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Halothane Effect on Formalin-Induced Paw Edema and Flinching in Rat

The formalin test is a model of injury-produced inflammatory pain. Anesthetics, in clinically relevant concentrations, affect neutrophils and immune suppression. This study was to determine whether halothane reliably inhibits inflammatory reaction and formalin induced pain behavior or does not. Rats were exposed to 100% oxygen (control) or halothane, respectively for 30 min and then 24 hr later five percent formalin test was assessed. The base values of the paw's diameter were obtained earlier, and then formalin induced edema was assessed by measuring diameters of the injected paws at 5 min, 1 hr, 4 hr and 24 hr after the injection. Nociceptive behavior was quantified by counting the number of times with the paw flinched at 5 min intervals for 60 min. The diameters of edema in the halothane group lessened more than those in the oxygen group at 1 and 24 hr in each following of the injection ($p<0.05$). The rats pre-administered with oxygen or halothane were similar appearances in nociceptive behaviors. It suggests that halothane anesthesia might inhibit slightly the inflammatory reaction with the formalin-induced edema but might not inhibit the formalin-induced pain behavior in the event of pre-administration halothane 24 hr earlier before the formalin test of rat.

Key Words : *Central nervous system agents; Anesthetics, general; Halothane; Vertebrates, rodentia, rats; Diagnostic techniques, neurological, neurologic examination; Pain measurement*

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INTRODUCTION

The rat formalin test, which causes local tissue injury of the paw, has been used as a model for tonic pain (1) and localized inflammatory pain (2, 3). Formalin-induced pain is caused primarily by peripheral tissue inflammation (4). The peripheral sensitization of primary afferents (5) and central sensitization of dorsal horn neurons (6) occurs during inflammatory pain.

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). Neutrophils stimulation also causes increased vascular permeability and produces edema (8), which is the hallmark of acute inflammation. Several studies have shown that, after injection of formalin, edema of the injected paw develops rapidly (9, 10).

Anesthesia and/or the stress of surgery has been associated with decreased lymphocytes recirculation (11), decreased delayed hypersensitivity reactions (12), and decreased neutrophils function (13). Although most studies demonstrate a decrease in neutrophils phagocytosis, in response to anesthetic exposure, there are conflicting reports (14, 15). Both anesthesia and surgery, individually and jointly, suppress a number of immune response components; however, the clinical ramifications of these changes may not be readily apparent. Such interactions may increase the risk of postoperative infection or tumor metastasis. Conversely, immune suppression may be beneficial in attenuating the deleterious effects of inflammatory or cellular immune response (16).

In recent years, there has been increasing interest in the possible effects of anesthetic agents on the process of nociceptive activity (17). Because acute inflammatory response is a reaction to initial localization of antigens, anesthesia-induced decreased primary non-specific immunity may influence acute inflammatory reaction (16). On the basis of the above mentioned experimental evidences, authors carried out the animal study for determining whether halothane anesthesia inhibits inflammatory reaction (edema) and for-

malin induced pain behavior (flinch) or does not.

MATERIALS AND METHODS

Animal preparation

Male Sprague-Dawley rats weighing between 250-300 g were used. With the approval of the Subcommittee on Research Animal Care, rats were kept in a cage at a barrier facility, maintained in a 12-hr light-dark cycle and allowed free access to food and water. Rats were divided into two groups of 100% oxygen (control, n=8) and 1% halothane (n=8). All studies were performed between 09:00 and 17:00 h. The testing room was maintained at 22-24℃ (18).

Control rats were placed in a plexiglass box flushed continuously with 100% oxygen at 5 L/min for 30 min. Anesthesia was induced by placing the animals in a Plexiglas box prefilled and flushed continuously at 99% oxygen and 1% halothane (Datex 222 anesthetic agent analyzer, Puritan Bennett, MA, U.S.A.). Halothane 1% was chosen to provide approximately 1.0 minimum alveolar concentration (MAC) anesthesia. This dose were calculated on the basis of reported MACs in the rat of 0.95±0.11% for halothane with 0% nitrous oxide (19). Animals were left undisturbed for 30 min and then were removed from the Plexiglass box, transferred to a clear cage bedded thinly with wood chips, and allowed to awaken.

Twenty four hours later, five percent formalin at it's test was prepared from a 37% formaldehyde solution by 1:19 dilution with 0.9% NaCl. Conscious rats received a subcutaneous injection of 100 μL of 5% formalin solution into the plantar surface of the left hind paw with a 27-gauge needle. The 24 hr interval from halothane anesthesia to the formalin test was referred to the literatures (20-22) for the time process of immune response.

Measuring of edema in the injected paw

The diameters of the hind paws were measured earlier for obtaining the base line value before the formalin injection and then those of the formalin injected paw edema were also measured for evaluating the dorsal plantar foot thickness of the metatarsal level by a caliper at 5 min, 1 hr, 4 hr and 24 hr in each after the injection. Both of the hind paws were measured simultaneously.

Nociceptive behavioral responses

After the formalin injection, rats were then placed in a 30 cm×30 cm×30 cm Plexiglass box to allow an unobstructed view of the paws. Observations for the purpose of generating nociceptive scores began immediately after formalin injection and were continued for 60 min. A nociceptive score was determined for each 5 min time block by measuring the sum of nociceptive behavior. Nociceptive behavior was quantified by counting the number of times as the injected paw flinched spontaneously, and it was counted as a unit of one flinch when the paw lifted one time. Formalin-induced nociceptive behavior was assessed by one observer who did not know the type of the group. The flinch data was recorded for the early acute phase (0-5 minutes after formalin injection, phase 1) and the late tonic phase (20-60 min after formalin injection, phase 2).

Statistical analyses

Data were presented as mean±SE. Analyses of the statistical significance of these flinch data between the groups were determined by t-test. Analyses of the statistical significance of these paw diameter were determined by t-test or Mann-Whitney rank sum test. Significance was measured at a level of $p<0.05$. All calculations were performed by use of a statistical software package (Sigmastat 2.0, Jandel Scientific, San Francisco, CA, U.S.A.).

RESULTS

The rats were so much alike following of recovery from halothane anesthesia that they were impossible to distinguish the oxygen group from the halothane group. At the time of the formalin injection, they did not react to it, and there were no evidence of abnormal ambulation and activity, altered bowel and bladder function, grooming and a change of appetite in all of subjects while the experimental period.

The diameters of the hind paws in halothane group showed less significantly than those in oxygen group at 1 hr and 24 hr in each after formalin injection ($p<0.05$) (Table 1, Fig. 1).

The nociceptive flinching behaviors were similar patterns between two groups following of the formalin injection even though the sum of the phase 2 in the halothane group showed a decrease of only 17% as comparing to that in oxygen group while the progressive biphasic flinching responses were induced (Table 2, Fig. 2).

DISCUSSION

As hypothesized, we would like to know whether halothane anesthesia might inhibit inflammatory reaction and if this inhibited inflammatory response might affect the pain perceptive pathway. Using a formalin-induced acute inflammatory pain model, we did not demonstrate a significant phase 2 inhibitory effect of halothane. Conversely, as expected, the

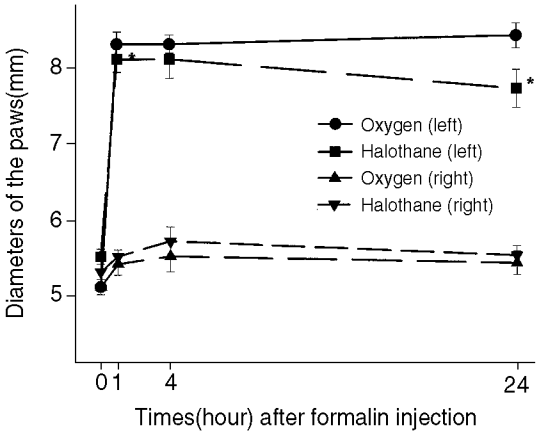

Fig. 1. Time course of the halothane effect on the paw edema after injection of 5% formalin into the plantar surface of left hind paw. The diameters of paws were measured at 5 minutes, 1 hr, 4 hr and 24 hr after the formalin injection. The increases of diameters in the halothane group were significantly less than those in the oxygen group at 1 hr and 24 hr each after formalin injection as compared with the baseline values of the pre-injection diameters. Data are mean±SE. *p< 0.05 compared with the oxygen group by t-test (n=8).

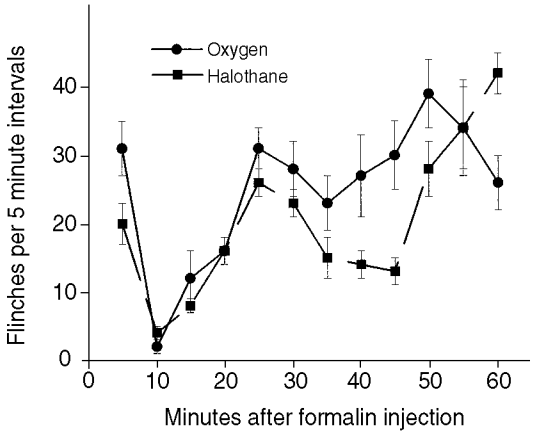

Fig. 2. The flinch response was measured after 5% formalin injection into the plantar surface of left hind paw at every 5 min intervals for 60 min. There was no difference between groups. Data are mean±SE (n=8).

Table 1. Effect of halothane on formalin-induced edema of the hind paws

		An increase of diameter (mm) after formalin injection				
Group	No.	Base	5 min	1 hour	4 hours	24 hours
Oxygen						
Right	8	5.1±1.0	0.2±0.1	0.3±0.1	0.5±0.2	0.3±0.2
Left	8	5.1±1.0	2.6±0.1	3.2±0.2	3.2±0.1	3.3±0.2
Halothane						
Right	8	5.3±0.7	0.1±0.1	0.2±0.1	0.4±0.2	0.2±0.1
Left	8	5.6±0.5	2.2±0.2	2.6±0.2*	2.6±0.3	2.2±0.3*

Data are mean±SE. Baseline values are pre-injection diameters.
*p<0.05 compared with the oxygen group by t-test.

Table 2. Effect of halothane on formalin-induced flinches

Group	No.	Phase 1	Phase 2
Oxygen	8	31±4	236±16
Halothane	8	20±3(-35)	196±14(-17)

Data are mean±SE. Numbers in parentheses are percentage in change of flinches from the oxygen group. There was no differ-ence between two groups.

halothane anesthesia was effective in reducing edema of the injected paw at 1 hr and 24 hr after formalin injection.

Immunity is a mechanism which recognizes a foreign substance and responds in an attempt to eliminate it. A primary response is non-specific in nature and requires no prior antigenic experience for immediate activation. Acute inflammatory response is mediated primarily by blood leukocytes, such as neutrophils and macrophages (16). Halothane, enflurane, nitrous oxide and high concentrations of isoflurane all inhibit superoxide generation by neutrophils (23). Halothane also inhibits chemotaxis of neutrophils (13), interferon-induced stimulation of natural killer cells cytotoxicity of murine splenic mononuclear cells after exposure and the duration of the anesthesia-induced inhibition lasts at least 11 days (24). Neutrophils stimulation also causes increased vascular permeability and produces edema (8). In the current study, formalin rapidly induced edema in the injected paw. We knew that formalin causes peripheral tissue inflammation. The edema peaked between 1 and 4 hr after injection in halothane, between 4 and 24 hr after injection in the oxygen group. The edema lasted 24 hr in both groups. The diameter was increased by 64% in oxygen and 46% ($p<0.05$) in the halothane group. This study does not indicate whether the effect of halothane was related to the inhibitory effect of neutrophils migration, the cytocidal activity of lymphocytes or the suppression of the immune response, because an immune-related study was not tested.

Factors that were related to inflammation include volume, pH and temperature (18, 25). In the current study, we used the same volume and concentration of formalin. A given room temperature was maintained continuously.

Formalin pain is caused by peripheral tissue inflammation (4). Normally fine afferent C- and A-fibers are activated by brief, high intensity stimuli, but the stimuli induce little or no tissue damage. However, during the inflammation, which was produced by mild tissue damage or infection, afferent fibers were activated by lower intensity stimuli and the produced pain differed in quality and may be more persistent. There is an associated phase of inflamma-

tion in which a variety of chemical mediators are able to alter the functions of peripheral afferent fibers. The inflammatory mediators released from the injured tissue play a central role in pain associated with injury (3, 5). In the current study, the formalin resulted in a progressive biphasic behavioral response such as flinching, favouring, licking and biting of the injected paw in both groups. Flinching was chosen as a measure of pain because it is more robust and spontaneous than other formalin pain-related behaviors (i.e., favouring, licking, and biting). The initial response begins immediately after injection and peaks for 5 min (phase 1) in both groups; following a 10-15 min quiescent period. A second response follows and lasts about 60 min with distinctive flinching, licking and biting (phase 2) in both groups too.

Recent studies suggest that phase 1 is caused predominantly by activation of C-fiber afferents by peripheral stimulus (26). Nociceptive sensitization, an increase in dorsal horn cell response to noxious stimulation, is thought to be responsible for phase 2 of the formalin test. Phase 2 is the result of central sensitization of nociceptive neurons induced by phase 1 and is thought to be mediated by excitatory amino acids such as glutamate (27). Therefore, blocking phase 1 stimulation and/or disruption of central neurochemical processes responsible for sensitization (i.e., potent analgesics, NMDA antagonists) attenuate the phase 2 hyperalgesic response (17). However, there are also other results that these agents suppress in phase 2, but not in phase 1 of the formalin response (28). In the current study, halothane produced a 35% reduction in phase 1 flinching, whereas this reduction was not sufficient to suppress phase 2 activity effectively.

Recent studies have suggested that inhalation anesthetic agents may affect spinal cord processing and modulate the response to nociceptive stimuli (29). However, the mechanism by which this might occur is a matter of debate. The effects of several inhalation anesthetics on the rat paw formalin test have been documented in several recent studies (17, 30). In all of these studies, the anesthetics were administered during and for a short period after formalin injection, and were discontinued before the onset of phase 2. They showed that administrating halothane during phase 1 demonstrated a 40% decrease in phase 2 activity (30) or no effect on phase 2 flinching (17). It is unclear why their data disagrees with each other. In the current study, we administered halothane 24 hr earlier before the formalin test. Halothane group produced only 17% reduction in phase 2 activity. Therefore, the time difference from the pre-administration of anesthetic to tissue injury is too long to reveal a antinociceptive response effectively, even if this choice of time interval was to inhibit the inflammatory reaction. Reduced paw edema by halothane therefore is questionable, but our finding reveals a trend of reducing edema at 24 hr after formalin injection ($p<0.05$). It indicates that the clinical importance of inflammation in acute tissue trauma requires further study. Inflammation alone seems insufficient to elicit the behavior as seen in phase 2, as inflammatory stimuli that induce an even greater degree of inflammation, such as yeast and carrageenan, produce little pain-like behavior in rats (25).

In summary, our data suggest that a pre-administration of halothane 24 hr before may reduce paw edema, but this benefit is limited and did not have any inhibitory effect on the nociceptive behavioral response of rats in the formalin test.

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