve (sodium nitrite solution), to determine the concentration of NO_2^- and NO_3^-.

Evaluation of the inflammatory exudate cells

The inflammatory exudate was obtained by MayGrunwald-Giemsa method for differential cell counts. The difference was determined by counting cells under a microscope of 400 cells per field, identified by standard morphological criteria, the morphology of the nuclei and cytoplasmic granulation. The cell count was expressed as a percentage of total cells and absolute values (10^6/ml).

Evaluation of PGE_2 levels

Exudate samples were diluted 1:10 in PBS. The level of PGE_2 was quantitated with ELISA kit specific for PGE_2 (RPN222, GE Healthcare UK Limited Amersham Place, Little Chalfont, Buckinghamshire, UK).

The absorbance values were measured at OD 570nm and the performance evaluation of the test method for immuno-adsorbed enzyme was used according to the manufacturer's instruction manual protocol.

Study of antinociceptive activity

Materials

The reagents used were: 3-benzoyl-propionic Acid (B13802), Acetic Acid (1005706), Indomethacin (I7378), Morphine Solution (M-0050) and Formalin Solution (HT501128) all purchased from Sigma Chemical Co. (St. Louis, MO, USA).

Animals

Mus musculus swiss male mice (40-45g) were purchased from the Animal Center of the Federal University of Pará (Belém, Pará, Brazil) and placed in experimental animal unit of Neuroinflammation Laboratory. The animals were housed under controlled temperature (22 ± 1°C) in cycle 12h day/night and water *ad libitum*.

Abdominal writhing test induced by acetic acid

Animals (n = 4) were treated with 3BPA in doses of 0.003 and 0.03 mg/kg (v.o). A group of animals received saline 0.9% (v.o., control group). The standard drug indomethacin (5 mg/kg, v.o.) was used as a positive control. Sixty minutes after each treatment was administered acetic acid (0.6%; 0.1ml solution/10g animal; i.p.). The animal's reaction to this stimulus was a repeated development contraction movements of the abdominal wall, body rotation and extension of the hind paws. This set of reactions is called abdominal writhing and the intensity of nociception is quantified as the total number of writhes during the 20 minute observation period starting at 10 minutes after administration of acetic acid. The animals during the period of observation were contained in a glass funnel with a diameter of about 22 centimeters[12].

Formalin test

Animals (n = 4) were treated with 3BPA in doses of 0.003 and 0.03mg/kg (v.o.). Sixty minutes after each treatment, mice (n = 5) received an intraplantar injection of 1% formalin (20µL/animal). After administration of formalin, the time (s) in which the animal spent licking the left hind paw was timed in two phases (the first phase was 0-5 minutes, the second, 20-30 minutes) being considered zero moment the point immediately after the administration of formalin. The control group received vehicle only (saline 0.9%, v.o.). The standard drug morphine (4mg/kg, i.p.), 30 min before Formalin, and Indomethacin (10mg/kg, v.o.) were used as positive controls[13].

Statistical analysis

All experimental data were presented in averages ± DP, being the study of the experimental type, where it was used descriptive statistics to characterize the sample, analysis of variance (ANOVA) of a criterion for the inter-group analysis, followed by multiple comparisons by the Tukey's test. The statistical analysis, as well as putting the results into spreadsheets and charting, were made by the GraphPad Prism, version 5.0 (GraphPad Software, San Diego California, USA). The value of $P < 0.05$ was considered statistically significant.

Results

Study of anti-inflammatory activity in vitro

Analysis of cell viability by the MTT method

To ensure that possible changes in oxidative profile are not due to cell killing, the cytotoxicity was tested in two types of macrophages, a lineage of J774-A1 cells and the peritoneal macrophages of mice, either treated with 3BPA at 24 and 48 hours, by the method described by Mosmann[7].

Cytotoxicity in J774-A1lineage

The toxicity of 3BPA was analyzed in lineage-A1 J774 cells, in order to verify whether the substance promoted cytotoxic effect when treated for 24 and 48 hours. It was observed that none of the concentrations used (1, 10, 25, 50 and 100µM) showed cytotoxic effect (Figure 1).Control (100.0% ± 13.3) 3BPA 1µM (102.1% ± 9.5), 10µM (110.1% ± 15.8), 25µM (107.6% ± 18.4), 50µM (106.8% ± 11.2) and 100µM (112.1% ± 14.1) in 24 hours (a). Control (100.0% ± 3.8), 3BPA 1µM (112.5% ± 2.0), 10µM (107.4% ± 11.9), 25µM (96.2% ± 11.1), 50µM (106.1% ± 5.6) and 100µM (104.8% ± 2.7) in 48 hours (b).

Cytotoxicity in peritoneal macrophages

The toxicity of 3BPA was analyzed in macrophages obtained from peritoneal lavage of mice. The treatment was performed over a period of 24 to 48 hours. It was found that the drug did not cause cell death in any of the concentrations used (1, 10, 25, 50 and 100µM) (Figure 2). Control (100.0% ± 6.9), 3BPA 1µM (82.7% ± 21.5), 10µM (94.0% ± 5.6), 25µM (110.2% ± 7.1), 50µM (101.6% ± 5.0) and 100µM (107.2% ± 10.1) in 24 hours (a). Control (99.5% ± 7.1), 3BPA 1µM (90.7% ± 8.0), 10µM (93.8% ± 4.9), 25µM (108.0% ± 9.4), 50µM (101.0% ± 5.0) and 100µM (105.8% ± 8.9) in 48 hours (b).

Dosage nitrite

The concentration of nitrite formed was quantified in the supernatant of peritoneal macrophages treated with 3BPA for 4 hours and stimulated with lipopolysaccharides from *Escherichia coli* 0111: B4 (LPS) (1µg/ml) for 24 hours. It was found that the group only stimulated with LPS produced an average of 3.42 times

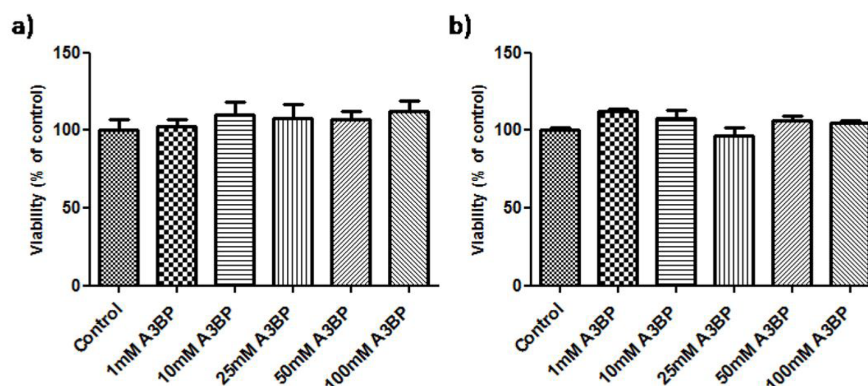

Figure 1. Viability of J774 cells under treatment with 3BPA during 24 (a) and 48 (b) hours. A $P<0.05$ compared with the control group and the group carrageenan 1% was adopted. Differences between groups were tested by analysis of variance (ANOVA) and the correction by the Tukey Multiple Comparison Test.

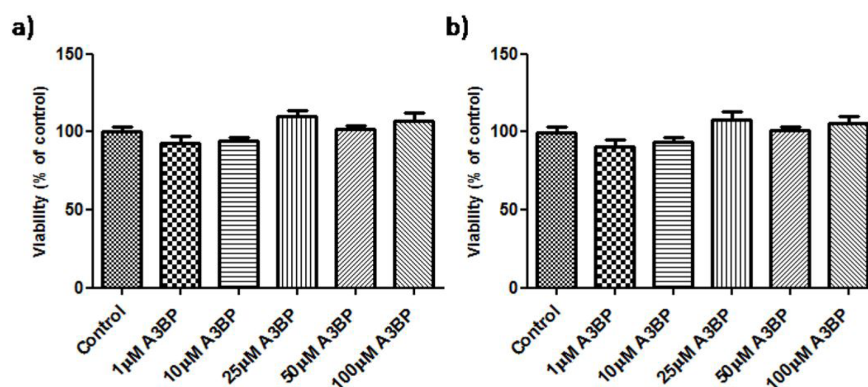

Figure 2. Peritoneal macrophage viability after treatment with 3BPA for 3h followed by incubation with LPS for 24 (a) and 48 (b) hours. A $P<0.05$ was adopted compared to the control group. Differences between groups were analyzed by analysis of variance (ANOVA) and the correction by the Tukey Multiple Comparison Test.

more than the control group (4.6µM ± 2.0 and 1.0µM ± 0.5, respectively). The 3BPA at concentration 25µM inhibited significantly the production of NO_2 49.32% in relation to the group only stimulated with LPS (2.3µM ± 1.3 and 4,6µM ± 2.0, respectively). In concentration 10µM the decrease was 45.6% compared to LPS (2.5µM ± 0.6 and 4.6µM ± 2.0, respectively) (Figure 3).

DNA damage assessment by the comet assay

To assess whether treatment with 3BPA can cause damage to DNA, we performed the alkaline comet assay version in MRC-5 cells (PD 19) treated for 72h with 3BPA at concentrations of 31, 62 and 124µM. As shown in Figure 4, the 3BPA treatment in any of the tested concentrations showed significant damage to the DNA content compared to the untreated control ($P>0.05$).Treatment with doxorubicin (Doxo 1.0µM) was performed as a positive control for DNA damage induction, which showed significant level in comparison to the negative control (*$P<0.05$).

Damage assessment to the plasma membrane - hemolytic test in erythrocytes

The 3BPA was subjected to the hemolytic test cells of mouse erythrocytes *Mus musculus swiss, in vitro,* to assess the feasibility of erythrocyte cells in the presence of the drug. Cells were incubated with 3BPA 250µg/mL for 1 hour. As shown in Figure 5, the material tested showed no hemolytic activity: control (saline 0.9%, 0.1nm ± 0.0), control + (Triton X-100, 0.3nm ± 0.0), control - (DMSO, 0.1nm ± 0.0) and 3BPA 250µg/mL (0.1nm ± 0.0).

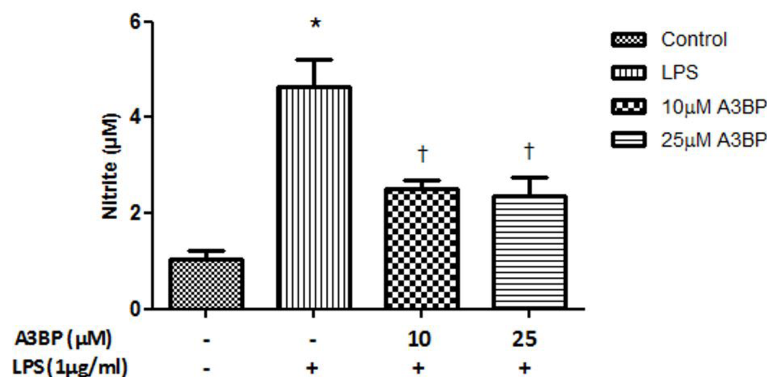

Figure 3. NO_2^- production in peritoneal macrophages treated with 3BPA and stimulated with LPS (1µg/ml). *$P<0.05$ compared with the control group and †$P<0.05$ compared with the control group. Differences between groups were analyzed by analysis of variance (ANOVA) and the correction by the Tukey Multiple Comparison Test.

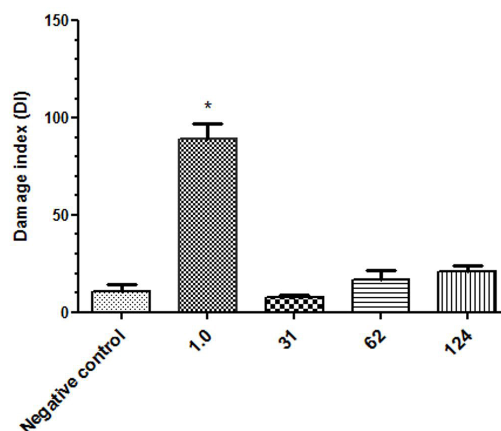

Figure 4. Damage Assessment index to the DNA of MRC-5 cells (PD19) treated with 3BPA analyzed for 72h by the Comet assay alkaline version. Treatment with 3BPA showed no significant difference compared to the negative control. Doxorubicin (Doxo 1.0µM) was used as a positive control. Differences between groups were analyzed by analysis of variance (ANOVA) and the correction by the Tukey Multiple Comparison Test with *P<0.05 compared to negative control.

Study of the anti-inflammatory activity in vivo

Via NO

In order to determine whether the via NO plays an important role in the inflammatory response induced by 1% carrageenan solution and the pre-treatment of inflammation with 3BPA at doses of 0.005, 0.05 and 0.5mg/kg., the production of NO_2^- and NO_3^- in the inflammatory exudate was measured.

As shown in Figure 6, carrageenan significantly increased (P<0.05) the concentration of NO_2^- and NO_3^- compared with the control group. All groups pre-treated with 3BPA and Ibuprofen led to a decrease in the levels of NO_2^- and NO_3^-: control (saline 0.9%) (0.87µM ± 0.87); carrageenan 1% (11.57µM ± 0.89); 3BPA 0.005mg/kg (3.64µM ± 0.34); 3BPA 0.05mg/kg (1.97µM ± 0.70); 3BPA 0.5mg/kg (1,01µM ± 0.30); Ibuprofen 10mg/kg (1.20µM ± 0.48). All treatment groups showed a statistically significant difference when compared with the carrageenan group (P >0.05).

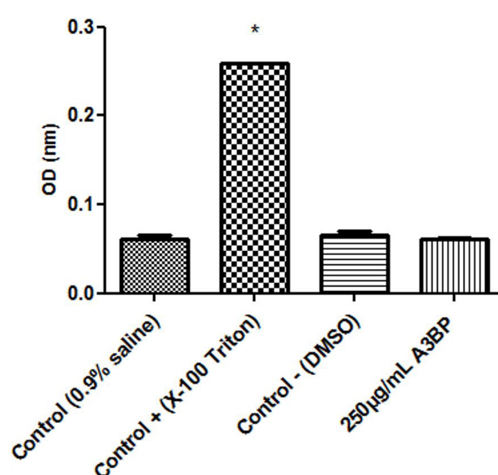

Figure 5. Hemolytic test with 3BPA in erythrocyte cells of *Mus Musculus Swiss* mice showed no hemolytic activity. The mixtures were centrifuged for detecting the presence of hemoglobin (red) by absorption at 541nm. *P<0.05 was adopted compared to the control group. Differences between groups were analyzed by analysis of variance (ANOVA) and the correction by the Tukey Multiple Comparison Test.

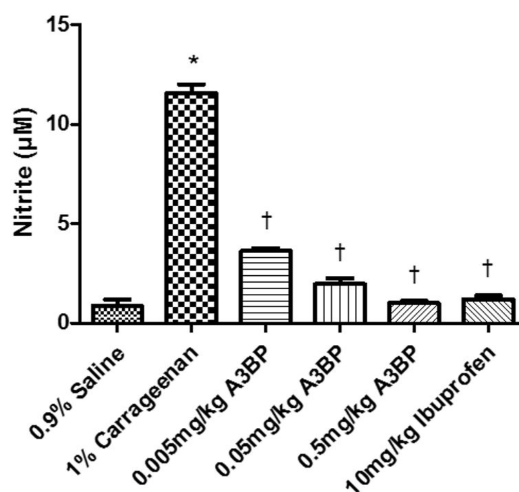

Figure 6. 3BPA inhibiting production of NO_2^- and NO_3^- in the carrageenan-induced inflammation 1%. Values are expressed as mean ± S.E.M. 4 mice each group. Control group (saline 0.9%); group carrageenan 1%; carrageenan group 1% pretreated with 0.005mg/kg 3BPA; carrageenan group 1% pretreated with 0.05mg kg 3BPA; carrageenan group 1% pretreated with 0.5mg/kg 3BPA. *P<0.05 compared with the control group and †P<0.05 the group carrageenan 1% was adopted. Differences between groups were analyzed by analysis of variance (ANOVA) and the correction by the Tukey Multiple Comparison Test.

Cell migration

In order to determine the migration of inflammatory cells occurred at the lesion site, the total number of cells present in the exudate induced with 1%carrageenan solution was assessed, as well as the pre-treatment of inflammation with 3BPA at doses of 0.005, 0.05 and 0.5mg/kg.

As shown in Figure 7, carrageenan significantly increased (P<0.05) cell migration compared with the control group. All groups pre-treated with 3BPA and Ibuprofen led to a decrease in cell migration: control (saline 0.9%) (18.50 x10^7 ± 1.91 cells/ml); 1% carrageenan (270.25 x10^7 ± 11.32 cells/ml); 3BPA 0.005mg/kg (86.33 x10^7 ± 23.29 cells/ml); 3BPA 0.05mg/kg (59.50 x10^7 ± 23.44 cells/ml); 3BPA 0.5mg/kg (36.00 x10^7 ± 28.18 cells/ml); Ibuprofen 10mg/kg (27.75 x10^7 ± 13.02 cells/ml). All treated groups showed a statistically significant difference when compared with the carrageenan group (P<0.05).

Differential count of leukocyte cell

The leukocyte count was performed to identify types of induced cells with the 1% carrageenan solution and the pre-treatment of inflammation with 3BPA at doses of 0.005, 0.05 and 0.5mg/kg. The data analysis of Figure 8 shows large population of neutrophils (Figure 8a), followed by lymphocytes (Figure 8b), monocytes (Figure 8c) and eosinophils (Figure 8d): Neutrophils (58.7 x10^6 ± 3.7 cells/ml); lymphocytes (14.5 x10^6 ± 1.3 cells/ml); monocytes (7.0 x10^6 ± 1.0 cells/ml); eosinophils (1.0 x10^6 0.4 ± cells/ml).

PGE_2 Values

The evaluation of 3BPA effect in the mediator values of inflammatory PGE_2 in inflammation was induced by 1% carrageenan. As shown in Figure 9, the analysis of PGE_2 values was performed on groups induced by carrageenan 1%. Carrageenan significantly increased (P<0.05) the PGE_2 values compared with the control group. All groups pre-treated with 3BPA and Ibuprofen led to a decrease in PGE_2 values: control (saline 0.9%) (460.96 pg/ml ± 62.64); carrageenan 1% (39845.55 pg/ml ± 2120.25); 3BPA 0.005mg/kg (35872.83 pg/ml ± 562.28); 3BPA 0.05mg/kg (32612.05 pg/ml ± 1738.89); 3BPA 0.5mg/kg (18336.12 pg/ml ± 6991.81); Ibuprofen 10mg/kg (14928.56 pg/ml ± 3113.35). The 3BPA groups treated with 0.5mg/kg and Ibuprofen 10mg/kg showed significant difference when compared with carrageenan (P <0.05).

Study of antinociceptive activity

Peripheral analgesic activity

The peripheral analgesic activity was evaluated by the number of abdominal writhes after acetic acid administration of 0.6% in the treatment with the doses 3BPA 0,003 and 0.03mg/kg (Figure 10). Doses 0.003 and 0.03mg/kg reduced the number of abdominal writhing in 54.5% and 65.2%, respectively, when

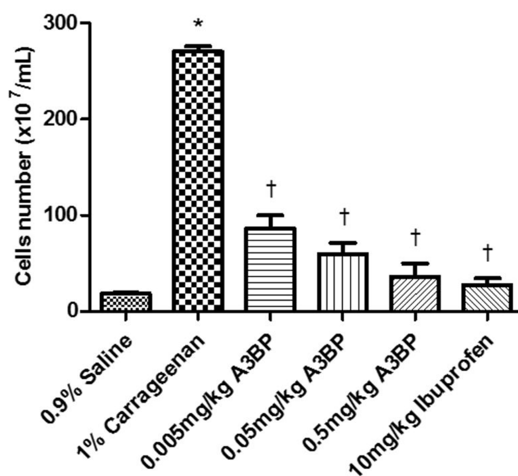

Figure 7. 3BPA inhibiting cell migration in inflammation induced by carrageenan 1%. Values are expressed as mean ± S.E.M. 4 mice each group. Control group (saline 0.9%); group carrageenan 1%; carrageenan group 1%pretreated with 0.005mg/kg of 3BPA; carrageenan group 1% pretreated with 0.05mg/kg 3BPA; carrageenan group 1% pretreated with 0.5mg/kg 3BPA. *P<0.05 compared with the control group and †P<0.05 the group carrageenan 1% was adopted. Differences between groups were analyzed by analysis of variance (ANOVA) and the correction by the Tukey Multiple Comparison Test.

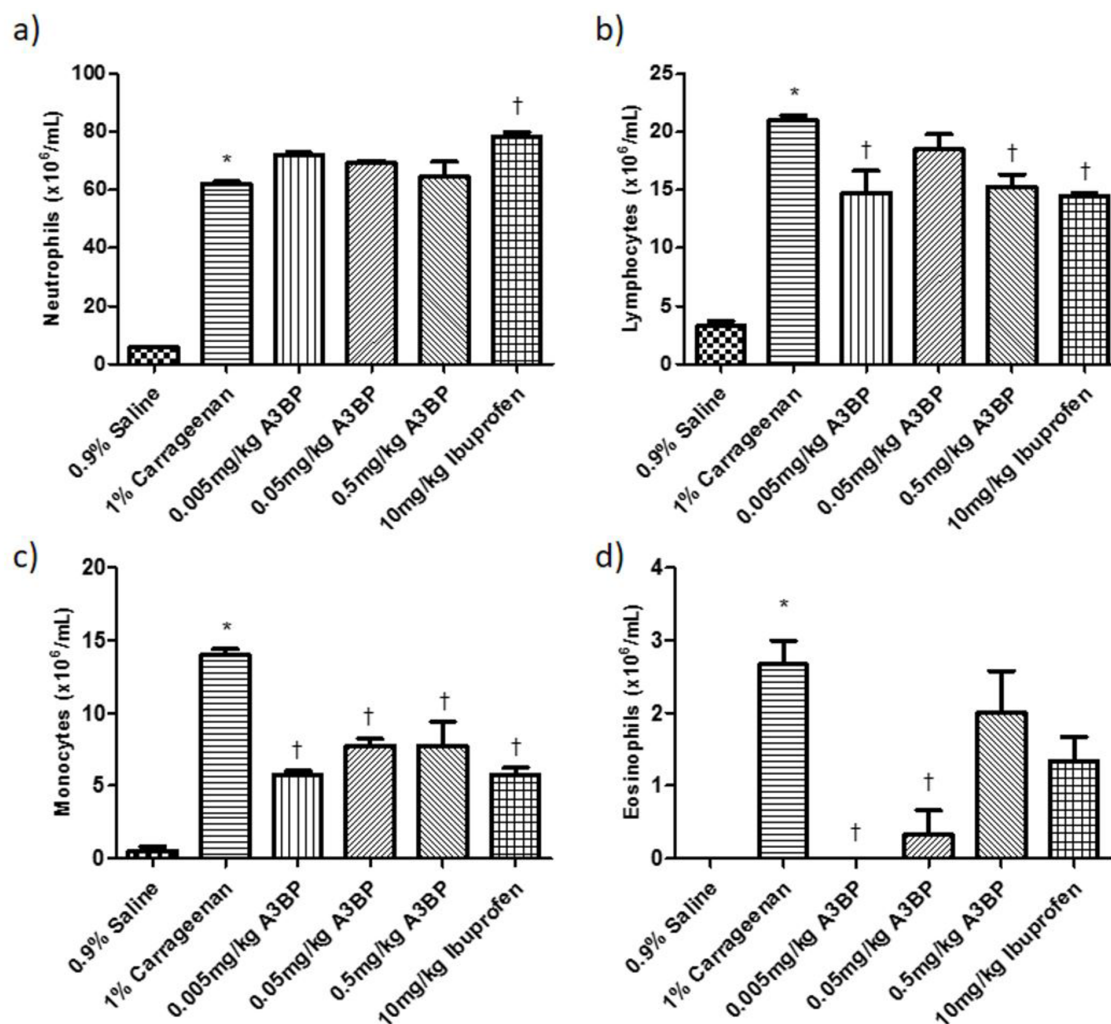

Figure 8. 3BPA in the differential count of leukocytes: neutrophils (a), lymphocytes (b), monocytes (c) and eosinophils (d) in the carrageenan-induced inflammation 1%. Values are expressed as mean ± S.E.M. of 4 mice each group. Control group (saline 0.9%); group carrageenan 1%; carrageenan group 1% pretreated with 0.005mg/kg 3BPA; 1% carrageenan group pretreated with 0.05mg/kg 3BPA; carrageenan group 1% pretreated with 0.5mg/kg 3BPA. **P*<0.05 compared with the control group and †*P*<0.05 the group carrageenan 1% was adopted. Differences between groups were analyzed by analysis of variance (ANOVA) and the correction by the Tukey Multiple Comparison Test.

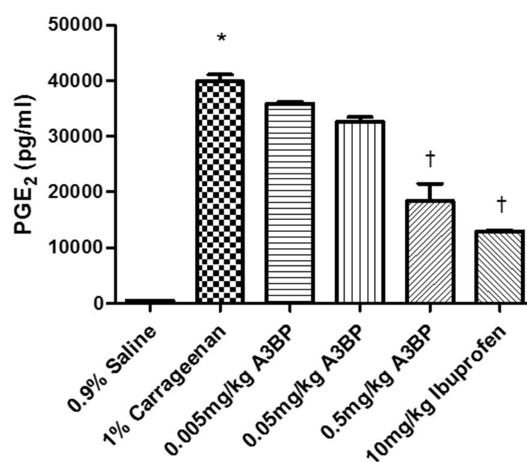

Figure 9. 3BPA values of PGE2 in the carrageenan-induced inflammation 1%. Values are expressed as mean ± S.E.M. 4 mice each group. Control group (saline 0.9%); group carrageenan 1%; carrageenan group 1% pretreated with 0.005mg/kg 3BPA; carrageenan group 1% pretreated with 0.05mg/kg 3BPA; carrageenan group 1% pretreated with 0.5mg/kg 3BPA; carrageenan group 1% pre-treated with 10mg/kg Ibuprofen. **P*<0.05 compared with the control group and †*P*<0.05 the group carrageenan 1% was adopted. Differences between groups were analyzed by analysis of variance (ANOVA) and the correction by the Tukey Multiple Comparison Test.

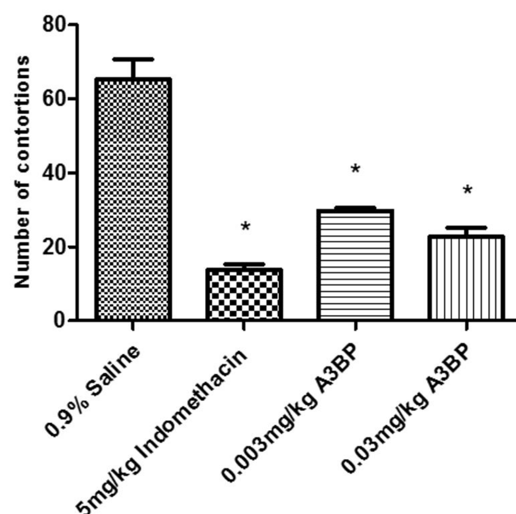

Figure 10. 3BPA and Indomethacin effect on nociceptive stimulus induced by intraperitoneal injection of acetic acid 0.6% in mice. Significant reduction is observed in 3BPA groups (0.003 and 0.03mg/kg) and indomethacin (5mg/kg) compared to the control group. **P*<0.05 was adopted compared to the control group. Differences between groups were analyzed by analysis of variance (ANOVA) and the correction by the Tukey Multiple Comparison Test.

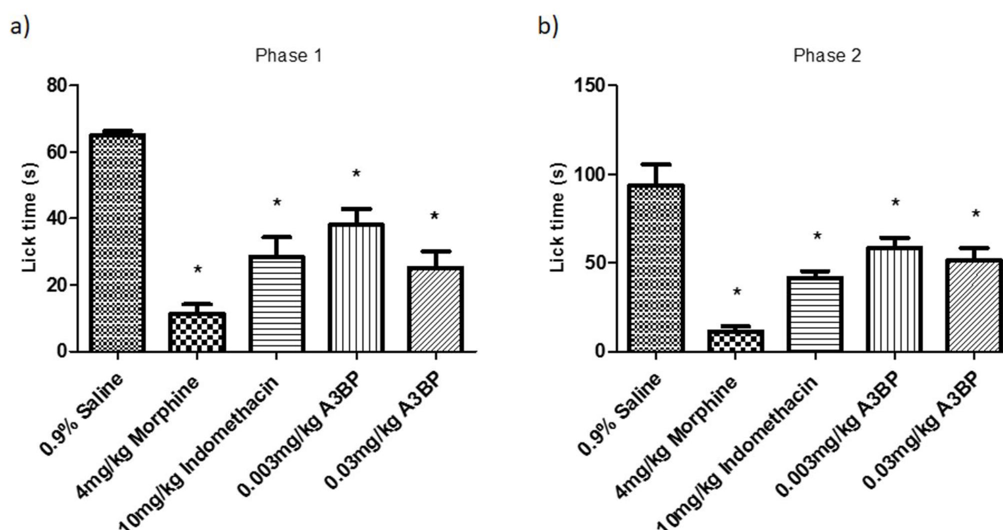

Figure 11. 3BPA Effect of Indomethacin and Morphine on nociceptive stimulus induced by intraplantar injection of formalin 1% in mice. It is observed a significant reduction in 3BPA groups (0.003 and 0.03mg/kg) and indomethacin (5mg/kg) compared to the control group. **P*<0.05 compared to the control group was adopted. Differences between groups were analyzed by analysis of variance (ANOVA) and the correction by the Tukey Multiple Comparison Test.

compared to the control group (saline 0.9%). Indomethacin (5mg/kg), NSAIDs used as a standard drug in this test, decreased by approximately 79.1% the number of writhes compared to the control group.

Formalin test

To confirm the anti-nociceptive effect of 3BPA it was used to test nociception induced by intraplantar formalin injection (Figure 11). The 3BPA in the doses 0.003 and 0.03mg/kg significantly reduced way the lick time compared to the control group of the formalin test in phase 1 (neurogenic phase) in 42 and 62%, respectively, and in phase 2 (inflammatory phase) at 39 and 45%, respectively. Morphine (4mg/kg), opioid analgesic, reduced significantly the two phases 82.6 and 85.3%, as well as indomethacin (10mg/kg) also reversed the effect to 1% formalin in both phases 36 and 40% (Figure 11a and 11b).

Discussion

Inflammation is, above all, a useful and beneficial process for the body, to compensate homeostasis break and restoring normal tissue. This defense and reparation process should only be fought when the acute clinical symptoms (classically tumor, heat, redness, pain and loss of function) are intense and uncomfortable, and when the process gets more systemic effects and subacute or chronic character, with demonstrations disabling symptomatic and cumulative tissue damage, such as deformities and functional loss[14].

From the pharmacological point of view, there must be caution in the treatment of inflammation. When there is systemic involvement, treatment may include steroidal and nonsteroidal anti-inflammatory and other types of drugs with specificity against elements of the inflammatory process[15]. In the arsenal of drugs acting on the inflammatory process, there is a class of NSAIDs that consists of chemically heterogeneous groups, but share analgesic, antipyretic and anti-inflammatory properties. These agents have only symptomatic effect on inflammatory diseases or processes to which are indicated. It is noteworthy that NSAIDs are classified as non-selective and selective COX-2 inhibitors with the most varied therapeutic applications, given the multitude of clinical manifestations of the diseases for which they are indicated[14].

The NSAIDs derivatives of 2-aryl-propionic acid are approved for use in the symptomatic treatment of rheumatoid arthritis, osteoarthritis and acute gouty arthritis, also being used as analgesics in the acute tendonitis and bursitis, as well as to treat primary dysmenorrhea[16]. Within this kind of anti-inflammatory drugs are included Ibuprofen, Ketoprofen, Naproxen, Fenbufen, Flurbiprofen, and other drugs with high analgesic and anti-rheumatic power[17].

The few researches with 3BPA reported their use in the treatment of cholecystolithiasis, while showing low toxicity[18], and mainly in the treatment of adjuvant-induced arthritis in rats, with good results[19,20]. However, its mechanism of action has been little studied and are not fully known, having thus the need for further clarifications, especially through the relationship with analogues derived from the 2-arylpropionic acid already well known.

In Brazil, several NSAIDs are found easily accessible to everybody in pharmacies, even Naproxen, Ibuprofen and Ketoprofen are listed among the Exempt Prescription Drugs (EPD) from 2003, despite they are agents with different potential cytotoxicity[21]. Cytotoxicity usually is associated with damage to the mitochondrial membrane, since these disrupt the lipid bilayer and affect different layers of polysaccharides, fatty acids and phospholipids[22].

In order to assess whether the 3BPA drug showed a low degree of cytotoxic risk, cytotoxicity assays were performed on mice peritoneal macrophages and secondary culture of J774 lineage cells as well as MRC-5 PD19 cells with fibroblasts characteristics derived from human embryonic lung.

Cirino et al.[23] on cell viability research with the J774 cell lineage, treated with Flurbiprofen NSAIDs, Aspirin®, Ketoprofen, Naproxen, Diclofenac and Ketorolac have showed that none of these compounds significantly affected mitochondrial membrane into the cell viability by the MTT method, suggesting that these NSAIDs are not cytotoxic to J774 cells, even at the highest concentration used, as well as NSAIDs mentioned above, the 3BPA also showed no cytotoxic effect at the concentrations used for 24 and 48 hours on the same cell lineage.

The analysis of the viability of peritoneal macrophages was performed as it is common to use this cell type *in vitro* tests to demonstrate the anti-inflammatory drug in culture, in which the LPS was used as macrophage activating agent and evaluated whether exposure to the test drug was able to inhibit this activation, which can be checked by measuring metabolites of the inflammatory process, such as the measurement of nitrite and nitrate release in the incubation medium[24]. However, if it is not confirmed the feasibility of macrophages after incubation, may have been a methodological error where an excessive concentration of the drug test could generate cytotoxic effect to the macrophage and, because of this action, provoke a decrease in metabolite rate of inflammation to be analyzed and so generate a false result as anti-inflammatory agent.

For this reason it was evaluated peritoneal macrophages viability by the MTT method, where our result showed that treatment with 3BPA for 3 hours at different concentrations, followed by incubation with LPS for 24 hours or 48 hours caused no loss in cell viability. In this test was performed in 3BPA incubation with different concentrations up to 100µM and thus show that such concentrations could be tested for ability to inhibit macrophage activation, without the risk of a false result as an anti-inflammatory agent.

In the measurement of metabolites of macrophages activation by LPS in the culture medium, such as nitrite and nitrate, the 3BPA concentrations used in the research significantly inhibited about 50% and 45%

nitrite production compared to the stimulated group LPS. Cirino et al.[23] in research related to the production of nitric oxide levels for (NSAIDs- NO) on the induction of nitric oxide (NO) synthase in cells treated with Flurbiprofen, Aspirin®, Ketoprofen, Naproxen, Diclofenac and Ketorolac, showed marked reduction, about 40% of the nitrite levels in J774cell lineage stimulated with LPS.

To confirm then whether the treatment with 3BPA could indeed cause the death of MRC-5 PD19 cells, comet assay was performed, which is capable to demonstrate damage to the core material. It is known that in various events of programmed cell death DNA fragmentation occurs, thus a high rate of DNA damage could imply a higher death rate by apoptosis caused by drug[25]. Another possibility of a high level of DNA damage would be by a genotoxic drug directly into the DNA of the cell[26]. Our results showed that treatment with 3BPA caused no increase in DNA damage index, thus being suggesting that the reduction in viability observed in the *Alamar Blue* assay was no for inducing apoptosis, which reinforces the idea that the observed decrease in viability mainly resulted from an effect on cell proliferation.

In general, the test for assessing hemolytic capacity *in vitro* is used like a toxic screening method to estimate the erythrocyte damage that may induce *in vivo*[27]. This parameter has been used by the scientific community for toxicological evaluation of different plants, drugs or compounds[28]. As Andrade[29], in research of the hemolytic activity of Ibuprofen associated with methylcellulose MCM-41 as analyzing the bioavailability of that derived from the 2-arylpropionic acid, showed no hemolytic activity of samples studied independently of Ibuprofen associated, the result with 3BPA also a derivative of 2-arylpropionic acid showed no hemolytic activity in erythrocyte cells.

In the study of the anti-inflammatory activity *in vivo* use of subcutaneous air pouch as the experimental model has been described in several works[11]. This model is used to perform a quantitative analysis of the factors involved in the inflammatory process through the formation of a symmetrical elliptical space, created by injecting air into the subcutaneous tissue of rats[30]. The examination of this cavity during the days following the first injection of air demonstrates the presence of a surface containing connective cells with structure and histochemical aspects similar to those found in synovial cavities. This similarity has been observed using an experimental model of 6 days at most[31].

Although there are other experimental models for studying the acute inflammatory response, such as the pleural cavity and peritoneal cavity, the subcutaneous air pouch presents the advantage of being easy technical application, and the analysis of the fluid is simple and can be repeated with minimal trauma. It is noteworthy that this model is useful for the removal of inflammatory cells, for measuring chemotaxis and production of inflammatory chemical mediators such as NO, the inflammatory cells and PGE_2[11].

NO is a small lipophilic molecule which can be rapidly diffused through the barrier of the cell membrane and thus can achieve the intracellular compartments of adjacent cells with diverse functions[32]. The NO produced by the NOS enzyme plays a role in inflammation and in the neuromodulation studied extensively[33], being part of various biological functions ranging from the formation of a protective mechanism against microorganisms, until the regulation of blood pressure and a process of neurotransmission[34].

Masukawa et al.[35] consider NO as a neuronal messenger of the central nervous system and modulator of various brain functions, so that various researches relate NOS enzyme to the use of Fenbufen in potential convulsive activities in rats. Sautebin[36] demonstrated that this free radical owns regulatory anti-inflammatory activity by the constitutive forms, it means, in basal concentrations; however, when NO is generated by the inducible form of NOS enzyme (iNOS), promotes inflammation and tissue dysfunction and therefore possesses pro-inflammatory properties and deleterious which to Costa et al.[37], pro-inflammatory effects of this moiety include increased vascular permeability of inflamed tissues.

The determination of the levels of NO final products in the inflammatory response induced by carrageenan solution was measured in the inflammatory exudate to show that in all groups pre-treated with 3BPA doses, as well as with another derivative of 2-aryl propionic acid, the ibuprofen, led to a decrease in the levels of NO_2^- and NO_3^-.

The recruitment of leukocytes from the bloodstream is a crucial reaction to the inflammatory process. This action occurs through several steps wherein the leukocytes interacts with the endothelium[38]. The leukocytes adhere to vascular endothelium, thus occurs transmigration of these cells through the vessel wall, and thus spill reaching the extra vascular tissue. Several drugs have aimed to stop cell migration as a way to ease the inflammatory process[39]. However, when it comes to inflammation is also important to remember that the transmigration of leukocytes across the vascular endothelium and its accumulation in inflamed tissues are the main events of an inflammatory effective response[40].

In the evaluation of the migration of inflammatory cells present in the exudate induced with carrageenan solution of all groups pre-treated with three doses 3BPA and Ibuprofen led to a decrease in cell migration, in agreement with Benez et al.[41], who reported the great capacity of Ibuprofen in reducing edema with consequent reduction in the migration of leukocytes, in a comparative study of cytotoxic activities and ulcerogenic of Ibuprofen forms of racemic and "S" in this inflammation in the air pouch.

The evaluation of cells that act in the inflammatory process has been the purpose of several studies[42]. The cell migration in response to inflammation is seen in a few hours after the stimulus that is causing tissue injury, with recruitment of neutrophils to the site where it is occurring tissue damage[43].

Noteworthy, it is very important to say that the effects of 3BPA showed no dose-response curve on the results of cell migration and from the assessment of the levels of NO, as the plateau of pharmacological curve present these results, and that this may be due to therapeutic index of the drug or the doses used in the research.

With respect to leucocyte differential count performed to identify types of induced cells with carrageenan solution into the pre-treatment of inflammation, 3BPA showed large population of neutrophils in all studied samples, followed by small amount of lymphocytes, monocytes and eosinophils. Agreeing with the study of Jiang et al.[44], who reported that in most forms of acute inflammation, neutrophils predominate in the inflammatory infiltrate during the first 6 to 24 hours.

Although neutrophils are the first cells in the removal of pathogenic agents such as bacteria, they also contribute to the inflammatory process by releasing inflammatory mediators, including mediators that attract macrophages to the site of inflammation[45]. The macrophages in turn release pro-inflammatory cytokines such as TNF-α, an iNOS stimulator in certain cell types, which induces chemotaxis of neutrophils and T lymphocytes and the expression of adhesion molecules[46].

The research on derivatives of 2-aryl-propionic acid, such as Ibuprofen and Ketoprofen are compared, particularly in levels of PGE_2, including forms "S" and racemic where the reduction in PGE_2 levels was found in inflammation present in the air pouch comparative study of the cytotoxic activities and ulcerogenic, both forms of the racemic Ibuprofen and S-Ibuprofen[41]. Another performedstudy, this time with the racemic and S-ketoprofen and Ketoprofen also showed reduction of PGE_2 activity in two ways in inflammation the intestinal mucosa in rats[47]. Just as in studies with two derivatives of 2-aryl-propionic acid shown above there was a reduction in the levels of PGE_2, the results shown here there was also a significant reduction in PGE_2 values with Ibuprofen dose and 3BPA at a dose of 0.5mg/kg.

It should be noted regarding to nociception that the pain stimulus can have a sufficient intensity to cause tissue injury that is associated with the release of many inflammatory mediators[48]. The countered pain has steadily as more prescribed medication for this purpose the NSAIDs. The action of these drugs is twofold: firstly interfere with the system of prostaglandins, group of substances that interact and are partly responsible for the sensation of pain. Secondly, most of these drugs reduces inflammation, edema and irritation that often surrounds a wound and increases the pain[49].

In order to evaluate the anti-nociceptive activity of 3BPA, various tests were performed, including the abdominal writhing induced by acetic acid 0.6% test, an experimental model used to evaluate possible peripheral anti-nociceptive effects of inflammatory nature, since the acetic acid, used in concentration induces indirect pain that occurs as a result of an acute inflammation of the peritoneum[50].

Therefore, the provoked inflammation causes the release of prostaglandins, sufficient to produce spasms translated into contortions. It is believed also that the acetic acid acts indirectly causing the release of endogenous mediators involved in the modulation of nociception, including bradykinin, serotonin, histamine and prostaglandins. Furthermore, nociception induced by acetic acid depends on the release of cytokines such as IL-1, TNF- and IL-8 from macrophages and basophils resident in the abdominal cavity and, together with other mediators, can induce the particular nociception observed in this model[50].

The tested doses of 3BPA reduced the number of abdominal writhes compared to the control group. The dose 0.03mg/kg was as effective as indomethacin an NSAID, used as the standard drugs in this assay, with the ability to reduce writhing in mice.

The formalin test is perhaps the most used model for clinical pain in which the first phase seems to be due to direct chemical stimulation of nociceptors, while the second phase is dependent on the peripheral inflammation and modifications in the central processing[51]. This test allows highlighting two phases of pain sensitivity: the first stage occurring during the first 5 minutes after injection of formalin (nociception of neurogenic origin) is a result of direct chemical stimulation of afferent fibers nociceptive myelinated

and unmyelinated, mainly C fiber, which can be suppressed by opioid analgesic drugs such as morphine[52]. Experimental results show that substance P and bradykinin participate in the first stage[53].

The second phase occurs between 15 and 30 minutes after injection of formalin, which inflammatory mediators formed in peripheral tissues such as prostaglandins, serotonin, histamine and bradykinin induce functional changes in the dorsal horn neurons that, over time, promote the easy transmission in the spinal level. This evidence suggests that peripheral inflammation process is involved in the second stage[54].

The 3BPA in the tested doses significantly reduced the lick time compared to the control group in the inflammatory phase of the formalin test. However, the 3BPA in 0.3mg/kg dose was not effective in neurogenic phase. Morphine, opioid analgesic, and indomethacin, an anti-inflammatory drug of non-steroidal nature, significantly reduced both phases.

In conclusion, this study has advanced substantially with respect to anti-inflammatory and analgesic properties of 3BPA by providing evidences of their probable mechanism of action, through the evaluation of anti-nociceptive activity, as well as the anti-inflammatory activity *in vitro* and *in vivo*, where the 3BPA showed no genotoxic or any hemolytic activity.

However, the results shown here are based on experimental data that need to be further detailed for therapeutic application; there is thus a need for new experiments in evaluating the possible reaction of the synthesis of 3-benzoyl-propionic acid in order to promote greater specificity of this drug.

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