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RESEARCH ARTICLE

Study of analgesic effect of amiloride compared to aspirin in chemically induced pain models in rats and mice

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ABSTRACT

Background: Acid-sensing ion channels (ASIC) contribute to acid-evoked pain in many painful inflammatory and ischemic conditions such as rheumatoid arthritis, cardiac ischemia, and exhausted skeletal muscles, which are accompanied by local tissue acidosis. Amiloride, a potassium-sparing diuretic was recently discovered to be a blocker of these ASIC. **Aims and Objectives:** The objective of present study is to evaluate the analgesic activity of amiloride in chemically induced pain models of rats and mice and compare it against the standard analgesic aspirin. **Materials and Methods:** A total of 24 mice and 24 rats were distributed into four groups of 6 each: Group A received distilled water as control, Group B received the test drug amiloride - low dose (20 mg/kg), Group C received amiloride-high dose (40 mg/kg), and Group D received standard drug aspirin (25 mg/kg); all drugs were given intraperitoneally. In formalin test, 0.025 ml of 1% formalin was injected under the plantar surface of hind paw and the mean time of paw licking (MTPL) was measured in both early (0-5 min) as well as late (20-40 min) phase. In acetic acid-induced writhing test, the animals were injected with 1 ml/100 g body weight of 0.6% acetic acid i.p. The number of writhings produced was recorded over a period of 20 min. **Results:** In the early phase of formalin test, the MTPL was significantly reduced ($P < 0.05$) in Group B (30.2%) and in Group C (59.1%) and highly significantly reduced ($P < 0.001$) in Group D (74.6%) in mice, whereas in rats there was a significant reduction in MTPL in Group B (48.4%) only and a highly significant reduction was observed in both Group C (62.8%) and Group D (76.9%). In the late phase, in both mice and rats, there was a highly significant reduction in MTPL in all the 3 groups. In writhing test, the number of writhes have been reduced significantly from 51.17 ± 3.28 (mean ± standard error of mean) in Group A to 32.33 ± 3.67 in Group B and to 26.16 ± 3.98 in Group C and highly significantly to 12.17 ± 2.52 in Group D. **Conclusion:** Amiloride showed a comparable but lower analgesic activity than aspirin in chemically induced pain models in rats and mice.

KEY WORDS: Acetic Acid-induced Writhing; Acid-sensing Ion Channels; Formalin Test; Mean Time of Paw Licking

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INTRODUCTION

Pain is one of the most common reason patients seek medical care.[1] Many painful inflammatories and ischemic conditions such as rheumatoid arthritis, cardiac ischemia, and exhausted skeletal muscles are accompanied by local tissue acidosis.[2] In such acidotic states, extracellular

protons provoke the pain by opening excitatory cation channels in nociceptors.[3]

Acid-sensing ion channels (ASICs) are leading acid sensors in nociceptors, and they are mainly activated in pathophysiological states of acidosis.[4,5] ASICs are members of epithelial sodium channel/degenerin (EnaC/DEG) superfamily of amiloride-sensitive EnaCs.[6] ASICs are distinct among the ENaC/DEGs in that they are proton-gated sodium channels proposed as transducers of acid-evoked pain.[7] They are widely expressed in both central and peripheral nervous systems (PNSs). Furthermore, ASICs contribute to pain processing and central sensitization during pathological states such as inflammation and ischemia, both of which are accompanied by moderate-to-severe tissue acidosis.[8]

Even the recent electrophysiological experiments have strongly suggested the involvement of ASICs[9] in nociception linked to acidoses. Sensory neurons from mice lacking ASICs are severely deficient in their responses to acidic stimuli *in vitro*.[10] A recent study in rats further suggests that peripheral ASIC is the essential sensors of cutaneous acidic pain in both normal and inflammatory conditions.[11] Many studies also point out that activating ASICs is sufficient to cause pain.[12,13] The strongest evidence to date that inhibiting ASICs in either the central nervous system or the PNS reduces pain was obtained using black mamba venom, which contains a three-finger peptide (mambalgin-1) that blocks ASIC currents. These observations suggest that wister rats ASIC antagonists may have distinct advantages over opioids for pain alleviation in acidotic conditions.[14]

Recently, it was discovered that amiloride, a potassium-sparing diuretic is a blocker of these ASICs.[15,16] Amiloride can be expected to have analgesic activity mainly in the conditions where local tissue acidosis is the main cause of pain. So, the present study was conducted to evaluate the analgesic effect of amiloride in chemically induced pain models in rats and mice and compare it against the standard drug Aspirin.

MATERIALS AND METHODS

Animals

Albino mice weighing 20-30 g and Wistar rats weighing 150-250 g of either sex were used in this study. The animals were procured from the central animal house of our institute. They were housed in cages in standard laboratory conditions with natural light and dark cycle and at room temperature. Food and water was given *ad-libitum*. The study protocol was approved by the Institutional Ethics Committee, and care of the animals was as per the "Guidelines for the care and use of laboratory animals."

Drugs and Chemicals

Amiloride (Midamor) 5 mg tablet manufactured by Merck & Co. and aspirin (ecosprin) 75 mg tablet manufactured by USV Limited were used in this study. All drug solutions were freshly prepared in distilled water at room temperature. Formalin was used in a concentration of 1%, and acetic acid was used in a concentration of 0.6% in mice and 3% in rats.

Experiment Protocol

A total of 24 mice and 24 rats were distributed into four groups of 6 each:

1. Group A received distilled water as control
2. Group B received amiloride - low dose (20 mg/kg)
3. Group C received amiloride - high dose (40 mg/kg)
4. Group D received standard drug aspirin (25 mg/kg).

All drugs were given intraperitoneally.

Assessment of Analgesic Activity

For assessing the analgesic activity, two chemical methods were used namely formalin test[17] and acetic acid-induced writhing test. In both the tests, rats and mice were first screened to eliminate non-responders, animals which do not show any response in the tests due to their inherent nature.

Formalin test

Amiloride was administered 45 min before, and aspirin was administered 1 h before the nociceptive stimulus which was 0.025 ml of 1% formalin, injected under the plantar surface of the right hind paw of mice. 0.1 ml of 1% formalin was used for rats. Left hind paw was injected with an equal volume of distilled water as control. The measured quantities of drugs were given according to the body weight of animals. Mice were then placed in cages and time spent by the animals licking or biting the injected paw or mean time of paw licking (MTPL) was recorded with the help of a stopwatch by an independent observer blind to the treatment given to animals. Two distinct periods of intensive licking and biting activity, i.e., an early phase (0-5 min) and late phase (20-40 min) were scored separately for each animal in the different groups. Decrease in the paw-licking and biting response was considered to be the positive analgesic response. Percentage reduction in the MTPL was calculated using the following formula.

$$\text{Percentage reduction} = \frac{\text{Mean time in test (or) Std} - \text{Mean time in control}}{\text{Mean time in control}} \times 100$$

Acetic acid-induced writhing test

After 45 min of drug administration to the four groups, the animals were injected with 1 ml/100 g body weight of 0.6% acetic acid intraperitoneally. The number of writhings

produced by each mouse was recorded over a period of 20 min. For scoring purposes, a writhe is characterized by stretching of the abdomen with simultaneous stretching of at least one hind limb.[18] The observations were tabulated, and the results were permitted to express the percentage of protection of writhes compared with the control by using the following formula:

$$\text{Percentage reduction} = \frac{\text{Mean no.of wriths in test/ Std - Mean time in control}}{\text{Mean time in control}} \times 100$$

The test is repeated with rats as experimental animals and with the same number of groups as in mice but in case of rats, 3% acetic acid solution is used instead of the 0.6% used in mice.

Statistical Analysis

All the results are presented in mean ± standard error of mean (SEM) and in percentage protection among test and standard groups in comparison to control group. Statistical analysis of data was performed using Student's *t*-test to study the differences among the means. A *P* value of <0.05 is considered significant and that of <0.001 is considered.

RESULTS

In mice, the MTPL was 126.72 in Group A, 88.50 in Group B, 51.83 in Group C, and 32.21 in Group D in the early phase, whereas in late phase it was 249.16 in Group A, 60.01 in Group B, 35.66 in Group C, and 16.83 in Group D (Tables 1 and 2).

In rats, the MTPL was 89.50 in Group A, 46.16 in Group B, 33.33 in Group C, and 20.66 in Group D in the early phase, whereas in late phase it was 213.66 in Group A, 53.00 in Group B, 25.83 in Group C, and 33.20 in Group D (Tables 3 and 4).

In the early phase of formalin test, the MTPL was significantly reduced ($P < 0.05$) in Group B (30.2%) and in Group C (59.1%) and highly significantly reduced ($P < 0.001$) in Group D (74.6%) in mice (Figure 1), whereas in rats, there was a significant reduction in MTPL in Group B (48.4%) only and a highly significant reduction was observed in both Group C (62.8%) and Group D (76.9%) (Figure 2).

In the late phase, in both mice and rats, there was a highly significant reduction in MTPL in all the three groups. Mice - B: 75.9%, C: 85.6%, and D: 93.3% (Table 2; Figures 1 and 2) rats - B: 75.2%, C: 87.9%, and D: 84.5%) (Table 4; Figures 1 and 2).

Table 1: Paw licking response in mice-early phase (0-5 min)

Group	Drug given	Dose (mg/kg)	MTPL (s) mean±SEM	Percentage protection
A	Distilled water (control)	1	126.72±15.47*	-
B	Amiloride	20	88.50±7.70†	30.16
C	Amiloride	40	51.83±5.75†	59.10
D	Aspirin (standard)	25	32.21±6.41‡	74.58

*P>0.05: Not statistically significant, †P<0.05: Statistically significant, ‡P<0.001: Statistically highly significant. MTPL: Mean time of paw licking, SEM: Standard error of mean

Table 2: Paw licking response in mice-late phase (20-40 min)

Group	Drug given	Dose (mg/kg)	MTPL (s) mean±SEM	Percentage protection
A	Distilled water (control)	1	249.16±17.11*	-
B	Amiloride	20	60.01±6.91‡	75.92
C	Amiloride	40	35.66±6.16‡	85.62
D	Aspirin (standard)	25	16.83±2.29‡	93.25

*P>0.05: Not statistically significant, ‡P<0.001: Statistically highly significant. MTPL: Mean time of paw licking, SEM: Standard error of mean

Table 3: Paw licking response in rats-early phase (0-5 min)

Group	Drug given	Dose	MTPL (mean±SEM)	Percentage protection
A	Distilled water (control)	1	89.50±12.68*	-
B	Amiloride	20	46.16±4.16†	48.42
C	Amiloride	40	33.33±4.86‡	62.76
D	Aspirin (standard)	25	20.66±3.28‡	76.92

*P>0.05: Not statistically significant, †P<0.05: Statistically significant, ‡P<0.001: Statistically highly significant. MTPL: Mean time of paw licking, SEM: Standard error of mean

Table 4: Paw licking response in rats-late phase (20-40 min)

Group	Drug given	Dose (mg/kg)	MTPL (mean±SEM)	Protection %
A	Distilled water (Control)	1	213.66±33.96*	-
B	Amiloride	20	53.00±8.05‡	75.19
C	Amiloride	40	25.83±9.32‡	87.91
D	Aspirin (standard)	25	33.20±6.95‡	84.46

*P>0.05: Not statistically significant, ‡P<0.001: Statistically highly significant. MTPL: Mean time of paw licking, SEM: Standard error of mean

With regard to writhing test in mice, the number of writhes has been reduced significantly from 51.17 ± 3.28 (mean ± SEM) in Group A to 32.33 ± 3.67 in Group B and to 26.16 ± 3.98 in Group C and highly significantly to 12.17 ± 2.52 in Group D (Table 5). The percentage decrease in the mean number of writhings for amiloride (20 mg/kg) was 36.82%, for Amiloride (40 mg/kg) was 48.88%, and for aspirin was 76.22% (Figure 3).

With regard to writhing test in rats, all the three groups, i.e., amiloride (20 mg/kg), amiloride (40 mg/kg), and aspirin (20 mg/kg) showed decrease in the mean number of writhings significantly ($P < 0.05$) (Table 6). The percentage decrease in the number of writhings for the above drugs when compared to control and are 41.78%, 52.21%, and 58.57%, respectively (Figure 4).

On the whole, the percentage decrease in mean number of writhings by both doses of amiloride in mice is lower when compared to that in rats, whereas the percentage decrease in mean number of writhes by aspirin is more in mice than that in rats (Figures 3 and 4).

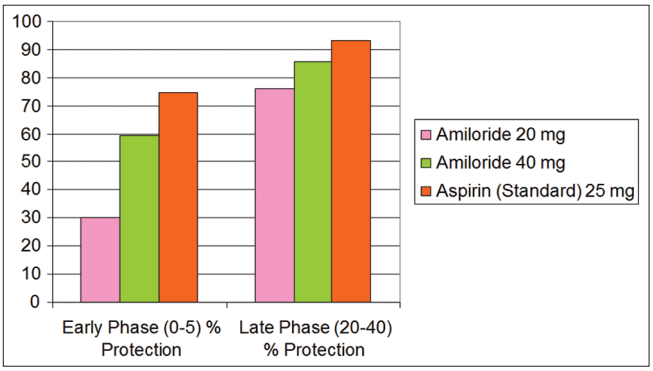

Figure 1: Percentage protection of paw licking response in mice

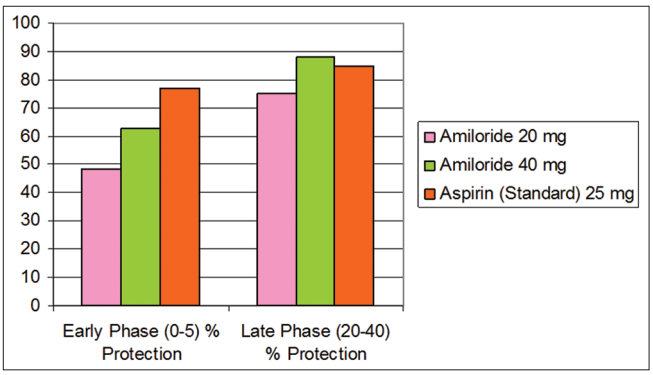

Figure 2: Percentage protection of paw licking response in rats

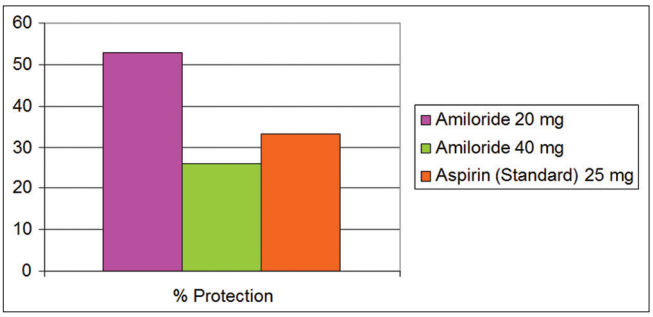

Figure 3: Percentage protection of writhing in mice

DISCUSSION

In this study, we evaluated the analgesic action of amiloride, a potassium sparring diuretic using two chemically induced pain models - formalin test and acetic acid-induced writhing test in rats and mice. The analgesic action was compared with aspirin, which is considered as the standard drug for treating chemically induced pain.

Overall, the percentage decrease in the MTPL, also known as percentage protection which indicates the degree of analgesia produced is comparatively more in the late phase (20-40 min) than in the early phase (0-5 min) in the formalin test. This indicates that both aspirin and amiloride showed greater efficacy in the late phase than the early phase. The early phase is also called as acute phase, and the late phase is called the chronic phase or inflammatory phase. The greater efficacy of amiloride in the late phase can be explained by the fact that acid/protons play a major role in causing pain in late phase than in the early phase. As amiloride is a potent blocker of ASICs, the acid-induced pain which is the main component in late phase is attenuated so that the percentage protection is more in the late phase compared to early phase. It has been shown that amiloride acts as a pore blocker and inhibits ASICs even in the micromolar concentration range - IC 50 ranging between 5 and 100 µM.[19,20]

As far as writhing test is concerned, in mice, the reduction in the mean number of writhes has been significant in Group B and Group C and highly significant in Group D, whereas in rats, it was significant in all the three groups. Here, the test drug amiloride showed a significant response, but it is lower

Table 5: Results of writhing response in mice

Group	Drug given	Dose (mg/kg)	Number of writhings (in 20 min) mean±SEM	Percentage decrease in the number of writhes
A	Distilled water (control)	1	51.17±3.28*	-
B	Amiloride	20	32.33±3.67†	36.82
C	Amiloride	40	26.16±3.98†	48.88
D	Aspirin (standard)	25	12.17±2.52‡	76.22

*P>0.05: Not statistically significant, †P<0.05: Statistically significant, ‡P<0.001: Statistically highly significant. SEM: Standard error of mean

Table 6: Results of writhing response in rats

Group	Drug given	Dose (mg/kg)	Number of writhings (in 20 min) mean±SEM	Percentage decrease in the number of writhes
A	Distilled water (control)	1	43.23±4.34*	-
B	Amiloride	20	25.17±2.93†	41.78
C	Amiloride	40	20.66±3.22†	52.21
D	Aspirin (Standard)	25	17.91±3.29†	58.57

*P>0.05: Not statistically significant, †P<0.05: Statistically significant. SEM: Standard error of mean

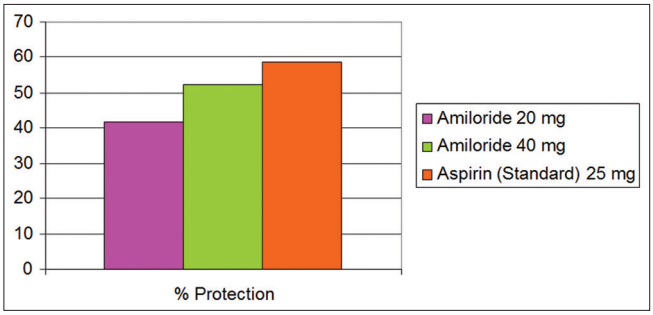

Figure 4: Percentage protection of writhing in rats

when compared to aspirin. The effectiveness of amiloride in this test can be explained by the fact that here, directly the acetic acid is injected into the peritoneal cavity which causes irritation and pain and the associated writhing movements. As amiloride is a blocker of ASICs, it decreases the acid/protons-evoked pain and thus associated writhings. The percentage decrease in mean number of writhes was less in rats compared to mice which might be due to the sensitive nature of mice to acid-induced writhing movements compared to rats.

Tissue acidosis occurs in various physiological and pathophysiological states, including inflammation, infection, ischemia, cancer, tissue injury, and metabolic stress.[21-24] Protons can modulate the activity of a number of receptors and ion channels expressed in nociceptors[25] among which ASICs are the most important.[7,26]

All the effects of amiloride in the above tests can be explained by its blockade of ASICs. ASICs are voltage-independent cation channels, which are gated by extracellular protons, and therefore, constitute effective proton sensors in both central and peripheral neurons.[27-29] The ASIC family comprises at least six members in rodents, namely, ASIC1a, ASIC1b, ASIC2a, ASIC2b, ASIC3, and ASIC4.[30,31] These heterologously expressed ASICs generate a biphasic inward current that is similar to the native proton-activated current in dorsal root ganglion neurons.[27] The ASIC is capable of reproducing the features of acid-evoked currents in cardiac nociceptors.[32] In addition, recently it has been reported that non-proton ligand sensor existing in the ASICs causes persistent activations of ASICs.[12,13] Thus, non-proton ligand sensor may contribute to the effect of amiloride. Amiloride can be a useful drug in painful conditions where tissue acidosis occurs.

CONCLUSION

In our study, amiloride showed higher analgesic activity in the late phase of formalin test compared to early phase both in rats and mice. In acetic acid-induced writhing test, it showed higher analgesic activity in mice compared to rats. In both the tests, amiloride showed a comparable but lower analgesic activity than aspirin.

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