Low dose aspirin like analgesic and anti-inflammatory activities of mono-hydroxybenzoic acids in stressed rodents

Saba Anjum Khan a, Shyam Sunder Chatterjee b,1, Vikas Kumar a,⁎

a Neuropharmacology Research Laboratory, Department of Pharmacognosy, Indian Institute of Technology, Banaras Hindu University, Varanasi, India
b Stettiner Straße 1, Karlsruhe, Germany

A R T I C L E   I N F O

Article history:
Received 1 August 2015
Received in revised form 25 January 2016
Accepted 9 February 2016
Available online 11 February 2016

Keywords:
Stress
Acetic acid induced writhing
Cotton pellet granuloma
Carrageenan induced paw edema
Hot plate test
Repeated dosing
Hydroxybenzoic acids

A B S T R A C T

Aims: To compare analgesic and anti-inflammatory activities of aspirin and mono-hydroxybenzoic acids after their daily oral doses.

Main methods: Efficacies of repeated daily stress response suppressing low oral doses (20 mg/kg) of aspirin and 2-, 3-, and 4-hydroxybenzoic acids in mice hot plate test for centrally acting analgesics, and in acetic acid induced writhing test were compared. Effects of their same daily doses and treatment regimen in cotton pellet granuloma and carrageenan edema test for anti-inflammatory drugs in stressed rats were compared in a second experiment. Effects of treatments on body weights, basal rectal temperatures, organ weights and plasma glucose, insulin and cortisol levels in stressed animals were compared also.

Key findings: Although stress response suppressing effects of aspirin and all the three hydroxybenzoic acids in both mice and rats were almost equal, effectiveness of 3- and 4-hydroxybenzoic acids as analgesic and anti-inflammatory agents were lower than those of aspirin or salicylic acid.

Significance: Observations made after single oral doses of aspirin or of mono-hydroxybenzoic acids are not very reliable predictors of their pharmacologically interesting bioactivity profiles and efficacies. Prostaglandin synthesis inhibition is not involved in low dose anti-inflammatory activities of 3- and 4-hydroxybenzoic acids. After their repeated daily low oral doses they are almost as potent stress response desensitizers as aspirin or salicylic acid.

© 2016 Elsevier Inc. All rights reserved.

1. Introduction

Long before analgesic and anti-inflammatory activities of aspirin became known, numerous plants enriched in 2-, 3- or 4-hydroxybenzoic acids and their metabolic precursors have been used for prevention and cure of diverse chronic diseases. Amongst them the 2-hydroxybenzoic acid (salicylic acid) is pharmacologically the most well studied one, and it is now well recognized that aspirin and other salicylic acid derived products can be used for prevention and cure of diverse metabolic disorders, including cancer, and associated mental health problems [1]. Salicylic acid is a plant hormone also involved in ecological survival processes of numerous plants [2], and together with 4-hydroxybenzoic acid it is often considered to be a bioactive constituent of numerous traditionally known medicinal plants often pharmacologically classified as adaptogenic or stress resistance improving herbs [3, 4]. Although the 3-hydroxybenzoic acid is also encountered in several plants often used in traditionally known systems of medicine and dietary therapies, it still remains to be one the least pharmacologically explored one [5, 6].

In all dietary and traditionally known herbal therapies, plant pheno- lics are regularly used for medicinal or health care purposes, and it is now well recognized that repeated daily oral doses of plant extracts enriched in them is necessary for observing their therapeutically interesting bioactivities in animal models [7, 8]. Observations in our laboratories have revealed that repeated daily oral doses of aspirin or of mono-hydroxybenzoic acids lower than 30 mg/kg can suppress diverse physiological stress responses triggered by daily handling and repeated testing, or on repeated exposures of experimental animals to stressful experimental conditions [3, 9]. Their daily dose dependant activity profiles in suppressing diverse adaptive stress responses quantified in those experiments, or as potential antidepressants or anxiolytics, were not always identical, and depended also on the numbers of treatment days.

Biological processes and mechanisms regulating adaptive stress responses, or allostatic load, are also involved in etiology, pathogenesis and progression of numerous chronic diseases commonly associated with inflammation and pain [10–12]. Preventive potential of regular intake of low dose aspirin against cardiovascular and other diseases are well recognized, and its diverse brain function modulating effects are now also well known [1]. However, as yet very little concentrated efforts have been made to assess anti-inflammatory and analgesic...
activities of low dose aspirin or to compare its efficacies with salicylic and other two naturally occurring mono-hydroxybenzoic acids. It has recently been reported though, that unlike several other phenolic acid with anti-inflammatory activities in animal models, the 3- or 4-hydroxybenzoic acids do not inhibit prostaglandin synthesis [13]. Results of experiments conducted to verify the possibility that stress response desensitizing low daily oral doses of the three mono-hydroxybenzoic acids and aspirin are also effective in conventionally known rodent bioassays for aspirin like anti-inflammatory drugs are described and discussed in this communication. Choices of the doses and treatment regimen used in the experiments were based on the observations made in our earlier exploratory experiments [3, 10]. Stress biomarkers and other parameter quantified were also same as those used in earlier studies with mono-hydroxybenzoic acids and other known bioactive plant metabolites and extracts [3, 10, 14, 15].

2. Materials and methods

2.1. Animals

Adult male Swiss mice (20 ± 5 g) and wistar rats (150 ± 50 g) were obtained from Central Animal House of Institute of Medical Sciences, Banaras Hindu University, Varanasi (Registration number Dean/2014/CAEC/607). At least one week before starting the experiment all animals were acclimatized to constant laboratory conditions. They were randomly selected and group-housed (six animals per cage) in polypropylene cages (28 × 19 × 12.5 cm) maintained at an ambient temperature (25 ± 1 °C) and relative humidity (50 ± 10%) with a 12:12 h light/dark cycle (light on at 06:00 and off at 18:00). Animal cages were provided with husk and they were routinely cleaned. Except when mentioned, all animals were always provided with standard rodent diet and water ad libitum. Prior approval from the Central Animal Ethical Committee of the University was obtained (Dean/2014/CAEC/344, dated May 30, 2014) for experimental protocols. In both the experiments, six randomly selected groups of six animals each were used, and all experimental groups in a given experiment were tested in parallel (i.e. on the same days of the experiments). A trained observer made the observations blind to the treatments given to the animals, and body weights and basal rectal temperatures of all animals were recorded (before drug administration) on all observational days. Except when mentioned, all tests were conducted 1 day after the day’s oral treatments.

2.2. Drugs, chemicals, and test kits

Aspirin, salicylic acid, 4-hydroxybenzoic acid and 3-hydroxybenzoic acid were purchased from HiMedia laboratories Pvt. Ltd. Mumbai, India; carboxymethyl cellulose (CMC) from Central Drug House Pvt. Ltd., New Delhi, India; acetic acid and carrageenan from Sigma Aldrich, Bengaluru, India. Plasma glucose level was estimated by biochemical enzyme test kit (ERBA diagnostics Mannheim GmbH, Germany) and plasma insulin level was estimated using Enzyme-Linked Immunosorbenbt Assay (ELISA) test kit (Chemux BioSciences, Inc, USA). Plasma cortisol was estimated using ELISA kit (DSI S.r.l., Italy). All other chemicals and reagents used were from other laboratory suppliers and of highest analytical quality available in India.

For oral administrations, aspirin and 2-, 3- and 4-hydroxybenzoic acid were suspended in 0.3% CMC, and the application volume was always 10 ml/kg. Except when mentioned, the control groups of the experiments were always similarly treated with 0.3% CMC only.

2.3. Design of the experiments

Male mice used for comparing analgesic activities of aspirin and the three mono-hydroxybenzoic acids were preselected (one day before the start of the experiment) for their reaction times on a hot plate maintained at 55 ± 1 °C. Only those mice reacting within 15 s after placing them on the hot plate on the pre-selection day, and which did not show large variation when tested on four separate occasions (each 15 min apart) on that day, were randomly assigned to the six test groups used in this experiment. Although this pre-selection procedure is necessary for improving reproducibility of observation, such animals are also mildly stressed and repeatedly handled ones. One of the experimental groups (the reference group) was further handled and daily treated orally with 0.3% CMC, but was not subjected to hot plate test on the days 1, 5, 7 and 10 of the experiment. The others were treated daily either with 0.3% CMC (control group), or with 20 mg/kg/day of aspirin, or 2-, or 3-, or 4-hydroxybenzoic acids for 12 consecutive days and subjected to hot plate test on those days of the experiment. Except for the reference group, all others were subjected to acetic acid writhing test on the 11th experimental day. On the 12th day of the experiment, all animals of all groups were subjected to the tail suspension test for evaluating antidepressant activity, and the day thereafter they were sacrificed (without treatments) for estimating their blood glucose, insulin, and cortisol levels and weights of their spleen and adrenal glands.

In another experiment conducted to compare anti-inflammatory activities of aspirin with mono-hydroxybenzoic acids in stressed rats, a similar experimental procedure was used (see Fig. 1b). Except for the animals of the CMC treated reference group used in this experiment, pre-weighed (50 ± 1 mg) cotton pellets (for cotton pellet granuloma test described later) were implanted (one day before the 1st day of the experiment) in all other animals of other groups, and the reference group was not subjected to foot shock stress triggered hyperthermia test on days 1, 5, 7 and 10 of the experiment. The other five groups were daily treated (orally) either with CMC (control group), or with 20 mg/kg/day aspirin, or 2-, or 3-, or 4-hydroxybenzoic acids for 12 consecutive days and subjected to foot shock stress induced hyperthermia test on those days of the experiment. On the 11th observational day, all animals of all groups were subjected to carrageenan induced paw edema test. On the next day (1 h after the days treatments) animals of the five cotton pellet implanted groups were weakly ether anesthetized for removing the their cotton pellets covered by granuloma tissue. On this last day of the experiment, all animals of all groups were sacrificed by decapitation for estimating their blood glucose, insulin and cortisol levels, and weights of their spleen and adrenal glands.

2.4. Experimental procedures

2.4.1. Hot plate test

This test was conducted 1 h after recording their basal core temperatures (using a rectal probe and calibrated electronic thermometer) and oral treatments on the 1st, 5th, 7th and 10th day of the experiments. In this test, an individual mouse of a group was placed on a hot plate maintained at 55 ± 1 °C and its reaction time in seconds for forepaw licking or jumping was recorded [16]. For preventing any thermal injury, maximum time the mouse was allowed to stay on the hot plate was 30 s. Immediately after the test, the mouse was returned to its home cage, and 10 min thereafter its core temperatures were recorded again. The animals of the CMC treated reference group were not subjected to hot plate test, but were also placed on a similar plate maintained at room temperature for 15 s and then returned to their home cage and 10 min thereafter their temperatures were also recorded again.

2.4.2. Acetic acid writhing test

On the 11th day of the experiments, and 1 h after oral treatments and basal core temperature measurements, all mice of all experimental groups (except those of the REF group) of the experiment were intraperitoneally injected with 0.7% (v/v) aqueous acetic acid (10 ml/kg). Number of writhes for the following 5 min of observational period was counted [17].
2.4.3. Tail suspension test
On the 12th day of the experiments, body weights and rectal temperatures of all the mice were recorded again, and 60 min after oral treatments an individual mouse of a group was hung by its tail (50 cm above the bench floor by using an adhesive tapes placed 1 cm from the tip of the tail on a wire) in an upside down posture. After initial vigorous movements, the mouse assumes an immobile posture and the period of immobility during the following 5 min of observation were recorded [18].

2.4.4. Stress induced hyperthermia test
This test was performed in rats on the 1st, 5th, 7th and 10th day of experiment. Body weights and rectal temperatures of all rats were recorded before drugs administration. 60 min after treatments an individual rat of a group was placed in a black box (24 × 29 × 40 cm) with a grid floor for 1 min. Electric foot shocks through the grid floor (2 mA, 50 Hz of 2 ms duration) was delivered for stress induction. Five consecutive 2 mA foot shocks at 10 s interval are given after the rat had stayed in the box for 10 s. At the end of a minute, the animals were placed back in their home cages, and foot shock stress triggered transient change in rectal temperature was recorded 10 min thereafter by using the same rectal thermometer used for recording basal core temperature [19].

2.4.5. Carrageenan induced edema
This test was performed on the 11th day of the rat experiment by injecting 0.1 ml of 1% w/v carrageenan sodium salt subcutaneously into the sub-plantar region of the rat right hind paw to all groups of an individual rat of a group was placed in a black box (24 × 29 × 40 cm) with a grid floor for 1 min. Electric foot shocks through the grid floor (2 mA, 50 Hz of 2 ms duration) was delivered for stress induction. Five consecutive 2 mA foot shocks at 10 s interval are given after the rat had stayed in the box for 10 s. At the end of a minute, the animals were placed back in their home cages, and foot shock stress triggered transient change in rectal temperature was recorded 10 min thereafter by using the same rectal thermometer used for recording basal core temperature [20].

2.4.6. Cotton pellet granuloma
The autoclaved cotton pellets weighing 50 ± 1 mg each were implanted subcutaneously (on day 0 of the experiment) through a small incision made along the axilla or flank region of the weakly ether anesthetized rats of all groups (reference group rats were similarly anesthetized with weak ether and had undergone sham surgical procedure). One day after implantation, the animals belonging to different test groups were orally treated with the test agents or the vehicle for 11 consecutive days. On the 12th day of the experiment, the cotton pellets covered by the granulomatous tissue were removed from all animals under weak ether anesthesia, and then dried in hot air oven (55 ± 5°C) until a constant weight was achieved. Granuloma weights were calculated by subtracting the weights of the implanted cotton pellets (before start of the experiment) from the weight of the cotton pellet on the 12th day [20].

2.4.7. Plasma glucose, insulin, cortisol level and organs weights
Immediately after the last temperature measurements on the 12th day of mice analgesic experiment, and on the 13th day of the rat anti-inflammatory experiment, all animals were sacrificed by decapitation and blood was collected by direct cardiac puncture in EDTA coated tubes kept in ice, and centrifuged at 1000 × g for 20 min at 4°C to separate plasma (Compufuge CPR-30 Plus, with Rotor No. 8; REMI, India). Plasma was separated and aliquots were stored at −70°C for biochemical estimations. All biochemical estimations were done by using an absorbance micro-plate reader (MarkTM-Bio-Rad Laboratories, California, USA) according to instructions manual of enzyme test kits. Immediately after blood collections, adrenal glands and spleen of the animals were dissected out, washed under slowly running tap water and weighed after removing adhered water by gently drying them on sheets of filter papers [21].

2.5. Statistical analysis
Means ± standard errors of means (SEM) were calculated for the observed values of each group in each experiment. Analysis of Variance (ANOVA) followed by Bonferroni post hoc test performed statistical analysis and t-test was performed when stated. Graph Pad Prism-5 (Graph Pad Software Inc., La Jolla, California, USA) was used for statistical analysis. Origin-Pro 8 software (Origin Lab Corporation, Massachusetts, USA) was used for drawing the graphs. p-Value less than 0.05 was considered as statistically significant.

3. Results

3.1. Analgesic activity in mice

3.1.1. Body weight and basal rectal temperature
Mean body weights of the CMC treated reference group not subjected to hot plate test on the 1st, 5th, 7th, and 10th day of the experiment continued to increase till the 11th day of the experiment when the acute acid writhing test was conducted, whereas those of the CMC treated control group continued to decrease steadily till that observational day. Mean body weights of the aspirin or mono-hydroxybenzoic acid treated groups did not change much till the 7th day of the experiment, and steadily increased on the following observational days. Percent changes in mean body weights of different groups observed on different observational days are summarized in Fig. 2a.

Another physiological biomarker of stress triggered responses quantified in this experiment was basal core temperature of the animals during the course of the experiment. These results are summarized in Fig.
2b. Mean basal core temperatures of the reference group increased very slightly on the 5th observational day, and remained almost constant on all subsequent days of the experiment. These values of the CMC treated and all other groups on the 5th observational day were also slightly higher than those recorded for them 1 h before subjecting them to hot plate test on the first day of the experiment. However, there were no statistically significant differences between the mean values of the treated groups subjected to hot plate test on any observational day of the experiment. It was interesting to note though, that the mean values of the CMC treated control group remained elevated till the last day of the experiment, whereas those of the aspirin or mono-hydroxybenzoic acid treated groups tended to return to the values recorded for them 1 h before they were subjected to hot plate test for the first time on day 1 of the experiment.

3.1.2. Transient hyperthermic response

Mean rectal temperatures of the reference group after the animals of the group were exposed to the plate maintained at room temperature increased only slightly, but not significantly, on all observational days, and this response of the group remained almost constant during the course of the experiment. Magnitude of this transient hyperthermic response of the vehicle treated control group observed 10 min after exposures to hot plate maintained at 55 ± 1 °C on the first observational day was somewhat lower than those observed for the group on the subsequent days of the experiment. However, on the first as well as on all subsequent observational days, these transient hyperthermic responses of the control group were always significantly (p < 0.05) higher than those of the reference group. Although, no statistically significant effects of a single oral dose (20 mg/kg) of aspirin or the three mono-hydroxybenzoic acids treated groups were observed on the first observational day, mean responses of the drug treated groups on the 5th and subsequent observational days were always lower than the control group on the day, and their such effects continued to increase with increasing numbers of treatment days. Except for the 3-hydroxybenzoic acid treated group on day 5, mean values of all other drug treated groups on all observational days were always significantly lower than the corresponding values of the control group, and after their 10 daily oral doses the mean values of the aspirin or salicylic acid treated groups were statistically not significantly different from the corresponding value of reference group. These results are summarized in Fig. 3.

3.1.3. Hot plate test

These results are summarized in Fig. 4a. As expected, no significant effects of a single 20 mg/kg of aspirin or of the three mono-hydroxybenzoic acids were observed in the hot plate test for central analgesics. However, when treated daily for 5 or 7 or 10 consecutive days the mean reaction times of the aspirin and salicylic acid treated groups on the hot plate were significantly higher than those of the control group on those days, and the observed effects continued to increase with increasing numbers of treatment days. Except for the 3-hydroxybenzoic acid treated group on day 5, mean values of all other drug treated groups on all observational days were always significantly lower than the corresponding values of the control group, and after their 10 daily oral doses the mean values of the aspirin or salicylic acid treated groups were statistically not significantly different from the corresponding value of reference group. These results are summarized in Fig. 3.
different from those of the corresponding control values, whereas those of the 4-hydroxybenzoic acid group on the 10th observational days was statistically significantly higher than that of the control on that day.

3.1.4. Acetic acid writhing test

Results of this test for non-steroidal anti-inflammatory drugs conducted 1 h after 11th daily oral doses of test agents are summarized in Fig. 4b. Mean numbers of writhes of aspirin or mono-hydroxybenzoic acid treated groups were significantly lower than that of the CMC treated control group. Observed effects of aspirin and salicylic acid in this test were also higher than those of the other two mono-hydroxybenzoic acids, and that of the 4-hydroxybenzoic acid was somewhat higher than that of 3-hydroxybenzoic acid.

3.1.5. Other observations

In contrast to the observations made earlier in foot shock stressed mice [9], mean immobility time in tail suspension test for antidepressants conducted 1 after 11 daily oral treatments of all groups (including the reference and control ones) were not statistically significantly different from one another. Such were also the mean values of plasma glucose, insulin and cortisol levels as well as adrenal gland and spleen weights quantified 24 h after the last oral treatments. Therefore, these results are not given in details in this communication. They are mentioned here only to point out that unlike body weight and core temperatures, repeated exposures to hot plate and other tests, or 11 daily oral 20 mg/kg doses of aspirin and mono-hydroxybenzoic acids, do not have any longer lasting effects on these physiological parameters of male mice often uses as easily quantifiable biomarkers of stress responses.

![Fig. 3. Effects of occasional thermal stress on transient hyperthermic responses of male mice treated once daily either with aspirin or with mono-hydroxybenzoic acids. Values are mean ± SEM (n = 6). * denotes statistically significant difference (Two way ANOVA followed by Bonferroni post hoc test) relative to CON group (* = p < 0.05). ¥ denotes statistically significant difference (Two way ANOVA followed by Bonferroni post hoc test) relative to REF group (¥ = p < 0.05).](image1)

![Fig. 4. Effects of daily oral doses of aspirin or mono-hydroxybenzoic acids on (a) hot plate reaction time and (b) number of acetic acid induced writhes in male mice. Values are mean ± SEM (n = 6). * denotes statistically significant difference (Two way ANOVA followed by Bonferroni post hoc test for figure [a] and one way ANOVA followed by Student t-test for figure [b]) relative to CON group (* = p < 0.05, ** = p < 0.01 & *** = p < 0.001).](image2)
3.2. Anti-inflammatory activity in rats

3.2.1. Body weight and basal rectal temperature

Mean body weights of the reference group increased during the first ten days of the experiment, whereas the control group lost body weights during the course of the experiment. On the last three days of the experiment, mean body weights of both these groups remained almost constant (see Fig. 5a). Mean body weights of the salicylic acid and all the three mono-hydroxybenzoic acids treated groups did not change much during the first 5 days of the experiment. Although these values of all these four groups increased till their 7th daily doses, after that day body weight gain rates of the salicylic and aspirin treated ones were higher than those of the 3- or 4-hydroxybenzoic acid treated ones. On the last observational day, mean body weights of the aspirin and salicylic acid treated groups on the last day of the experiment were closer to, but statistically significantly higher than, that of the reference group.

Mean basal rectal temperature of the reference group not subjected to foot shocks increased a bit till the 10th observational day, and returned to the mean value of the group recorded on the 1st day of the experiment, whereas mean body weights of both these groups remained almost constant (see Fig. 5a). Mean body weights of the aspirin and salicylic acid treated groups were not statistically different from that of the reference group on that day, whereas those of the 3- or 4-hydroxybenzoic acid treated ones were a bit lower.

Mean basal rectal temperature of the reference group not subjected to foot shocks increased a bit till the 10th observational day, and returned to the mean value of the group recorded on the 1st day of the experiment, whereas mean body weights of both these groups remained almost constant (see Fig. 5a). Mean body weights of the aspirin and salicylic acid treated groups were not statistically different from that of the reference group on that day, whereas those of the 3- or 4-hydroxybenzoic acid treated ones were a bit lower.

3.2.2. Foot stress induced transient hyperthermia

Results summarized in Fig. 6 revealed that after 10 min of exposures of the CMC treated control group to unavoidable and unpleasant foot shocks for less than one minute on all observational days increased the mean rectal temperature of the group, and that this stress triggered transient hyperthermic response of the group increased further on the 7th and 10th observational days. Except for the 3-hydroxybenzoic acid treated group, this response of the other three test agents treated groups on the 5th observational day were statistically lower than that of the CMC treated control group. Such effects of all the four test agents increased on the 7th and 10th treatment days. The corresponding mean values of the reference groups not subjected to foot shocks were always statistically higher than that of the control groups. Except on the 10th observational day, the mean values of all the test agents treated groups were also statistically significantly higher than the corresponding values of the reference group.

3.2.3. Carrageenan induced paw inflammation

Mean increases of the carrageenan treated paw volumes of the foot shock stressed and cotton pallet implanted control group and the reference group were almost identical at all time points of the assay (Fig. 7a). Up to the 2nd hour after carrageenan injections, the increases of carrageenan treated paw volumes of the 3- or 4-hydroxybenzoic acid treated
groups were also similar to those of the control or reference groups. Thereafter, these volumes of both these groups continued to decrease and were significantly lower than those of the control or reference groups. Increases in paw volumes of the aspirin or salicylic acid treated groups on all time points of the assay were always significantly lower than those of the control or reference groups.

Fig. 6. Effects of occasional footshock stress on transient hyperthermic responses of male rats treated once daily either with aspirin or with mono-hydroxybenzoic acids. Values are mean ± SEM (n = 6). * denotes statistically significant difference (Two way ANOVA followed by Bonferroni post hoc test) relative to CON group (* = p < 0.05). ¥ denotes statistically significant difference (Two way ANOVA followed by Bonferroni post hoc test) relative to REF group (¥ = p < 0.05).

Fig. 7. Effects of daily oral doses of aspirin or mono-hydroxybenzoic acids on (a) mean paw volume in carrageenan induced paw edema test and (b) weight of granuloma in cotton pellet test on male rats. Values are mean ± SEM (n = 6). * denotes statistically significant difference (Two way ANOVA followed by Bonferroni post hoc test for figure [a] and one way ANOVA followed by Student t-test for figure [b]) relative to CON group (* = p < 0.05 & *** = p < 0.001). ¥ denotes statistically significant difference (Two way ANOVA followed by Bonferroni post hoc test for figure [a]) relative to REF group (¥ = p < 0.05).
3.2.4. Cotton pellet granuloma

Mean granuloma weights of the control group was significantly higher than those of all other test agents treated groups (Fig. 7b). However, the observed effects of 12 daily 20 mg/kg oral doses of 3- or 4-hydroxybenzoic acid against this more slowly evolving and chronic inflammatory response was only marginal, and much lower than those of similar treatments with aspirin or salicylic acid.

3.2.5. Plasma glucose, insulin, cortisol level and organs weights

These results are summarized in Tables 1 and 2. As expected, mean plasma glucose and cortisol levels of the control group were significantly higher, and their mean plasma insulin level significantly lower than those of the reference group. Although the mean adrenal gland weight of the control group was higher and its mean spleen weight lower than those of the reference group, there were no statistically significant differences between the relative organ weights (expressed as mg organ weight per g body of the rats) of the two groups. These observations indicate that the observed differences in the plasma glucose, insulin and cortisol levels of the two groups are not due to adrenal hypertrophy or spleen hypertrophy in the stressed rats with cotton pellet granuloma, and that most probably all these alterations are due to altered extracellular fluid accumulation in the two stress sensitive organs studied.

Mean values of all these stress biomarkers in aspirin or mono-hydroxybenzoic acid treated groups were statistically significantly different from those of the control group. These mean values of the aspirin or salicylic acid treated groups were always closer or identical to those of the reference group. Observed effects of 3- or 4-hydroxybenzoic acid treatments on all these biomarkers were always lower than those of aspirin or salicylic acid.

4. Discussion

Reported observations reaffirm that like diverse other organic acids and plant phenolics [3, 14, 22–25], all three mono-hydroxybenzoic acids and aspirin are also stress response desensitizing agents, and reveal that 3- or 4-hydroxybenzoic acids also possess aspirin and salicylic acid like activity profiles in conventionally known rodent bioassays for non-steroidal anti-inflammatory drugs (NSAID) with analgesic activities. Quantitatively though, all observed effects of 3- and 4-hydroxybenzoic acids were always lower than those of aspirin or salicylic acid, whereupon the observed effects of the tested dose of 3- and 4-hydroxybenzoic acids in the granuloma test for chronic inflammation were marginal only, and the activity profiles of aspirin or salicylic acid and 3- and 4-hydroxybenzoic acids in carrageenan edema test were also not identical. Observations made in the hot plate test suggest further that aspirin as well as all mono-hydroxybenzoic acids also suppress central hyper-sensitivity to pain induced by repeated exposures of mice to peripheral thermal stimuli.

Table 1

<table>
<thead>
<tr>
<th>Treatment groups</th>
<th>Glucose (mg/dl)</th>
<th>Insulin (μU/ml)</th>
<th>Cortisol (ng/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>REF</td>
<td>89.90 ± 3.84†</td>
<td>17.50 ± 1.11†</td>
<td>94.50 ± 1.12†</td>
</tr>
<tr>
<td>CON (0.3% CMC)</td>
<td>135.00 ± 3.10†</td>
<td>9.96 ± 0.8†</td>
<td>113.20 ± 1.17†</td>
</tr>
<tr>
<td>ASA (20 mg/kg)</td>
<td>90.50 ± 3.81†</td>
<td>14.90 ± 0.76†</td>
<td>94.33 ± 1.12†</td>
</tr>
<tr>
<td>SA (20 mg/kg)</td>
<td>91.20 ± 2.77†</td>
<td>14.50 ± 0.60†</td>
<td>94.80 ± 1.51†</td>
</tr>
<tr>
<td>4-HBA (20 mg/kg)</td>
<td>114.00 ± 4.38†</td>
<td>12.50 ± 1.06†</td>
<td>99.90 ± 2.86†</td>
</tr>
<tr>
<td>3-HBA (20 mg/kg)</td>
<td>114.60 ± 4.76†</td>
<td>13.20 ± 0.56†</td>
<td>98.90 ± 4.67†</td>
</tr>
</tbody>
</table>

Values are mean ± SEM (n = 6).

† Denotes statistically significant difference (One way ANOVA followed by Student t-test) relative to CON (0.3% CMC) group (p < 0.05).

This test is often used for identifying centrally acting analgesics, and it has been reported that even repeated daily intraperitoneal administrations of fairly high aspirin doses (100 mg/kg/day) to mice do not alter their responses in this test [26]. Since blood or brain levels of aspirin and salicylic acid achievable 1 after 5 or more daily 20 mg/kg oral dose of aspirin or salicylic acid can be expected to be lower than those possible after its single 100 mg/kg intraperitoneally administered dose of aspirin, we had expected that aspirin and salicylic acid will only have protective effects against central hypertensitivity to pain induced by repeated exposures of animals on hot plate maintained at 55 °C, and that the mean reaction time of treated groups will remain constant during the course of the experiment. However, such were not the observations summarized in Fig. 4a. Mean reaction times of the aspirin and salicylic acid treated groups on day 5 onwards were constantly higher than those of all experimental groups recorded on the first observation- day, and analogous were the effects of 3- and 4-hydrobenzoic acids treated groups from 7th observational day onwards. These observations suggest that in addition to their protective effects against central hyper-sensitivity to pain triggered by repeated exposures to noxious thermal stimuli, they might also have centrally acting analgesic like activities after their prolonged oral intake. However, further experiments using higher daily oral doses and other pain models will be necessary for reaffirming this possibility.

It remains certain though, that 20 mg/kg daily oral doses of aspirin and all mono-hydroxybenzoic acids are high enough not only for increasing stress resistance, but also for suppressing inflammatory re- sponses in stressed rodents. Results of the carrageenan edema test strongly suggest that the pharmacological targets and modes of actions of 3- and 4-hydrobenzoic acids are not identical to those of aspirin or salicylic acid. As expected for non-steroidal anti-inflammatory drugs (NSAIDs) and other prostaglandin synthesis inhibitors, both aspirin and salicylic acid inhibited all phases of carrageenan-induced edema. However, unlike aspirin and salicylic acid, both 3- and 4-hydrobenzoic acids had no effects on the early phases of the edema and had protective effects against the late phases of the edema only (Fig. 7a). These observations strongly suggest that biological processes and mechanisms involved in the inflammatory cascade increasing paw volume during the first few hours after carrageenan injection [27] are not involved in the modes of actions of 3- and 4-hydrobenzoic acids, and that unlike NSAIDs they do not inhibit pros-taglandin synthesis. This inference is in agreement with the recently reported observations [13, 28] that unlike several other plant phenolics with anti-inflammatory activities, they do not inhibit prostaglandin biosynthesis in an in vitro cellular bioassay.

Early phases of carrageenan induced paw edema are inhibited by anti-TNF-α antibody [28], and it has been reported that aspirin also inhibits TNF-α synthesis in endothelial cells, and that this observed effect
of the drug increases with increasing duration of incubation [29, 30]. Several reports revealing protective effects of aspirin against elevated circulating levels of TNF-α in carrageenan challenged rodents have appeared during more recent years, and NSAIDs like effects of antidepressants against early and late phases of carrageenan edema and TNF-α levels have been also reported [31, 32]. Although antidepressants like activities of daily oral doses of aspirin and all the three mono-hydroxybenzoic acids in foot shock stressed mice were observed in our earlier study [9], in the present study no effects of 3- and 4-hydroxybenzoic acids on the early phases of carrageenan induced paw edema, or in the tail suspension test for antidepressants were observed. Therefore, it seems reasonable to assume that the pharmacological targets involved in the observed effects of 3- and 4-hydroxybenzoic acids in carrageenan edema test are not identical to those of conventionally known NSAIDs and anti-depressant drugs.

A growing body of evidence suggests that the etiology of anxiety and depression related conditions can be derived from the sensitization of particular stress-related circuits that are “primed” following exposure to a short-term stressor [33], and involvement of nitric system has been implicated in regulating both short and long-term activation of the stress response [34]. Nitric oxide is also a key inflammatory mediator in the early and late phases of carrageenan induced edema, and that specific inhibitors of the inducible isoform of nitric acid synthase (iNOS) selectively block the evolution of second phase of the edema only [35]. Since, 3- and 4-hydroxybenzoic acids suppress stress responses and inhibit only the late phases of carrageenan induced edema, it could as well be that they are more specific inhibitors of iNOS, or iNOS induction, than aspirin and other NSAIDs and antidepressants with anti-inflammatory activities in carrageenan edema test. Efforts to experimentally verify this possibility could be useful not only for better understanding of the role of nitric system in regulating stress responses and inflammation but also for obtaining functionally novel drug leads against such disorders.

Although, numerous observations made with plant phenolics and their metabolites are now attracting considerable attention of modern nutritional and other researchers, most such preclinical reports dealing with brain function modulating and other therapeutically interesting bioactivities of mono-hydroxybenzoic acids concentrate mainly on those bioactivities of salicylic and 4-hydroxybenzoic acids only [36–38]. It has recently been reported though, that like nicotinic, lactic, 3-hydroxybutyric and numerous other acids, the 3-hydroxybenzoic acid is also an unspecific agonist of hydroxy-carboxylic-acid receptors (HCA-receptors) [39, 40]. HCA activation inhibits lipolysis dictating availability of polyunsaturated fatty acids necessary for biosynthesis of prostaglan-
dins and other lipid mediators regulating inflammation and pain [41]. Therefore, it could as well be that modulation of lipid homeostasis are also involved in the analgesic, anti-inflammatory, and other therapeutically interesting bioactivities of 3-hydroxybenzoic acid revealed by our efforts. Efforts to experimentally verify this possibility could not only be useful for better understanding of the biological functions of HCA-receptors, but also for better understanding of the role of this acid in tradi-
tionally known medicinal uses of numerous edible and other plants enriched in it or in its metabolic precursors.

As yet no very definitive statements on oral bioavailability and meta-
abolite fate of 3- and 4-hydroxybenzoic acids can be made. It is now ap-
parent though that blood levels of salicylic acid achievable after consumption of plant derived food can be as high as those achieved by low dose aspirin, and that salicylic acid is also involved in numerous (but not all) health benefits of low dose aspirin [42]. Observed similarities in activity profiles of low dose salicylic acid in rodents and those of the other two mono-hydroxybenzoic acids encourage us suggest that herbal extracts enriched in them, or in their metabolic precursors, or their combinations, could also be therapeutic alternative to aspirin. Efforts to experimentally verify such possibilities will now be made in our laboratories.

5. Conclusion

Like aspirin and salicylic acids, low daily oral doses of 3- or 4-
hydroxybenzoic acids are also effective in increasing stress resistance in laboratory rodents and also possess anti-inflammatory and analgesic activities in conventionally known animal models for NSAIDs. Pharma-
cological targets (s) and mechanism(s) involved in anti-inflammatory and analgesic activities of 3- and 4-hydroxybenzoic acids are not neces-
sarily identical to those aspirin and other NSAIDs and antidepressants. Low daily oral doses of aspirin or of mono-hydroxybenzoic acids are also effective in suppressing central hypersensitivity to pain induced by occasional exposures to noxious stimuli. Stress sensitive homeostatic processes and mechanisms regulating body weights and temperatures are involved in their modes of action. Efforts to define the pharmacolog-
ical sites and modes of actions of 3- and 4-hydroxybenzoic acids could lead to novel pharmacological targets and drug leads urgently needed for prevention and cure of uncontrollable stress triggered physical and mental health problems.

Conflict of interest

Authors declare no conflict of interest.

References


[8] A. Panossian, H. Wagner, Shifting effect of adaptogens: an overview with partic-

[9] S.A. Khan, S.S. Chatterjee, V. Kumar, Potential anti-stress, anxiolytic and antidepressant like activities of mono-hydroxybenzoic acids and aspirin in rodents: a compar-


[19] T.J.J. Zethof, J.A.M. VanderHeyden, J.T.B.M. Tolboom, B. Olivier, Stress-induced hyper-


N. Shivavedi, S.S. Chatterjee, V. Kumar, Stress response modulating effects of lactic acid in mice, Ther. Targets Neurol. Dis. 1 (2014), e418.


S.A. Khan et al. / Life Sciences 148 (2016) 53–62