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Variation of the Anti-Inflammatory Effect from Indomethacin on Acute and Chronic Inflammation Models

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Abstract

The anti-inflammatory activity of three samples of Indomethacin (IND-S1, IND-S2, IND-F) was evaluated in two models of acute inflammation (systemic and topical) and in the model of chronic inflammation. The three sample was solubilized in Tween 80:H_2O 1:9, was tested at 20 mg/kg and was administered by intragastric route. In first evaluation, the sample IND-S1 at 20 mg/kg showed 57.09% inhibition on carrageenan-Induced plantar edema model. The evaluation at this same dose of Indomethacin from 3 different samples (IND-S1, IND-S2, IND-F) showed a variation in the % inhibition with 34.61, 30.13 and 32.69%, respectively. On the other hand; topical anti-inflammatory activity was evaluated in the TPA model acute in female Balb/C mice. IND-S1 showed an ED_{50} = 1.31 mg/ear and at a dose of 2 mg/ear showed 56.41% of inhibition. IND-S1, IND-S2 and IND-F at a dose of 2 mg/ear were more active with 87.39, 90.13 and 75.87%, respectively. In the chronic model of ear edema-TPA, when the IND-S1 was administered by i.g. route at 10 mg/kg, it showed high mortality (10/10), however at dose of 8 mg/kg, IND-S1 showed a 60.63% inhibition of ear edema, but in this assay 4/10 mice head. In same assay (TPA) but the IND was administered by topic route at repeated dose of 2 and 1 mg/ear, it cuased the death of all animals and at dose of 0.5 mg/ear caused about 50% of animals deaths.

Keywords: Acute and chronic anti-inflammatory activity, carrageenan assay, TPA assay, Indomethacin, NSAIDs

Introduction

Non-steroidal anti-inflammatory drugs (NSAIDs) have antipyretic, analgesic, anti-inflammatory and anti-cancer properties, and are widely used in inflammatory processes These drugs were approved by the FDA for the treatment of chronic and acute musculoskeletal and inflammatory conditions [1,2].

IND is one of the most widely used anti-inflammatory drugs, it is an NSAID derived from Indoleacetic acid. This drug was presented at the American Rheumatism Association meeting in 1963 as the drug of first choice for treat rheumatoid arthritis [3,4]; also used for the treatment of was recommended osteoarthritis and gout arthritis, among other inflammatory process [5-7]. The mechanism of action is through reversible binding with the active site of cyclooxygenase-1 (COX-1) and COX-2; by competing with the binding of the substrate (arachidonic acid), to catalyze the synthesis of prostaglandins (PG), through bis-dioxygenation and the reduction of arachidonic acid (AA) [8].

COX-1 enzyme catalyze the formation PGs that participate in gastric protection and in hemostasis, meanwhile the enzyme COX-2 is associated of generates PGs that participate in inflammation and tumorigenesis. IND has a 15-fold selectivity for COX-1 relative to COX-2. However, these two isoforms contribute to the defense of the gastric mucosa [9].

The most common complications from the use of NSAIDs such as INDs are the risk of gastric ulcers, marked damage of the gastric mucosal surface, and kidney and liver toxicity. These types of drugs are the most commonly prescribed and sold over-the--counter for the treatment of inflammatory diseases (acute or chronic) and, because they are non-selective, they cause severe adverse effects [10,11].

Clinical studies warn that the continuous use of NSAIDs can cause cardiovascular and cerebrovascular alterations, also alter blood pressure and generate gastric and/or duodenal ulcers and affect the Central Nervous System [12-14].

In Nigeria, IND has been used since 1995 as a potent rodenticide [15,16]. High doses of IND (83, 166, and 250 mg/kg) administered intragastrically (i.g.) caused 100% mortality, within a time of 36 and 82 h in rats and mice.Olusegun Taiwo et al. demonstrated that the rodenticide action of IND is due to the fact that it generates serious injury to the gastric mucosa, endothelial damage, hemorrhage and disseminated intravascular coagulation; mainly organ necrosis: stomach, intestine, liver, kidneys, heart and brain through thrombosis and ischemia [17].

IND is used for the treatment of several inflammatory processes, so this drug is selected as a positive control in the search for natural and/or synthetic substances with an anti-inflammatory effect [18-20].

One of the most used tests to evaluate the development of new NSAID drugs is the model murine of acute inflammation of plantar edema INDuced by the phlogistic agent carrageenan; this test is biphasic, in the first phase of the inflammatory process vasoactive amines participate and during the second phase (3-5 h) COX enzymes mainly participate [21,22]. However, with this test it is not possible to evaluate the secondary effects that could be generated in the long term in a chronic inflammatory process and variation in the biological effect may occur in each test.

Recently, Suresha et al. [23] reported that IND (10 mg/kg) did not show consistent anti-inflammatory activity in three inflammation models (two acute inflammation and one subacute inflammation) performed in albino Swiss rats of both sexes. In the carrageenan assay (acute inflammation) IND showed 83.3% inhibition and in the turpentine-induced arthritis model (acute inflammation) it showed 48% inhibition, and in the third subacute inflammation model, where they measured the percentage of inflammation of the granuloma induced with cotton pellets showed 41.16%.

Another widely used test to evaluate acute and chronic inflammation of natural and synthetic substances is the murine model of ear edema induced with 12-O-tetradecanoylphorbol 13-acetate (TPA). This compound induces the production of prostaglandin; a molecule that mediates epidermal hyperpasia of the mouse ear and it has been shown that COX inhibitors can be effective topically in this model [24].

This work reports the variation of the anti-inflammatory effect of IND in two models of acute inflammation, one systemic (carrageenan assay) and the other topical (TPA assay). In addition, a model of chronic inflammation was performed using repeated doses of TPA and IND, administered every third day during 9 days by systemic and topic route, respectively.

Materials and Method

Reagents and HPLC Condition by Analysis of Samples

Two sample of IND was acquired from Sigma-Aldrich® (IND-S1 and IND-S2) and one sample was acquired from Fluka® brand (IND-F). Each sample was analyzed by HPLC, this analysis was carried out in a Waters equipment (Waters, USA) with a diode array detector (996). Equipment control, data acquisition, and information processing were performed using Empower 3 software (Waters). Phenomenex Luna® C18 (2) 100 Å, LC (250 x 4.6 mm, 5 µm) column was used with mobile phase (isocratic) phosphoric acid (50%) as phase A and acetonitrile (50%) as phase B, this was maintained for 10 min. The temperature programed was 40 °C, the Flow rate was 0.3 mL/min and the injection volume was 10 µL. The compounds were detected at 273 nm, with windows spectral 210-600 nm. The analytical conditions used were taking into account those previously described by Novákova et al., 2005 and Pai and Sawant, 2017 [25,26].

Experimental Animals

Female Balb/C mice (20 ± 2 g) were provided by the Bioterium of the Centro Médico Nacional Siglo XXI, IMSS, which were housed in light-dark conditions for 12 h, at a controlled temperature (25 ± 2°C), with a humidity percentage of 55-80%, with food and water ad libitum. The handling of the animals was carried out in accordance with the technical specifications for the production, care and use of laboratory animals (NOM-062-ZOO-1999, revised in August 2016).

Evaluation of anti-inflammatory Activity *in vivo*

Paw Edema Induced with Carrageenan

This experiment was carried out according to the methodology previously described by Juárez-Vázquez et al. [27]. In this experiment, 4 groups (n=5) of mice were used, which were injected with carrageenan (20 µL at 2%) in the sub-plantar region of the right hIND paw. Three experimental groups were administered by intragasgtric (i.g.) route with IND at doses of 10, 15 and 20 mg/kg, respectively one hour before the administration of carrageenan and a control group received only vehicle. IND was solubilized in vehicle (Tween 80:H_2O, 1:9).

Each lot of IND was evaluated independently. Plantar edema was measured with digital micrometer (Mitutoyo brand) at four time (1, 3, 5 and 7 hours). The percentage of edema inhibition was determined by comparing the edema measurements at the different times (Et) with respect to the basal measurement at time zero before injecting the carrageenan (Eo). The percentage of edema inhibition was calculated according to the following formula:

$$\%\ inhibition\ =\ \left[(Et-Eo)_{carrageenan}-(Et-Eo)_{treated}/(Et-Eo)_{carrageenan}\right]\ X\ 100$$

Ear edema induced with TPA

This experiment was carried out according to the methodology previously described by Juárez-Vázquez et al. [27]. Four groups (n= 5) of mice were used, and TPA (2.5 µg/ear) was dissolved in acetone (25 µL) (Ws') and was applied topically on the internal and external surface of the pinna of the left ear, using the right ear as a control (25 µL, acetone, Wo). Once TPA was applied, three groups (Ws) received IND at doses of 2, 1 and 0.5 mg/ear, 30 min later. Six hours after the application of TPA, the animals were sacrificed by cervical dislocation and auricular tissue (6 mm in diameter) was obtained from both ears, these tissue were weighed. The size of the edema was determined between the difference in weight (mg) of the treated tissue (left ear) compared to the untreated tissue (right ear). The percentage of edema inhibition was calculated according to the following formula:

$$\% \ Inhibition = \ [(Ws - Wo)_{TPA} - (Ws' - Wo)_{treated}/(Ws - Wo)_{TPA}] \ X \ 100$$

Ear edema induced with repeated doses of TPA (chronic inflammation model)

Chronic inflammation was induced in the pinnae of mice with topical application of TPA using the methodology described by Stanley et al. [28] with some modifications. Groups of 10 mice were used in this assay. In a first experiment, IND was administered by i.g. route at 10 and 8 mg/kg and 30 min later, TPA (2.5 µg/ear, disolved in 25 µL) was applied to the pinnae. IND and TPA were administered every third day for 9 days and 24 h after the last administration, the animals were sacrificed by dislocation.

In a second experiment, TPA was applied to the auricle every third day during 9 days and IND (at 2, 1, and 0.5 mg/ear, dissolved in acetone) was applied topically to the ear 30 min after TPA application. Six hours after the last application, the animals were sacrificed by cervical dislocation and auricular tissue (6 mm diameter) was obtained from both ears and were weighed. The percentage of edema inhibition was determined using the formula for the acute anti-inflammatory activity of TPA.

Results and Discussion

Acute anti-inflammatory activity assayed by carrageenan model

Systemic anti-inflammatory activity was evaluated in the carrageenan-induced plantar edema model in female Balb/C mice. The group that received only carrageenan showed a maximum inflammation of the edema at 5 h (0.79 ±0.07 mm), representing 100% inflammation. IND-S1 evaluated at doses of 10, 15 and 20 mg/kg (administered i.g. route) showed 36.15, 32.07 and 57.09% inhibition, respectively (Table 1).

In this trial, the IND at the highest dose showed a good anti-inflammatory effect. Gutiérrez-Rebolledo et al. [29] reported an ED_{50} = 10 mg/kg for IND Sigma-Aldrich brand, being 57.36% inhibition of edema when was evaluated in male Balb/C mice. The percentage of inhibiton was similar (57.09% vs 57.36%) but a different doses (20 mg/kg vs 10 mg/Kg). Some authors have been reported that in the carrageenan assay, IND is used at 20 mg/mL for Balb/C or ICR mice [30,31]. Zaa et al. [30] reported that IND at this same dose inhibited the inflammatory process with values of 20.93 and 33.49% at 4 and 6 hours, respectively, in Balb/C mice.

Table 1: Anti-inflammatory activity of IND-S1 in female Balb/C mice (carrageenan model)

Treatment	Dose(mg/kg)	Paw edema(mm)*	Inhibition(%)
Carrageenan	-	0.79 ±0.07	-
	10	0.50 ±0.05[a]	36.15
IND-S1	15	0.54 ±0.02[a]	32.07

	20	0.34 ±0.05[a]	57.09

Each group represents the mean (±) and the standard error (SE). The ANOVA statistical analysis followed by a Dunett's test, ($p \leq 0.05$) [a]vs carrageenan; n=5. *Time: 5 hours.

According to these reports, the systemic anti-inflammatory activity of different batches of IND (IND-S1, IND-S2 and IND-F) was evaluated in female Balb/C mice at 20 mg/kg. The results showed for each batch an inhibition of inflammation of 34.61, 30.13 and 32.69%, respectively (Table 2), this percentage of inhibition was low compared to the first evaluation of IND-S1, where was observed 57.09% inhibition at 20 mg/kg (Table 1) and the results at the same dose of the three batches of IND were low (<34.61%). The variation in the results obtained could indicate that the cause is related to the stability of the drug.

Table 2: Anti-inflammatory activity of three samples of IND in female Balb/C mice (carrageenan model)

Treatment	Dose(mg/kg)	Paw edema (mm)*	Inhibition (%)
Carrageenan		0.71 ±0.04	-
IND-S1 IND-S2 IND-F	20 20 20	0.46 ±0.04[a] 0.50 ±0.08[a] 0.48 ±0.03[a]	34.61 30.13 32.69

Each group represents the mean (±) and the standard error (SE). The ANOVA statistical analysis followed by a Dunett's test, ($p \leq 0.05$) avs carrageenan; n=5. *Time: 5 hours.

In the scientific literature it has been described that IND undergoes photolytic degradation when exposed to light and their color change can be observed [32]. It is important to mention that the three IND samples evaluated in this study were stored at room temperature and in the dark, these IND samples were analyzed by HPLC (Figure 1).

In the IND-S1 chromatogram (A) a major peak was observed with retention time (Rt)= 6.83 min (78.19%) and two peaks with Rt= 1.73 min (10.47%) and 1.64 min (8.26%). IND-S2 (B) showed a major peak with Rt=6.94 (67.43%) and two peaks with Rt=1.79 min (15.40) and Rt=1.63 min (13%).

Finally, for IND-F (C) sample a major peak with Rt= 6.81 min (73.09%) and two peaks with Rt=1.79 min (12.84%) and 1.64 min (10.12%) were observed. The main peak of each chromatogram corresponds to IND (**1**) (Rt= 6.83, 6.94 and 6.81 min, respectively); the other two peaks observed correspond to the main hydrolysis products of IND; 4-chlorobenzoic acid (**2**) and 5-methoxy-2-methyl-3-INDoleacetic acid (**3**) (Figure 2); these components INDicate a slow decomposition of the product during its storage, especially if it is in a humid environment [33,34].

According to Krasowska [35], who determined some pharmacokinetic parameters of IND. Non-ionic polysorbates (such as Tween 80) increase their solubility through the formation of micelles and do not affect their gastrointestinal absorption in rats when used at 5 or at 10% in buffer solution administered intraperitoneally with a maximum time, Tmax=1.5 hours. Nevertheless; this drug (IND) is readily absorbed in ionized form at pH 4 and 8 with maximum plasma concentration (Cmax) = 34.84 μg/mL, almost the same as at pH 8 with Cmax = 36.43 μg/mL. Therefore, the route of administration and the vehicle can also affect the bioavailability of the drug in biological assays.

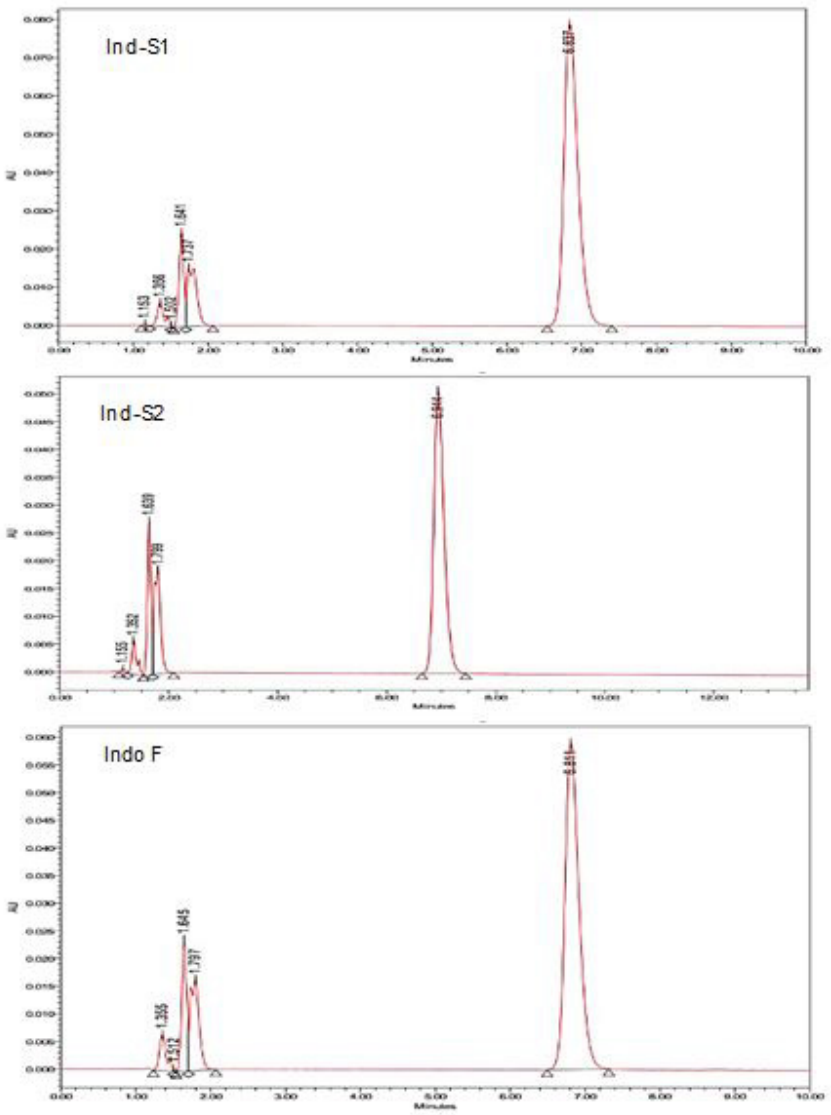

Figure 1: HPLC chromatogram of three sample of IND

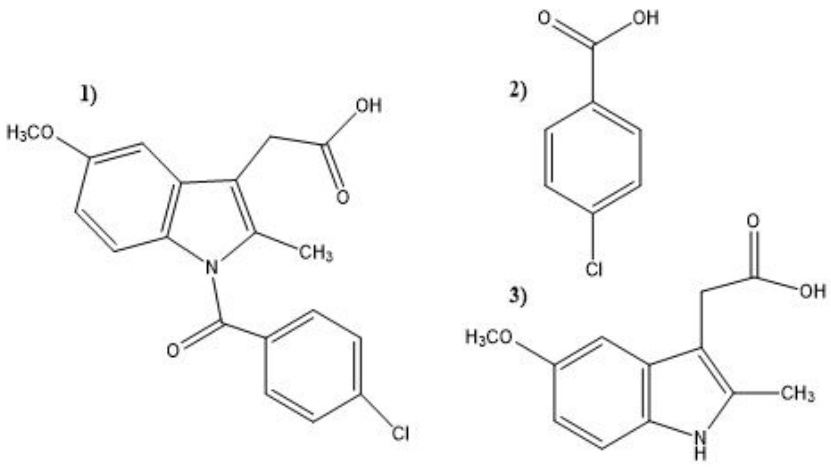

Figure 2: Chemical structure of the IND and its metabolites

Indomethacine (1), 4-chlorobenzoic acid (2) and 5-methoxy-2-methyl-3-Indoleacetic acid (3)

Acute anti-inflammatory Activity Assayed by TPA Model

On the other hand, topical anti-inflammatory activity was evaluated in the TPA model in female Balb/C mice; in this test the following results were obtained. IND-S1 showed an ED_{50}= 1.31 mg/ear (Table 3); however, at 2 mg/ear, 56.41% inhibition was obtained, so it is decided to use this dose to evaluate the three batches of IND. The results obtained shown that the percentage of inhibition of ear edema of IND-S1, IND-S2 and IND-F (2 mg/ear) was 87.39, 90.13 and 75.87%, respectively (Table 4). It was observed that the percentage of inhibition was not constant in this second test and the percentage of inhibition obtained was greater than that of the first test (56.41%, at 2 mg/ear). Gutiérrez-Rebolledo et al. [29] reported an ED_{50}=2.05 mg/ear, with 53.62% inhibition at a dose of 2 mg/ear. In the TPA test, acetone was used as a vehicle, the IND was totally soluble according to its lipophilic characteristics; this vehicle is used in all TPA test and is known not to cause skin irritation [36]. IND has been shown to be more effective in the topical model of inflammation. In histological sections of the ears treated with TPA, it is observed that IND decreases cell migration, reduces myeloperoxidase levels, which are increased in the inflammatory process [37].

Table 3: Anti-inflammatory activity of IND-S1 in female Balb/C mice (TPA model)

Treatment	Dose(mg/ear)	Auricular edema (mg)	Inhibition%	ED_{50}(mg/ear)
TPA	-	14.87 ±0.85	-	-
IND-S1	2 1 0.5	6.48 ±0.59[a] 8.02 ±0.84[a] 10.06 ±1.42[a]	56.41 46.08 32.32	1.31 R^2=0.99

Each group represents the mean (±) and the standard error (SE). The ANOVA statistical analysis followed by a Dunett's test, ($p \leq 0.05$) avs TPA; n=5

Table 4: Anti-inflammatory activity of the sample of IND in female Balb/C mice (TPA model)

Treatment	Dose (mg/ear)	Auricular edema (mg)	Inhibition%
TPA		9.12 ±0.98	-
IND-S1 IND-S2 IND-F	2 2 2	1.15 ±0.27[a] 0.9 ±0.28[a] 1.65 ±0.48[a]	87.39 90.13 75.87

Each group represents the mean (±) and the standard error (SE). The ANOVA statistical analysis followed by a Dunett's test, ($p \leq 0.05$) avs TPA; n=5

However, the inconsistency in the results may be due to some experimental limitations. In this work, the topical application was carried out in the ear of the animal without anesthetizing, but due to the animal's reflex, the drug can be removed from the ear; some authors use anesthesia during the procedure [37]. Therefore, it is recommended to anesthetize the animals to perform this experiment. For this assay it is recommended to use 2 mg/ear.

In this assay, chronic inflammation was induced in the pinnae of mice by applying TPA in repeated doses (2.5 µg/ear, 25 µL) every third day for 9 days and one day later the animals were sacrificed. In a first experiment, IND-S1 at 10 mg/kg was administered by i.g. route every third day for 9 days, in this assay all animals (10/10) died one day after the first administration. A second experiment was carried out with a dose of 8 mg/kg, administered by the same route (i.g.), in this case 4/10 animals died in a time of 9 days. At this dose, IND showed a 60.63% inhibition of edema ear (n=6). It should be noted that when IND was administered at 8 mg/kg by i.g. route, a good anti-inflammatory activity was observed, but almost 50% of the mice died.

Subsequently, an other experiment was carried out, in which TPA and IND were administered topically. IND at 2, 1 and 0.5 mg/ear was administered during 9 days. In this assay, IND at 2 mg/ear caused the death of the animals, 1/10 mice died on day 1, 4/10 mice died on day 3 and 5 mice died on day 6; only a one mouse survived. In this assay, all mice also died when IND was administered at 1 mg/ear, in this case 4/10 mice died on day 3 and 6/10 died on day 6, and at a dose of 0.5 mg/ear a mortality of 3/6 animals was observed on day 3 and 3/6 mice died at day 6. At these three doses tested, the animals presented stooping, evident piloerection and loss of body weight; in addition, they were observed to be lethargic during the study period (9 days). In this study, the percentage of inhibition could not be calculated because practically all animals died.

It is important to point out, that IND (20 mg/kg) has been reported to inhibit gastric COX by 95% in normal rats; 15 min after administration, it causes changes in the integrity of the vascular endothelium[38]. In addition, IND (5 mg/kg) administered i.g. in rats during 14 consecutive days causes sluggishness, diarrhea with some mortalities and reduced food intake; eventually leads to hepatic necrosis with increased serum ALP and AST [39]. Omogbai et al. [15] reported that IND showed an LD_{50} = 12.58 mg/kg in rats when was adminstered by i.g. route duting 7 days. On the other hand, Olusegun and Lawal [17] described that IND caused anorexia, dehidratation and weakness and 100% mortalitiy was observed between 24 to 96 hours in rats and mice and found that the IND showed a LD_{50} = 21.5 and 15.2 mg/kg by Norway rats and albino mice, respectively

Finaly, Stanley et al. [28] reported that for this test, IND applied topically at 0.1% (w/v) and other COX inhibitors did not prove to be effective when an inflammatory lesion is already established in the chronic process of TPA. Alonso-Castro et al. [40] reported a 55% edema inhibition in the chronic model of TPA (2.5 μg) when IND at 8 mg/kg was administered by i.g. in CD-1 mice. In this assay the drug IND were orally administered 30 min prior to the application of TPA every 48 h during 10 days.On the other hand, IND applied topically at a dose of 1% in gel (equivalent to 10 mg/kg of the active substance) daily for 3 weeks reduces knee inflammation and IL-6 levels in a model of knee-INDuced osteoarthritis in female Wister rats [41].

Conclusion

IND is a drug widely used in inflammatory processes such as arthritis, osteoartritirs and other inflamatory disease. In addition, it is widely used as a control in *in vivo* models of acute and chronic inflammation, such as carrageenan and TPA, to search compounds (natural and/or synthetic) with anti-inflammatory activity. However, variation in effectiveness may occur in each experiment due to the chemical nature of this drug; Therefore, it is suggested to store in airtight containers in a place free of moisture, under refrigeration, and monitor its stability by means of HPLC analysis.

The systemic model (carrageenan assay) and the topical model with TPA, as well as the chronic model of TPA (repeat doses), are suitable for the search for molecules with anti-inflammatory potential. These experimental models allow knowing the effect of a substance by topical and systemic route. However, it is necessary to take into account the vehicle to be used and the route of administration, as well as keeping the IND (reference drug) in adequate conditions. For the chronic model, it is necessary to standardize this technique because the doses of IND used are not recommended to be administered in repeated doses.

Conflict of Interest

The authors declare that they have no conflicts of interest.

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