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Role of adenosine receptors in the anti-nociceptive effects of allopurinol in mice

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Abstract. – BACKGROUND: Inhibition of xanthine oxidase by allopurinol increases hypoxanthine and xanthine, which are converted to purines, including the inhibitory neuromodulator adenosine.

AIM: We aimed to investigate the antinociceptive effects of allopurinol in thermal and chemical pain models in mice and to evaluate its possible antinociceptive mechanism by using selective adenosine receptors A1, A2A antagonists in mice.

MATERIALS AND METHODS: Sixty four adult male mice were used. Mice received an intraperitoneal injection of vehicle or allopurinol (50-200 mg/kg). Assessment of antinociceptive effects and locomotor activity were performed in three models of acute pain; a thermal model and two chemical model.

RESULTS: Allopurinol presented dose-dependent antinociceptive effects in all models with no obvious motor deficits. The opioid antagonist naloxone did not reverse these effects. The selective A1 antagonist, DPCPX, and the selective A2A antagonist, ZM241385, completely prevented allopurinol-induced antinociception.

CONCLUSIONS: Allopurinol-induced antinociception may be related to adenosine accumulation. Allopurinol seems to be well tolerated with no locomotor side effects at high doses and it may be useful to treat pain syndromes.

Key Words:
Allopurinol, Pain, Anti-nociceptive effect, DPCPX, ZM241385, Adenosine.

Introduction

Allopurinol is the first line treatment for gout. Allopurinol and its major metabolite oxypurinol inhibit xanthine oxidase (XO), the enzyme responsible for the formation of uric acid from hypoxanthine and xanthine[1].

In addition to blocking uric acid production, inhibition of XO causes an increase in hypoxanthine and xanthine, which are converted to closely related purines[1], including the inhibitory neuromodulator adenosine. It is believed that adenosine plays a role in promoting sleep[2], regulating synaptic activity and release of neurotransmitters such as noradrenaline, dopamine, serotonin, acetylcholine and glutamate[3]. These effects may contribute to its beneficial anticonvulsant and antipsychotic effects[4].

Cellular signaling by adenosine and its metabolite inosine occurs through four known adenosine receptor subtypes A_1, A_{2A}, A_{2B}, and A_3. Anti-nociceptive effect of adenosine may be related to inhibition of intrinsic neurons by an increase in potassium conductance and pre-synaptic inhibition of sensory nerve terminals, decreasing the release of substance P and glutamate[5]. They also inhibit the release of pro-inflammatory cytokines and chemokines in activated macrophages and protect against lung tissue damage and skeletal muscle reperfusion injury in mice. Inosine also has immunomodulatory and neuroprotective effects[6].

It was proved that adenosine A_1 receptor agonists produce a pronounced antinociception[7]. The role of the A_{2A} receptors in nociception has been intensely debated. It has been demonstrated that A_{2A} receptor antagonists showed consistent antinociceptive activity[8], while Borghi et al[9] proved that some A_{2A} receptor agonists can induce antinociceptive actions in mice. A_{2A} receptors have also an antinociceptive role against inflammatory and neuropathic pain tests[10]. Previous studies showed that A_{2B} and A_3 receptors are not involved in the antinociceptive effects of adenosine[11].

Based on the facts regarding the role of allopurinol in reducing purine degradation, it could be a method in managing pain associated with many disorders. Allopurinol may be a useful agent to combine with other analgesics which act predominantly on non-adenosine systems thus lowering their dosage and limiting the implication of their unwanted effects.

For these reasons, we aimed to investigate the antinociceptive effects of allopurinol against thermal and chemical models of pain. In addi-

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tion, this study evaluated the possible mechanism of action of allopurinol in pain relief by using selective adenosine A_1, A_{2A} antagonists in mice.

Materials and Methods

Animals

Sixty four adult male albino mice (25-30 g) were obtained from The Egyptian Company for Production of Vaccines (Cairo, Egypt) and used in this study. Animals were housed under controlled environmental conditions; normal light-dark schedule, temperature of 22 ± 1°C, in stainless steel cages (eight per cage), with free access to food and water and allowed for acclimatization before the start of the study for one week.

The study was approved by the Institutional Animal Care and Use Committee and carried out in accordance with the current guidelines for the care of laboratory animals and the ethical guidelines for investigations of experimental pain in conscious animals[12]. The number of animals used and intensities of noxious stimuli used were the minimum necessary to demonstrate the consistent effects of the drug treatments. All behavioral procedures were conducted between 8:00 and 10:00 a.m. In all experiments of nociceptive behavioral, the animals were acclimatized to the laboratory for at least 1 h before testing.

Drugs and Chemicals

Allopurinol was purchased from {GlaxoWellcome, GlaxoSmithKline (GSK.CO. UK)}. Normal saline solution (sodium chloride 0.9%) was purchased from (Nile Co., Cairo, Egypt). Morphine sulphate 10 ml of 20% ampoules and naloxone, 1, 3-dipropyl-8-cyclopentylxanthine (DPCPX) and 4-(2-[7-amino-2-(2-furyl)[1,2,4]triazolo[2,3-a][1,3,5]triazin-5-ylamino]ethyl)-phenol (ZM241385) were purchased from (Sigma Chemical Co., St. Louis, MO, U.S.A.). Capsaicin, acetic acid and dimethyl sulphoxide (DMSO) were purchased from the Egyptian International Pharmaceutical Industrial Company (EIPICO).

Thermal Pain Model (Hot-Plate Test)

In this experiment, the hot-plate apparatus (Ugo Basile, model-DS 37, Varese, Italy) was maintained at 55 ± 0.5°C. Animals were placed into a glass cylinder of 24 cm diameter on the heated surface, and the time between placing of the animal on the hot-plate and the occurrence of licking of hind paws or jumping off the surface was recorded as response latency. On day one, the animals were first habituated to the apparatus. On day two, mice were tested and animals displaying baseline latencies of more than 15 seconds were excluded from the study. An automatic 20 seconds cut-off was used to prevent tissue damage[13]. Percent analgesia was also calculated with the help of following formula[13]:

$$\% \text{ Analgesia} = \frac{(\text{Test latency} - \text{control latency})}{(\text{Cut-off time} - \text{control latency})} \times 100$$

Chemical Pain Model (Capsaicin-Induced Nociception)

Twenty μL of capsaicin dissolved in 5% DMSO (1.6 μg per paw) was injected intra-plantarly (i.pl), under the plantar skin of the right hind paw (Hamilton microsyringe with a 26-gauge needle). Animals were observed individually for five minutes after capsaicin administration for the time spent licking the injected paw, which was recorded and considered a measure of nociception[14].

Writhing Test

Abdominal constriction is a contraction of the abdominal muscle together with a stretching of the hind limbs in response to an i.p. injection of 0.6% acetic acid (1 ml/kg body weight) at the time of the test. After the challenge, mice were individually placed into glass cylinders 20 cm in diameter, and abdominal constrictions were counted cumulatively over a period of 20 minutes[15]. Antinociceptive activity was expressed as the reduction in number of abdominal constrictions compared with those of the control groups. Percent protection against pain was calculated with the help of following formula:

$$\% \text{ Protection} = \frac{(1 - \text{Mean no. of abdominal constrictions of treated drug})}{\text{Mean number of abdominal constrictions of control)}} \times 100$$

Measurement of Motor Performance

In order to evaluate non-specific effects of allopurinol on locomotor activity, we evaluated its effects in the rotarod test and in spontaneous locomotor activity test 30 minutes after i.p. treatments with i.p. allopurinol or vehicle.

The Rotarod Test

Rotarod apparatus (Ugo Basile, Varese, Italy) consists of a rotating (18 r.p.m.) bar (2.5 cm di-

ameter), subdivided by disks into six compartments. As previously described,[16] mice were initially trained to remain on the rotarod apparatus. On the day of experiment, the latency to fall from the rotarod (one trial with a maximum of 60 seconds) was determined.

Spontaneous Locomotor Activity Test

The open-field test was used to rule out the possibility that the antinociceptive action of allopurinol could be related to nonspecific disturbances of the locomotor activity of the animals. The ambulatory behavior was assessed in an open-field test as described previously[17]. The apparatus consisted of a wooden box measuring 40 × 60 × 100 50 cm. The floor of the arena was divided into 16 squares. The number of squares crossed with all paws (crossing) was counted in a 6 min session. The apparatus was cleaned with a solution of 10% ethanol between tests to hide animal clues.

Study Design

Twenty minutes before the experiment, animals were placed in polyethylene cages, which also served as observation chambers. After this adaptation period, animals were divided into eight groups (eight animals each) and treatments were performed by i.p. injection.

Groups one: Mice received (10 ml/kg) of vehicle (saline with 5% DMSO), negative control group.

Group two: Mice received morphine sulphate (6 mg/kg), positive control group[18].

Groups three, **four and five**: Mice received allopurinol (50, 100 and 200 mg/kg respectively) dissolved in normal saline (0.9% sodium chloride) thirty minutes before the experiments. All animals were exposed to all of the three tests but on different occasions.[19]

Groups six, **seven and eight**: In order to investigate the antinociceptive mechanism of action of allopurinol, animals in these groups were pre-treated (15 minutes in advance) with naloxone; a non-selective opioid receptor antagonist (1 mg/kg)[18], DPCPX; a selective adenosine A_1receptor antagonist (0.1 mg/kg) or ZM241385; a selective adenosine A_{2A} receptor antagonist (3 mg/kg)[11] respectively, then mice were given allopurinol (200 mg/kg).

Thirty minutes later, after drug administration, all animals were subjected to hot plate test, capsaicin and acetic acid injections in three different occasions. DPCPX and ZM241385 were dissolved in saline with 5% DMSO. The final concentration of DMSO did not exceed 5% and did not cause any effect per se[11].

Statistical Analysis

All data were expressed as mean ± S.E.M. and analyzed using the Statistical Package of Social Sciences program (SPSS Inc., Chicago, IL, USA), version 17. All the comparisons between groups were carried out using one-way analysis of variance (ANOVA) followed by post-hoc multiple comparison; bonferroni test, to test the significance difference among group means. $p < 0.05$ was considered statistically significant at confidence interval 95%.

Results

Antinociceptive Effect of Allopurinol

In hot plate test, mice treated with allopurinol (50-200 mg/kg) or morphine showed a significant ($p < 0.05$) increase in the latency time in seconds (hot plate test) (Figure 1A) compared with mice treated with vehicle (control). In accordance, the percent protection against pain in hot plate test was increased significantly ($p < 0.05$) from 22.6% ± 1.9%, 33.25% ± 2.7% and 43.9% ± 3.1% in allopurinol-treated mice respectively to 77.18% ± 4.5% in the positive controls.

In capsaicin test, the results of the current work showed the ability of allopurinol to induce a significant ($p < 0.05$) and dose-related inhibition of the capsaicin-induced nociception as manifested by licking time compared with mice treated with vehicle or morphine (Figure 1B).

Regarding writhing test, the results in (Figure 1C) showed also that allopurinol produced a dose-related significant ($p < 0.05$) decrease in the number of acetic acid-induced abdominal constrictions in mice compared to the control and morphine groups. The percentage of pain inhibition was significantly increased from 32.7% ± 2.4%, 43.9% ± 3.9% and 53.1% ± 4.2% according to allopurinol doses respectively to 86.1% ± 4.5% for morphine.

Effect of Naloxone and Involvement of Adenosine Receptors in Antinociceptive Effect of Allopurinol

Figure 2 shows that the non-selective opioid-receptor antagonist naloxone significantly prevented morphine induced anti-nociception, without affecting anti-nociception induced by allop-

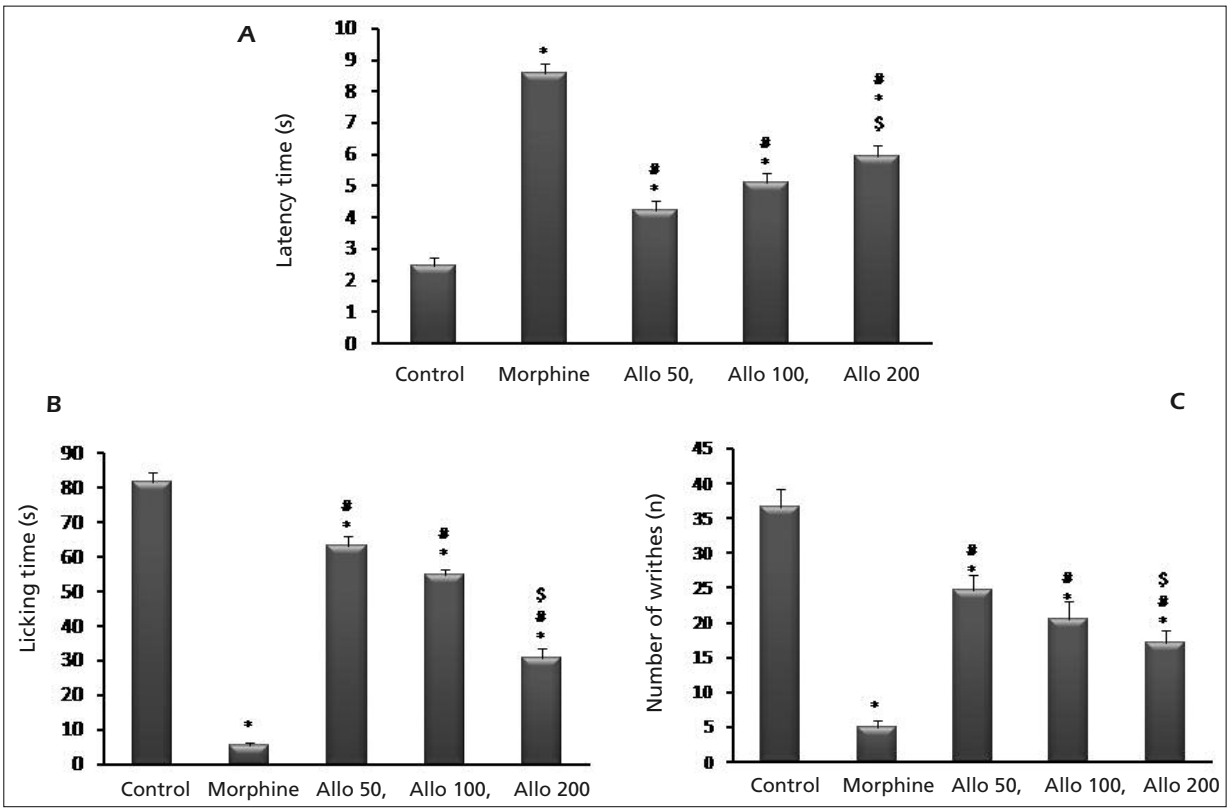

Figure 1. Anti-nociceptive effect of allopurinol (Allo 50, 100, 200 mg/kg) in ***(A)*** Latency time in hot plate (s), ***(B)*** Licking time after intraplanter capsaicin injection (s) and ***(C)*** Acetic acid induced writhes (n). C = control group. Data were expressed as mean ± SEM, analyzed by one-way ANOVA followed by Bonferroni test. $^{*}p < 0.05$ vs. control group, $^{\#}p < 0.05$ vs. morphine group, $^{\$}p < 0.05$ vs. Allo 50 group. n=8.

urinol in hot plate test (Figure 2A), in capsaicin-induced pain (Figure 2B) and in writhing test (Figure 2C).

The results depicted in (Figure 2) showed also that previous treatment of mice with DPCPX; a selective adenosine A_1 receptor antagonist or ZM241385; a selective adenosine A_{2A} receptor antagonist significantly ($p < 0.05$) reversed the antinociception caused by allopurinol in hot plate test (Figure 2A), in capsaicin induced chemical pain (Figure 2B) and in writhing test (Figure 2C) compared to allopurinol alone treated mice.

Effect of Allopurinol on Locomotor Activity

Allopurinol did not affect locomotor activity of the mice, as evaluated by the performance in the rotarod test and in the open field test compared with control group received vehicle (Table I).

Discussion

The aim of the current study was to elucidate the possible antinociceptive effect of the xanthine oxidase inhibitor, allopurinol, on different pain models in mice. Mice received vehicle, morphine or allopurinol at doses of (50-200 mg/kg). To investigate the mechanism of action of allopurinol, mice were injected in advance with naloxone, DPCPX or ZM241385.

The present study demonstrated that allopurinol produced a dose-dependent anti-nociceptive effects in the hot-plate, intraplantar capsaicin and intraperitoneal acetic acid pain models in mice. Although these animal models are essentially based on acute, short-lasting noxious stimuli, some differences between tests can be found.

Hot-plate test is a complex thermal pain model that produce two behavioral components (i.e. paw licking and jumping) considered to be supraspinally integrated responses[20]. Our results were in agreement with Inkster et al[19] who showed that allopurinol attenuated thermal pain and this effect may be related to a reduction in xanthine oxidase activity and purines accumulation. Adenosine can alter pain transmission by acting on both nociceptive afferent and transmis-

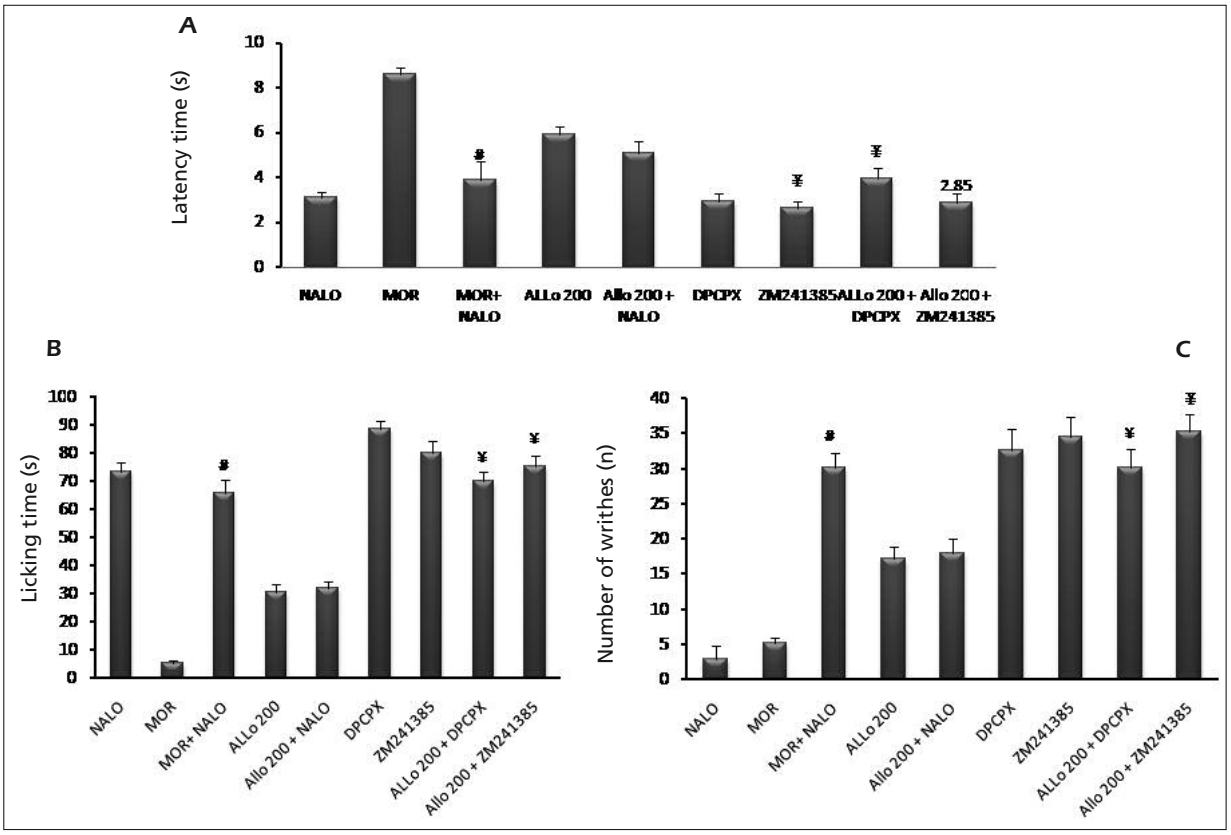

Figure 2. Effect of naloxone (NALO), on the anti-nociceptive effect of morphine (MORF) or allopurinol 200 mg/kg (Allo 200) and effect of or 8-cyclopentyl-1, 3-dipropylxanthine, (DPCPX) and {4-(2-[7-amino-2-(2-furyl)[1,2,4]triazolo[2,3-a][1,3,5]triazin-5-ylamino]ethyl)-phenol} (ZM241385) on the anti-nociceptive effect of Allo 200 in ***(A)*** Latency time in hot plate (s), ***(B)*** Licking time after intraplanter capsaicin injection (s) and ***(C)*** Acetic acid induced writhes (n). Data were expressed as mean ± SEM, analyzed by one-way ANOVA followed by post hoc Bonferroni test. $^{\#}p < 0.05$ vs. morphine group, $^{¥}p < 0.05$ vs. allopurinol treated group. n=8.

sion neurons, and these actions are mediated primarily by adenosine A_1 receptors[6]. Additional effects on inflammatory cells at peripheral sites are mediated by adenosine A_{2A}, A_{2B} and A_3 receptors also occur, and these potentially can produce indirect effects on pain transmission[20,21].

In agreement with Sakurada et al[14] who stated that intraplantar injection of capsaicin usually produces nociceptive responses, through a mechanism mediated by tachykinin and NMDA (N-methyl-D-aspartate) receptors, and morphine can ameliorate these responses, the results of the current investigation proved that allopurinol – in a dose dependent manner – decreases licking time after capsaicin injection while morphine exhibited the maximum atinociceptive effect. This can

Table I. Effect of allopurinol on the rotarod and spontaneous locomotor activity tests in mice.

Groups	Latency to fall (s)	Squares crossed (n)
Control	54.2 ± 3.1	71.9 ± 3.6
Allopurinol 50 mg/kg	55.1 ± 1.5	69.0 ± 3.1
Allopurinol 100 mg/kg	57.6 ± 2.0	68.9 ± 5.4
Allopurinol 200 mg/kg	58.7 ± 1.9	67.6 ± 2.8

Vehicle or allopurinol was given 30 min prior to the locomotor assessment: latency to fall in seconds (s) (rotarod) and number (n) of crossings (spontaneous locomotor activity). Data are expressed as mean ± SEM and analyzed by one-way ANOVA followed by Bonferroni test. $p < 0.05$ was considered significant. n = 8.

be explained as adenosines and its metabolites accumulation decrease the release of substance P and induce glutamate attenuation by NMDA-induced production of nitric oxide[1,22].

In accordance, the results reported in this study indicated significant and dose related effect of allopurinol when assessed in acetic acid-induced visceral nociception[11]. Pain sensation in acetic acid-induced writhing method is elicited by triggering localized inflammatory response resulting in release of free arachidonic acid via cyclooxygenase (COX) and so increased levels of PGE2 and PGF2□ in peritoneal fluids as well as lipoxygenase products which enhances inflammatory pain by increasing capillary permeability[23]. Together, these results suggest that the antinociceptive action of allopurinol in capsaicin and acetic acid-induced pain could be caused by the inhibition of the release of pro-inflammatory mediators, such as prostaglandins, glutamate and histamine[24].

The beneficial effect of allopurinol can be explained according to its mechanism of action; allopurinol and its metabolite oxypurinol inhibit xanthine oxidase enzyme. This leads, in addition to decrease systemic level of uric acid, to an increase in the concentration of the precursors, hypoxanthine and xanthine[25]. Hypoxanthine can be converted to inosine, then, to adenosine and guanosine both in central nervous system and periphery[26]. Anti-nociceptive effects of adenosine and its metabolites may be related to the inhibition of intrinsic neurons by an increase in K^+ conductance and pre-synaptic inhibition of sensory nerve terminals, influencing synaptic transmission and modulating the activity of the nervous system[27]. Allopurinol has no direct agonist or antagonist effect on adenosine receptors[25].

In our study, the antinociceptive effect of allopurinol was emphasized through comparing the outcome of the groups given allopurinol alone with the groups that were given allopurinol plus naloxone, DPCPX or ZM241385.

In agreement, our findings demonstrated that while naloxone completely reversed morphine-induced antinociception, it had no effect against antinociception of allopurinol which proved that opioid pathway is unlikely to be involved in the antinociception caused by allopurinol. However, pretreatment of mice with DPCPX or ZM241385 – at doses that did not cause any effect by themselves – significantly reversed the antinociception caused by allopurinol. These results indicated that A_1 and A_{2A} adenosine are most probably involved in these effects.

Our data agree with those reported by other Authors, indicating that adenosine A_1 receptor agonists produce a pronounced antinociception[28,29]. Also, our findings are in agreement with Authors who have shown that the activation of the adenosine A_{2A} receptor has an antinociceptive role against the chemical (writhing test) and thermal model of pain[10].

In contrast to our results, it has been demonstrated that A_{2A} receptor antagonists showed consistent antinociceptive activity in mice lacking the adenosine A_{2A} receptor[8]. In the formalin test, A_{2A} receptor agonist, produced antinociceptive actions in mice[9]. Thus, involvement of the A_{2A} receptor could depend on the intensity and modality of the stimulus.

The antinociceptive effect of allopurinol can also be explained as inhibition of xanthine oxidase can decrease formation of free radicals. Free radicals can mediate acute pain transmission and maintain chronic pain[30].

Conversely, our results showed that allopurinol at any dose did not produce any motor incoordination (rotarod) and did not reduce spontaneous motor activity (open field test), while another study proved that administration of adenosine was associated with significant side effects, such as hypotension, sedation and impaired motor function that can explain its antinociceptive effects[28]. This may be due to difference in the used dosage regimen. Our results are in accordance to Nascimento et al[11] who stated that inosine didn't alter the locomotor activity of mice in the open-field test compared with controls.

Conclusions

Allopurinol produces a pronounced antinociceptive effect against the pain induced by hot plate, capsaicin and acetic acid and this effect is not related to inhibition of motor function. The mechanisms through which allopurinol exerts its action need more investigations, but an involvement of adenosine A_1 and A_{2A} receptors seem largely to contribute to its antinociceptive effect.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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