Effects of Different Concentrations of Formalin on Paw Edema and Pain Behaviors in Rats

The aim of this study was to determine whether formalin reliably provokes a paw edema and pain behavior. The paw of male Sprague-Dawley rats were injected with 100 μL of formalin with 2.5% (F2.5), 5% (F5), and 10% (F10) concentrations. Following the formalin (n=8) or saline (control, n=6) injection, the flinching or licking of the paw was recorded for the phase 1 response (0-5 min after injection) and phase 2 response (20-60 min). The formalin-induced paw edema was assessed by measuring the diameters of the injected paws at 4 hr after injection. As for flinching, phase 1 and 2 of all three groups showed higher frequency than those of the control group (p<0.05). As for licking, phase 1 cumulative time of the F2.5 and F10 groups, and phase 2 cumulative time of the F2.5 and F5 groups showed a longer duration than those in the control group (p<0.05). The diameters of the paw in the F10 group were significantly larger than those in the control group (p<0.05). Flinching behavior was more reliably expressed the biphasic response than licking response at all formalin concentrations. Peak of the licking was reached at 2.5% and that of flinching was reached at 5%, whereas the paw edema peaked at 10% concentration. This suggests that there may be some dissociation of nociception from the edema formation.

Key Words : Pain Measurement; Nociceptors; Formalin Concentration; Inflammation; Edema

INTRODUCTION

The rat formalin test, which causes a local tissue injury of the paw, has been used as a model for tonic pain (1) and localized inflammatory pain (2, 3). There are two phases of the responses. While the stimulus during the early phase is a direct chemical stimulation of the nociceptors, that during the late phase involves inflammation (4). It is an interesting aspect of this test that two principally different stimuli are employed in the same test. Formalin-induced pain is caused primarily by peripheral tissue inflammation (5). A central sensitization of dorsal horn neurons occurs during the inflammatory pain. In this respect, the formalin test has been regarded as being a more satisfactory model of clinical pain than hot plate tests (6).

Acute inflammation lasts a relatively short duration; only for minutes, several hours, or a few days, and its main characteristics are the exudation of fluid and plasma proteins and the emigration of leukocytes, predominantly neutrophils (7). Several studies have shown that, after an injection of formalin, of the injected paw develops edema rapidly (8, 9).

There have been reports of formalin tests using various volumes and concentrations of formalin (10, 11), which have varied from 20 to 150 μL and from 0.02% to 15%, respectively (12). The behavior of experimental animals during the formalin test indicated that low- and high-intensity nociceptive stimuli could dict different effects on the parameters. Therefore, we examined whether the concentration of formalin is an important factor in generating a biphasic nociceptive response. In particular, we tried to verify if there was a positive relationship between the frequencies of flinching and licking the biphasic response and the extent of paw edema. We also examined whether or not the extent of paw edema is correlated to the biphasic nociceptive response.

METHODS

Animal preparation

The experimental animals were 34 male Sprague-Dawley rats (250-300 g in body weight). They were housed in plastic boxes in 4 groups and fed food and water ad libitum in a room under natural light. All tests were conducted between 09:00 am and 15:00 p.m. To habituate them to the formalin test environment, rats were placed in the test chambers in 3 groups for 15 min a day for 4 days, and alone on the 5th day. Each animal was used only once and sacrificed at the end of
the experiment. The testing room was maintained at 22-24°C. The guidelines on ethical standards for investigations of experimental pain in animals were followed (13). The following experiments were performed under protocols approved by the Institutional Animal Care Committee of the Clinical Research Institute.

**Formalin test**

On the day of testing, animals were randomly assigned to 3 groups: formalin 2.5%-100 µL (F2.5, n=8), formalin 5%-100 µL (F5, n=8), and formalin 10%-100 µL (F10, n=8). Sham-injected rats (n=4) underwent a subcutaneous insertion of the needle, through which no substance was injected. One hundred microliters of saline was injected in the control group (n=6). Each animal was kept singly in the experimental room. The formalin test was carried out in a 30 × 30 × 60 cm-sized clear transparent plastic chamber. A mirror placed behind the box allowed for an unobstructed view of the rat's body and the rat's behavior was recorded on a videotape. The formalin was made of commercially available 37% formaldehyde solution further diluted in isotonic saline. Conscious rats received a subcutaneous injection of formalin solution into the plantar surface of the right hind paw with a 26-gauge needle. The rats were then placed in an individual cage.

**Measurement of pain behavior**

In our analysis, the pain-related behaviors were quantified by determining the incidence of spontaneous flinching or licking of the injected paw or the cumulative time of licking of the injected paw. Flinching is one of the pain-related behaviors in a formalin model and is characterized by spontaneous, rapid, brief shaking or lifting of the paw. A flinch is defined as a sudden reflexive retraction of the injected paw. Licking of the hindquarters, sometimes including most of the body. A nociceptive score was determined for each 5 min block by measuring the sum of duration or frequencies of the behavior. Formalin-induced nociceptive behavior was assessed in an observer-blind manner. Data were recorded for the early acute phase (phase 1) observed during 0-5 min after the injection and the late tonic phase (phase 2) observed during 20-60 min after the injection.

**Measurement of edema of the injected paw**

The baseline diameters of the hind paws were measured before the formalin injection using a caliper; at the metatarsal level. Those of the hind paws that developed edema were determined at 4 hr after the injection by measuring the dorsal plantar foot thickness at the metatarsal. Both of the hind paws were measured simultaneously. The 4 hr interval from the formalin injection to the measurement of the paw edema was set from the literatures for the maximum time to develop an edema (15, 16).

**Statistical analysis**

For the formalin test, time-response data were presented as the sum of the frequencies of flinches or sum of the duration of licking during the observation periods. The statistical significance of the behavioral or paw edema data among the groups with different formalin concentrations was analyzed by one-way analysis of variance (ANOVA) or Kruskal-Wallis one-way analysis of variance on ranks followed by Tukey or Dunn's test for multiple comparisons, respectively. P value less than 0.05 was considered statistically significant.

**RESULTS**

The rats showed no typical reaction at the time of injection and no evidence of abnormal ambulation, activity, bowel or bladder function, grooming, and appetite. The injection of formalin resulted in a progressive biphasic response of the injected paw. The first phase (phase 1) began immediately after injection and peaked during the 5 min after a 10-15 min quiescent period. The second response (phase 2) followed and lasted about 60 min. Sham-injected animals showed no pain responses and therefore were not included in the statistical analysis of the parameters.

All formalin-treated (F2.5, F5, and F10) groups showed more phase 1 and 2 flinching response than the control group (p<0.05). In F5 group, the phase 2 flinching was more frequent than in F2.5 group whereas in F10 group, the frequency was not further increased (Table 1 and Fig. 1A). Licking behaviors showed a different profile from the flinching. The cumulative time of phase 1 licking response of the F2.5 and F10 groups and that of phase 2 of the F2.5 and F5 groups was much longer than the control group (p<0.05) (Table 1 and Fig. 1B).

During the phase 1 of all formalin-treated groups, brief reflex-like retractions of the injected paw frequently preceded transitions from normal weight bearing to favoring, or from favoring to lifting. In addition, during the peak of the phase 2 of those groups, the most prominent flinching was repetitive high-frequency shakes of the paw. At a higher concentration of formalin (5%), most rats showed almost continuous paw flinching between 30 and 50 min after formalin. The F2.5 group showed higher frequency of licking.
behavior than other formalin-treated groups. Licking was
not prominent at higher concentrations (phase 1 of F5
and phase 2 of F10) and since flinching is incompatible
with licking, the time-course of flinching at these formalin
concentrations may reflect a competition between flinching
and paw licking. The highest concentration (10%) did not
further enhance either flinching or licking.
An examination of the consistency of the behavior between

Table 1. The effect of different concentrations of formalin on behaviors

| Group  | Phase 1 response | Phase 2 response |
|--------|------------------|------------------|
|        | Flinching (Freq.) | Licking (Sec.)   | Flinching (Freq.) | Licking (Sec.)   |
| Control (n=6) | 2 ± 2            | 0 (0-0)          | 0 (0-1)            | 0 (0-0)          |
| F2.5 (n=8)    | 20 ± 14*         | 60 (24-108)*     | 152 (118-223)*     | 269 (160-359)*   |
| F5 (n=8)      | 23 ± 8*          | 28 (14-49)       | 227 (174-280)*     | 125 (77-204)*    |
| F10 (n=8)     | 19 ± 12*         | 29 (2-55)*       | 147 (89-230)*      | 100 (55-150)     |

Data are mean ± S.D. or median (25-75%). The numbers are frequencies (Freq.) of flinching or total time (seconds; Sec.) spent licking the injected paw. The behavioral response was measured at a total of 60 min after plantar injection, at 5-min intervals. F2.5=100 μL of 2.5% formalin, F5=100 μL of 5% formalin, and F10=100 μL of 10% formalin. *p<0.05, compared with the control group (n=6) (ANOVA with Tukey test or Kruskal-Wallis ANOVA on ranks with Dunn’s test).

Table 2. The effect of different concentrations of formalin on edema at 4 hours after formalin injection

| Group  | Diameters (mm) of paws |
|--------|------------------------|
|        | Baseline | 4 hours after injection |
|        | Left     | Right     | Left     | Right     |
| Control (n=6) | 5.1 ± 0.2 | 5.1 ± 0.2  | 5.3 ± 0.7 | 5.9 ± 0.2  |
| F2.5 (n=8)    | 5.2 ± 0.3 | 5.2 ± 0.2  | 5.6 ± 0.5 | 7.6 ± 0.4  |
| F5 (n=8)      | 5.1 ± 0.2 | 5.1 ± 0.5  | 5.5 ± 0.7 | 8.1 ± 0.5  |
| F10 (n=8)     | 5.2 ± 0.2 | 5.3 ± 0.8  | 5.6 ± 0.9 | 10.0 ± 0.5*|

Data are mean ± S.D. Baseline values represented pre-injection diameters of paws. The paw edema was measured by evaluating the dorsal plantar foot thickness at the metatarsal level using a caliper. F2.5=100 μL of 2.5% formalin, F5=100 μL of 5% formalin, and F10=100 μL of 10% formalin. *p<0.05, compared with the control group (n=6) (ANOVA with Tukey test).
DISCUSSION

Normally, the fine afferent C- and A-δ fibers are activated by brief, high intensity stimuli, which induce little or no tissue damage. However, during the inflammation, induced by a mild tissue damage or infection, the afferent fibers can be activated by lower intensity stimuli and the pain produced differs in quality and may be more persistent. Formalin-induced pain is caused by peripheral tissue inflammation (5). It involves a phase of inflammation in which a variety of chemical mediators alter the functions of peripheral afferent fibers. In the current study, the formalin resulted in a progressive biphasic behavioral response such as flinching, favoring, licking, and biting of the injected paw in all experimental groups. The beginning time point of the phase 2 response in these experimental groups was 15 min after the formalin injection.

Although the frequencies of flinching response were highest in the F5 group, all formalin-treated groups showed significantly increased frequencies of the response. The increasing concentration of formalin was not associated with increasing levels of flinching behavior in the present experiment. There was no a significant increase of biphasic licking response of F5 and F10 groups. Some rats exhibited 'backward walking' while shaking the paw, a behavior observed occasionally in F10 group. Overall, it was suggested that if a single parameter is to be chosen at any concentration (even if higher than 5%) of formalin in rats, the paw flinching is.

With an assumption that the increasing concentrations of formalin are associated with increasing levels of pain, the dose-response relation of formalin can be used to establish which behavioral index is the best measure of pain. The main focus of this study was to determine the factor to yield the biphasic pain response in the formalin test at different concentrations. A similar positive relationship between formalin concentration and the frequencies of flinching was also observed up to F5. However, the administration of the highest formalin concentration (F10) was associated with a decrease in flinching. One explanation for this ceiling effect is that higher formalin concentrations induce other behavioral reactions that may interfere with the primary behavior (10). In our experiment, for example, long duration of backward walking during the shaking of the paw and freezing during the immobile posture were observed.

Because formalin induces swelling and inflammation, it may produce sensations other than pain, which may evoke behaviors scored in the formalin test. We tested the effect of concentration of formalin that would be expected to produce dose-related edema in the paw. Factors related to the inflammation include volume, pH, and temperature (17, 18). The formalin pain response increases with increasing environmental temperature (19). In this study, we performed the formalin test at room temperature (22-24 °C). We tested the formalin pain response during the daytime to give rats time for familiarization with the environment to minimize other factors that may lead to a depression of pain scores or inflammatory reaction.

Another main focus of this study was to determine whether the increasing concentrations of formalin are associated with the extent of edema formation, and whether the dose-effect relation of formalin can be used to establish the optimal dose for measuring pain. The extent of edema was significantly increased in the F10 group. There was a discrepancy between the extent of the formalin-induced edema, which was prominent at the highest concentration (10%), and behavioral response, which is prominent at lower concentrations (2.5% and 5%). A possible explanation is that the firing of primary afferents subsides after 60 min due to the toxic effects of formalin on the peripheral nerve fibers, while the inflammation still evolves in the peripheral tissues. In addition, edema alone seemed insufficient to elicit the biphasic behavior, as inflammatory stimuli that induce an even greater degree of inflammation, such as yeast and carrageenan, hardly produce pain-like behavior in rats (18).

In summary, a good measure of pain should be also a good measure of inhibitory effect of hyperalgesia, and the analgesic agent should be systematically related to behavioral indices of pain. Applying this criterion to the present study, we suggest that flinching behavior is a better index than licking because it is more robust in terms of generating the biphasic response at all concentrations of formalin. We also suggest that the formalin 2.5-5% are better doses than 10% because they evoke maximal responses. The formalin 10% is not recommended for a pain test, even though it enhances paw edema prominently, but this effect did not correlate with nociceptive behavioral responses. All kinds of stress may influence peripheral blood flow and edema formation and thereby the test results without correlation to nociception itself.

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