

# Study of copaiba oil on behavioral testing in rodent models by inducing neuropathic and inflammatory pain

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## ABSTRACT

**Objective:** The *Copaiba* sp. oil has been known to possess anti-inflammatory, antiulcer, antiviral and others activities. The copaiba oil pharmacological activity was assessed using models of nociception in mice. The involvement of both megakaryocytes and enzyme markers for muscle damage under acute copaiba oil treatment on acetic acid (AA)-induced abdominal constriction was investigated. **Materials and Methods:** Swiss mice and/or Wistar rats were pretreated orally (2 h) before all experimental procedure with *Copaifera officinalis* oil or vehicle. The abdominal constriction response was induced by intra-peritoneal injection of AA 0.6% and the number of abdominal constrictions was counted cumulatively over 60 min. Behavioral nociception models, i.e., hot plate and tail-flick test, were also performed. Plasma enzymes (creatine kinase [CK]; aspartate aminotransferases [AST] and alanine aminotransferases; [ALT]) and organs histology were analyzed too. **Results:** Copaiba oil administration caused a significant reduction on the AA-induced writhing in the rodents. It also presented significant antinociceptive activity in the behavioral models. Plasma CK levels and megakaryocyte production were significantly augmented in the copaiba oil-pretreated animals when compared with control group. AST and ALT remained unchanged. **Conclusion:** Our results indicate that copaiba oil may act as an anti-nociceptive agent and induce plasmatic CK increase by a mechanism involving probably the splenic overproduction of megakaryocytes, suggesting this way the participation of immature platelets in the process.

**KEY WORDS:** Anti-nociceptive, *Copaifera*, *Copaiba*, megakaryocyte, muscular enzymes, writhing

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## INTRODUCTION

Various animal models of neuropathic and inflammatory pain are established and acute visceral pain induced by acetic acid (AA) is an animal model that mimics some of the acute inflammatory responses seen in muscular injury [1-5]. *Copaifera* sp., popularly known as “copaiba,” is a large tree that grows abundantly in the Amazonas. The oil-resin of this plant is a popular remedy, in its natural form. *Copaifera officinalis* was the first species in the genus *Copaifera* to be described [6-8]. Many studies using experimental pharmacological models have established the anti-inflammatory, gastroprotective, wound healing and others activities of copaiba oil [9-13].

Many muscular, neuromuscular [14-17] and mitochondrial diseases [18-22] either can affect or are dependent under the control of respiration and bioenergetics during muscle contraction. The pathogenesis of many these disorders is not yet fully understood. However, neurological symptoms and signs and the underlying neuropathological changes have

been related to altered composition of cellular membranes. Therefore, it is conceivable that by different mechanisms, the damage due to free radicals results in impairment of mitochondrial functionality and adenosine triphosphate (ATP) production [23-25], and the endurance muscle performance is highly dependent on ATP production from mitochondrial oxidative phosphorylation [26-28]. A majority of ATP in the brain is formed in the mitochondria through oxidative phosphorylation of adenosine diphosphate (ADP) with the  $F_1F_0$ -ATPase enzyme. This ATP production rate plays central roles in brain bioenergetics, function and neurodegeneration [29]. Moreover, dysfunction of the blood-brain barrier (BBB) causes many pathological conditions and is modified by diseases that are not primarily caused by BBB dysfunction. Very little is known about how metabolism influences the BBB at the molecular level [30,31].

Brain vasculature is anatomically distinct from that of other organs and comprises, in addition to endothelial cells, pericytes and astrocytes which collectively form the neurovascular unit

(NVU). The molecular mechanism involved in the development of the brain vasculature and human genetics diseases are primarily affected by dysfunction of components of the NVU. Therefore, the study of NVU supplies intimate knowledge about the physical and functional connection between the brain tissue and blood vessels. Basement membrane is an important regulator of epithelial cells and is essential for maintaining tissue integrity of brain vasculature. Mouse genetic studies have shown that basement membrane is important for maintaining vessel stability and the lack of individual matrix components is a cause of hemorrhages due vascular fragility [32]; In human, genetic defects in the gene encoding a component of collagen IV lead to nephropathy and hemorrhagic stroke [33,34].

Furthermore, muscular dystrophy is a group of genetic diseases in which muscle fibers are unusually susceptible to damage. These damaged muscles become progressively weaker. Magnetic resonance imaging analysis of the brain in patients with merosin-deficient congenital dystrophy has shown the presence of vasogenic edema [35,36]. Most people who have muscular dystrophy will eventually need to use a wheelchair. There are many different kinds of muscular dystrophy. Symptoms of the most common variety begin in childhood, primarily in boys. Other types of muscular dystrophy don't surface until adulthood. People who have muscular dystrophy may have trouble breathing or swallowing. Their limbs may also draw inward and become fixed in that position: A problem called contracture. Some varieties of the disease can also affect the heart and other organs. Although, there is no cure for muscular dystrophy, medications and therapy can slow the course of the disease [37-41].

This way, the anti-nociceptive activity of copaiba oil was assessed by using models of behavioral tests. It was also investigated the involvement of both enzymes markers for muscle damage and megakaryocyte overproduction regarding to suggest copaiba oil as a potential therapy for muscular diseases.

## MATERIALS AND METHODS

### Animals

Male Swiss mice (25-30 g) or male Wistar rats (250-300 g) were obtained from the Potiguar University, Natal, Brazil. Animals were housed in environmentally-controlled conditions ( $22 \pm 2^\circ\text{C}$ , 12 h light-dark cycle), with free access to the standard diet and tepid water. All the experimental protocols were approved by the local Ethics Committee for animal experimentation (number 001|2012) and performed in accordance with the Guidelines of the Brazilian College for Animal Experimentation (COBEA) and the Law 11,794/08; experiments adhered to the Ethical Guidelines for investigations of Experimental Pain in Conscious Animals [42].

### Plant Material, Chemicals and Drugs

The oil-resin of *C. officinalis* (copaiba oil) was obtained from all chemistry (Sao Paulo, Brazil). Sodium dipyrone was

obtained from Sanofi-Aventis (Sao Paulo, Brazil) and morphine hydrochloride from Merck (Darmstadt, Germany). All other chemicals used were of analytical grade.

### Drug Pre-treatment and Behavioral Testing

Mice or rats were pretreated orally with copaiba oil (2 ml/kg) or vehicle (0.9% NaCl) 2 h before induction of acute visceral pain and other anti-nociceptive activity models, and with dipyrone (500  $\mu\text{g}/\text{Kg}$ , oral) or morphine (5 mg/kg, intra-peritoneal) 0.5 h before the tests [13,43]

To examine acid-evoked pain responses, we used the "AA-induced writhing test." Writhing was induced by intra-peritoneal injection (100  $\mu\text{l}$ ) of AA 0.6% [44]. Plasma and tissue samples were taken from mice at the end of all experimental protocol. "Hot plate" and "tail-flick" methods were used to test thermal hyperalgesia. Rats or mice were habituated to the experimental environment for 30 min in their home cage. Then they were placed on a hot plate ( $55 \pm 1^\circ\text{C}$ ) and the time until the rodent jumped or licked either of its hind paws was recorded as a hot plate latency. Following a response, the test animal was immediately removed from the plate [45,46].

Mechano-nociception was evaluated by the tail-flick test. Animals were placed on a box and its tail under a bulb. A radiant heat tail-flick analgesiometer was used to measure response latencies according to the method described previously [47,48]. The reaction time was recorded for control (saline injection) animals or in animals pretreated with copaiba oil (2 ml/kg, p.o.) or with morphine (5 mg/kg, i.p.) 0.5 h before the tests. All animals were selected 24 h before the test on the basis of their reactivity in the model by eliminating those that remained on the apparatus for up to 8 s. A latency period (cut-off) of 20 s was defined as complete analgesia.

### Histology

Tissues were collected from control and treated mice ( $n = 3$ ) for each experimental group, fixed in 10% neutral formalin, and stained by either hematoxylin and eosin or Masson's trichrome. The organs architecture and inflammatory infiltration were evaluated.

### Biochemical Analysis

Aspartate and alanine aminotransferase (AST and ALT) and creatine kinase (CK or CPK) plasmatic enzyme activities were evaluated at Clinical Analysis Laboratory from UNP and determined by commercial kits from Roche (Germany).

### Statistical Analysis

Data are expressed as means  $\pm$  standard error of mean and comparisons among groups were performed using one-way analysis of variance, followed by the Student-Newman-Keuls test for multiple comparisons. Statistical significance level was considered as  $P < 0.05$ .

## RESULTS

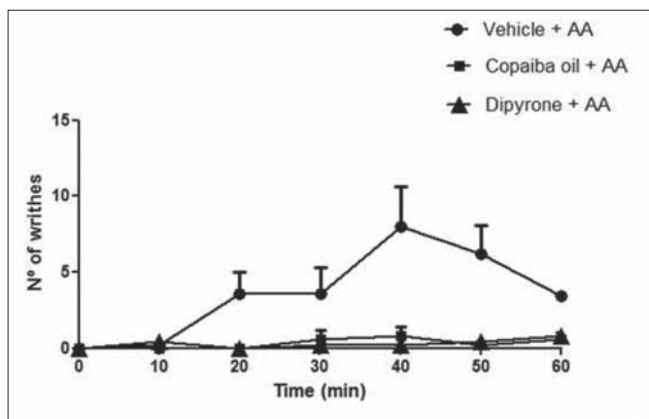
### Behavioral Testing Models

#### AA-induced Writhing

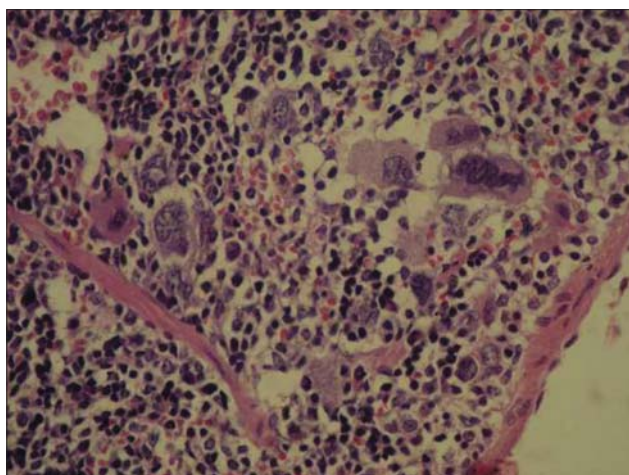
The injection of AA (100  $\mu$ l/mice) markedly increased the writhing in mice than that observed in the vehicle group ( $P < 0.05$ ), thus reproducing a real acute abdominal constriction response [Figure 1], which was accompanied by inflammatory activity confirmed by congestion and intracellular edema in all organs collected. Copaiba oil pre-treatment significantly ( $P < 0.05$ ) reduced AA-induced writhing to level near with the vehicle group; moreover, an overproduction of megakaryocytes by spleen was observed only in this group [Figure 2].

#### Hot Plate and tail-flick Assays

Copaiba oil pre-treatment caused a marked increase in the pain latency in both algometer assays, hot plate and tail-



**Figure 1:** Acetic acid (AA)-induced writhing. Copaiba oil and dipyrene pretreatment significantly reduced AA-induced writhing in mice when compared to levels to those of the AA plus vehicle group ( $n=5$ ). Both treatment modalities significantly differed from the vehicle-treated group ( $P < 0.01$ )



**Figure 2:** Spleen histology. Overproduction of megakaryocytes by spleen (H and E,  $\times 400$ ) in copaiba oil pretreated group and submitted to acetic acid-induced writhing in mice ( $n = 3$ )

flick, compared to levels observed in vehicle-treated group [Figures 3 and 4].

### Plasma Enzymes AST, ALT and CK Activity

The CK activity was significantly increased in both copaiba oil and dipyrene-treated group when compared with levels observed in vehicle-treated group. No statistically different change was observed in AST and ALT activity for all experimental groups [Figure 5 and Table 1].

## DISCUSSION

AST (EC 2.6.1.1) and ALT (EC 2.6.1.2) are enzymes found mainly in the liver, but also present in red blood cells, heart cells, muscle tissue and other organs, such as the pancreas and kidneys. AST and ALT formerly are called serum glutamate oxalacetate transaminase and serum glutamate pyruvate transaminase, respectively. The levels of AST and ALT in serum can also help in the diagnosis of muscular injury [49,50].

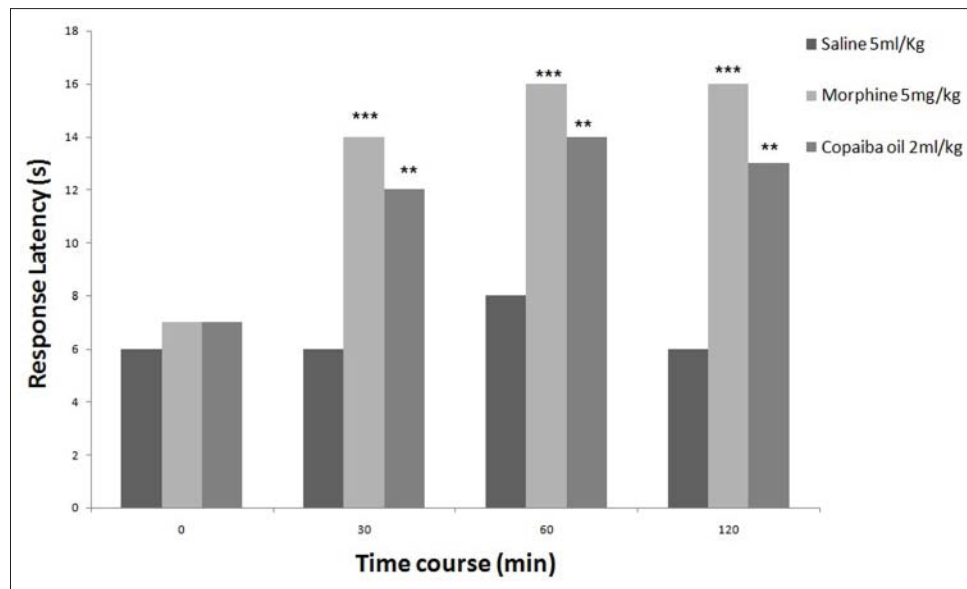
CK is an intracellular enzyme found in a variety of striated and smooth muscles and brain; it is an important enzyme regulator of high-energy phosphate production and utilization in contractile tissues. Normally, very little CK is found in the circulating blood. Elevated levels indicate damage to either muscle or brain; possibly from a myocardial infarction (heart attack), muscle disease or stroke [51-53]. Although not all myopathies produce a rise in CK activity, it is found to be increased as a result of muscle fiber destruction after mechanical trauma, toxic injury or alteration of enzymatic or structural proteins. In addition, sera from patients with muscle disease contain an inhibitor of CK that may lead to underestimation of CK activity. This suggests that the CK inhibitor may be released into serum from injured muscle [54]. CK elevation varies within disorders, with increases that may range from 2 to 100-fold of the reference value. Blood can be sampled from animals using different techniques with differing impacts on animal discomfort due to differences in handling, restraining, anesthesia, invasiveness and the volume taken. The method of blood sampling can also affect the outcome of blood analysis [55].

In the current study, the copaiba oil administration caused a significant reduction on the AA-induced writhing in mice, and

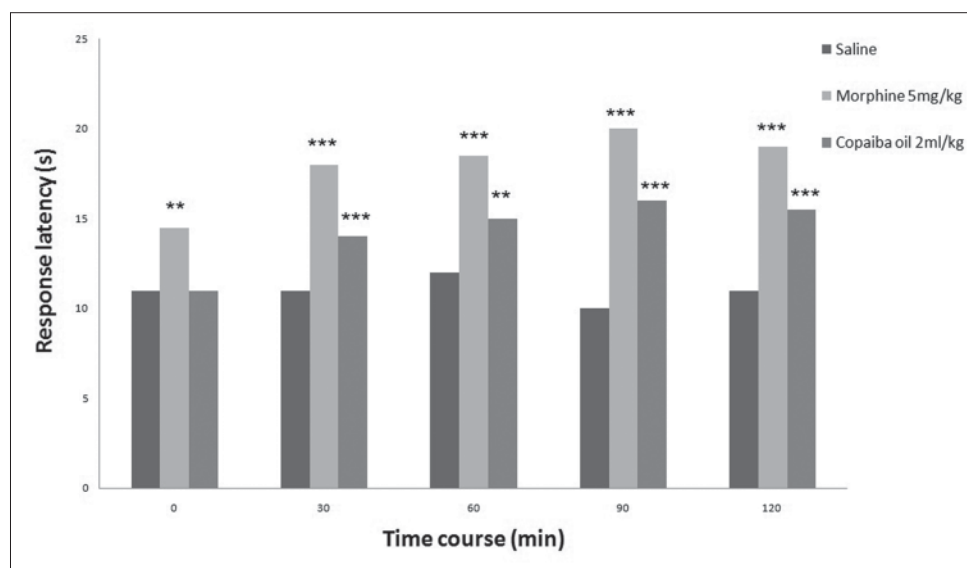
**Table 1:** Measurements of AST, ALT and CK enzyme plasma activity in AA-induced writhing in mice ( $n=5$ ) and pretreated with copaiba oil

Groups	AST (UL)	ALT (UL)	CK (UL)
AA	151 $\pm$ 15.25	62 $\pm$ 7.844	547.8 $\pm$ 55.49
AA+copaiba oil	248.6 $\pm$ 36.08	55.13 $\pm$ 5.31	1332 $\pm$ 302.1***
Copaiba oil	144.4 $\pm$ 21.36	44.57 $\pm$ 2.78	485.2 $\pm$ 145.6
AA+dipyrene	219.1 $\pm$ 43.91	49.83 $\pm$ 6.204	1100 $\pm$ 142.5***
Dypirone	145.6 $\pm$ 26.84	46.40 $\pm$ 7.42	414 $\pm$ 128.6
Vehicle	137.8 $\pm$ 23.64	72.17 $\pm$ 12.73	522.8 $\pm$ 83.39

Data are expressed as mean $\pm$ SEM of 5 mice. \*\*\* $P < 0.001$  compared to mice injected with saline (vehicle) AST: Aspartate aminotransferases, AA: Acetic acid, ALT: Alanine aminotransferases, CK: Creatine kinase, SEM: Standard error of the mean



**Figure 3:** Hot plate assay. Copaiba oil pretreatment caused a significant increase in pain latency response in hot plate test similar to level observed to morphine in algometer assay ( $n = 5$ ).  $**P < 0.01$  and  $***P < 0.001$  compared to the vehicle-treated group

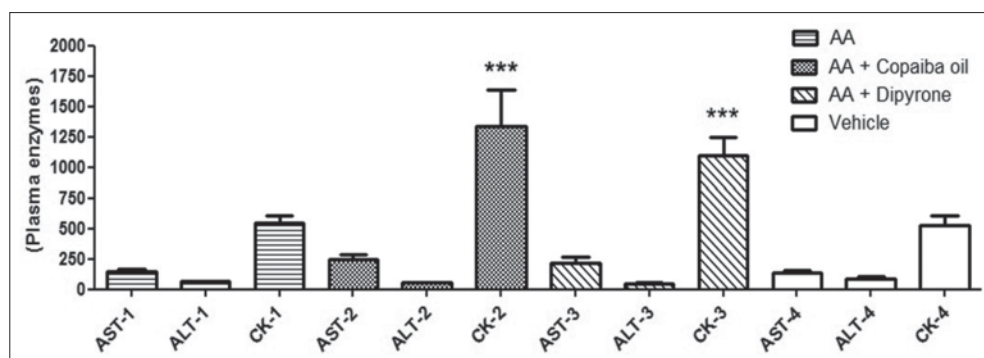


**Figure 4:** Tail-flick assay. Copaiba oil pretreatment caused a significant increase in pain latency response in tail flick test similar to level observed to morphine in algometer assay ( $n = 5$ ).  $**P < 0.01$  and  $***P < 0.001$  compared to the vehicle-treated group

on the other hand it has a significant anti-nociceptive activity under behavioral testing models in mice and rats. The plasma CK levels and the megakaryocytes production were augmented in both copaiba oil and dipyrone-pretreated group submitted to AA-induced writhing when compared to the vehicle control group. However, AST and ALT presented no significant change in any of the pretreated groups compared with vehicle control group. Shibata and Kobayashi [56] demonstrated that substantial amounts of CK leaked out of rat platelets and suggested that CK might contribute to some reactions, which consume ATP during aggregation, presumably donating the energy for shape change. The elevation of CK activities in whole blood was attributed to the liberation of these enzymes from platelets during the clotting process before centrifugation,

and also occurs to lactate dehydrogenase, AST and others [57,58]. This increase might not be observed in other species such as dogs, as previously reported [59]. The elevated CK from platelets might be of the brain-type (CK-BB), as Meltzer and Guschwan [60] only found this isoenzyme in rat platelets. Some authors recommend the use of plasma in studies using laboratory animals, particularly rodents, in view of the marked release of intracellular substances from blood corpuscles, platelets in particular, during blood coagulation [61,62].

There are three types, or isoforms, of CK: CK-I or BB, is produced primarily by brain and smooth muscle; CK-II or MB, is produced primarily by heart muscle, and CK-III or MM, is produced primarily by skeletal muscle [52,63]. Supporting “the



**Figure 5:** Aspartate aminotransferases (AST), alanine aminotransferases (ALT) and creatine kinase (CK) plasma enzymes. Mice submitted to acetic acid-induced writhing and pretreated with copaiba oil or dipyrrone presented increased CK levels when compared to levels observed in vehicle group. No significant changes were detected in AST, ALT ( $n = 5$ ). \*\*\* $P < 0.001$  compared to the vehicle-treated group

privileged access” of ATP to the enzyme, it has been shown that mitochondrial CK reacts slowly with externally added ATP, but rapidly utilizes ATP newly synthesized by oxidative phosphorylation [64].

In current respiratory and bioenergetics muscle control models, the analysis has been focused on the influence of ADP, ATP, nicotinamide adenine dinucleotide dehydrogenase or  $O_2$  on  $VO_2$  in either the kinetic or thermodynamic formulation, especially with respect to a rate-limiting substrate or enzyme activity. The analysis provides continuity between past and present studies, but overlooks a fundamental issue: The tight interaction of cellular metabolite flux militates against a simple reduction of all enzyme reactions to a rate-limiting step. Indeed, metabolic control analysis has pointed out this weakness and has introduced control coefficient and elasticity terms to characterize metabolic flux during muscle contraction [65-69].

In addition, megakaryocytes express all the known eight subtypes of P2Y receptors and constitutively release ADP. Balduini *et al.* [70] made the first demonstration that ADP released by megakaryocytes regulates their function by interacting with P2Y<sub>13</sub>. They demonstrated that the platelet count of patients with congenital delta-storage pool deficiency lacking secretable ADP was significantly lower than that of patients with other platelet function disorders, confirming the important role of secretable ADP in platelet formation. Platelets also release substances that promote tissue repair and influence the reactivity of vascular and the other blood cells in angiogenesis and inflammation [71]. In addition, red blood cells are understood to enhance interactions between leukocytes and endothelial adhesion molecules by pushing the larger leukocytes to the periphery [72]. Leukocyte migration to the sites of inflammation occurs through specific interactions with the vascular endothelium under conditions of shear stress [73]. Previously our team demonstrated the capacity of copaiba oil treatment to reduce leukocyte migration and edema in caerulein-induced pancreatitis in mice [13].

The medical impact of pain, mainly muscular pain, is such that much effort is being applied to develop novel analgesic drugs directed toward new targets and to investigate the analgesic efficacy of known drugs. Ongoing research requires

cost-saving tools to translate basic science knowledge into clinically effective analgesic compounds. Experimental models can be developed into truly predictive tools saving costs for analgesic drug development, and provide expert knowledge about; (i) the pharmacological actions of analgesic drugs, (ii) physiological bases of the experimental pain models and (iii) the pathophysiology and pathopsychology of clinical pain [74-79]. Our results indicate that copaiba oil may act as an anti-nociceptive agent and induce the plasmatic CK increase by a mechanism involving, probably, megakaryocytes overproduction by the spleen, suggesting the involvement of immature platelets in this process; however, these data need to be better investigated in the future.

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