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Local Cryoanalgesia Is Effective for Tail-Tip Biopsy in Mice

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Tail-tip biopsy for genotyping of genetically modified mice older than 21 d typically is performed by using isoflurane anesthesia. Isoflurane-induced changes in behavior and metabolism can result in unexpected complications and death. We investigated whether cryoanalgesia by using ethylene chloride spray would be an effective local anesthetic for tail-tip biopsies in mice. C57BL/6J mice were allocated randomly into 4 groups (n = 10 each) to receive isoflurane anesthesia with tail biopsy, ethylene chloride spray on the tip of the tail before biopsy, ethylene chloride spray without biopsy, or no treatment. Blood glucose was measured periodically in both groups undergoing tail biopsy, and the tail-pinch assay was performed in all mice that received ethylene chloride spray. Body weight, water, and food intake were measured daily for 2 wk. In both groups undergoing tail biopsy, blood glucose levels at 15 min were significantly higher than those after 2 min. This elevation was greater and more prolonged after 30 min in mice that received isoflurane compared with ethylene chloride spray. Tail-pinch latency at 20 min was greater than that after 2 min in all mice that received ethylene chloride spray. All mice gained weight, and there was no difference in food and water intake among groups. We conclude that ethylene chloride spray is an effective local anesthetic and a valuable alternative to isoflurane.

Tail biopsy is performed routinely for genotyping genetically modified mice. Although alternative methods to obtain DNA that use the ear pinna, blood, saliva, hair, and buccal and rectal mucosa are available,[3,14,18,22] tail biopsy remains the method of choice.[12,11] According to the NIH, "Obtaining tissue via tail biopsy is a safe, effective, and humane procedure that causes minimal or transient pain and distress when performed properly."[20] According to institutional recommendations, mice older than 21 d require anesthesia for this procedure; isoflurane often is used.[11] This method requires the use of either a vaporizer or, if performed by using an open-drop method, a ducted hood. However, these types of equipment may not be readily available in some facilities. In addition, laboratory personnel need to be trained in using and performing general anesthesia. Furthermore, the influence of isoflurane on behavior and metabolism, combined with the risk of general anesthesia, could result in unexpected complications and loss of animals, especially in particularly sensitive strains. For example, some strains are susceptible to isoflurane-induced seizures.[16] Therefore, local anesthesia may be preferable in these strains.

Cryoanalgesia is used in humans as a minimally invasive method for producing local anesthesia and is efficient for short-term use.[9,13,15,21] Ethylene chloride spray is a vapocoolant that is intended to control pain associated with minor surgical procedures, dermabrasion, injections, and minor sports injuries in humans.[15] This product contains nontoxic, nonflammable, ecologically friendly, and inexpensive gases developed as refrigerants. Another vapocoolant, ethyl chloride, is flammable and has a greater ecologic effect than does ethylene chloride.[15] Therefore ethylene chloride spray is preferable to ethyl chloride.

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In the current study, we investigated whether the use of ethylene chloride spray as a local anesthetic is feasible, efficient, and effective at eliminating pain during tail biopsies in mice older than 21 d.

Materials and Methods

Animals. This IACUC-approved study involved C57BL/6J mice (n = 40; The Jackson Laboratory, Bar Harbor, ME) that were treated in accordance with guidelines of The University of Texas Health Science Center (Houston, TX). Mice were bred and group-housed, given water and rodent diet (Purina, St Louis, MO) ad libitum, and maintained on a 12:12-h light:dark cycle. They were housed in individually ventilated cages on corncob bedding. Sentinels exposed to dirty bedding from all cages on the rack were negative for Sendai virus, pneumonia virus of mice, mouse hepatitis virus, *Mycoplasma pulmonis*, mouse poliovirus (GD7), minute virus of mice, reovirus 3, mouse adenovirus, ectromelia virus, polyoma virus, lymphocytic choriomeningitis virus, mouse parvovirus, epizootic diarrhea of infant mice, pinworms, and fur mites. Offspring (male and female) were allocated randomly into 4 groups (n = 10 each) at 25 to 28 d of age to receive isoflurane anesthesia with tail biopsy, ethylene chloride spray on the tip of the tail before biopsy, ethylene chloride spray without biopsy, or no treatment.

Anesthesia–analgesia and tail-tip biopsy. After anesthesia was induced with isoflurane via a vaporizer, mice in this group were placed in sternal recumbency. The tail was prepared for biopsy by using 2% chlorhexidine gluconate (Vedco, St Joseph, MO). A 5-mm portion was cut from the tip of the tail by using a no. 10 scalpel blade. Hemostasis was achieved by cautery (Bovie, Clearwater, FL).

Mice that received the ethylene chloride spray (Fluor–Ethyl Spray, Gebauer, Cleveland, OH) spray were assessed by using the tail pinch assay before application of the ethylene chloride spray. Each mouse then was placed in a rodent plastic tube restrainer, with its tail exposed. The ethylene chloride spray was

Figure 1. Effect of ethylene chloride spray on a mouse tail. Left panel: The tail before application of the ethylene chloride spray. Right panel: the skin of the tail turned white after application of the ethylene chloride spray.

applied from a distance of 8 to 10 cm for 4 to 10 s until the skin turned white (Figure 1). Mice were returned to their home cages for observation. After 2 min, the tail-pinch assay was repeated. Mice undergoing tail biopsy were returned to the restrainer for the procedure, which was performed as for the isoflurane group.

Blood samples were collected from the tail tips of all biopsied mice at 2, 15, and 30 min after biopsy for measurement of blood glucose levels (Alpha Trak 2 Glucose Test, Abbott, Chicago, IL). Mice were returned to their individual cages and were assessed by using a mentation scoring system; in addition, behaviors such as attending to the tail, licking, and respiratory effort were recorded every 5 min for 2 h. Mentation was scored as follows: 3, mouse was bright, alert, and responsive; 2, mouse was quiet, alert, and responsive; 1, mouse was depressed but responsive; and 0, mouse was depressed and nonresponsive. Breathing was scored as: 2, normal and regular breathing; 1, increased breathing but no respiratory effort; 0, increased breathing and abdominal effort.[24] The mice were observed for 14 d, and body weight and food and water intake were assessed and recorded daily.

Tail-pinch assay. The tail pinch assay was performed by placing a bulldog clamp (V Mueller, Kleve, Germany) on the tip of the tail. If no response occurred after 15 s, the clamp was released to avoid tissue damage. This assay was performed at 0 and 2 min after provision of isoflurane or ethylene chloride spray in mice that underwent biopsy or at 0, 2, 10, and 20 min in mice that did not receive the biopsy.

For statistical analysis (SPSS version 16.0, IBM, New York, NY), Student *t* tests and ANOVA were performed on normally distributed data, and rank scores (breathing, mentation) were assessed by using comparable nonparametric statistics. Data are presented as mean ± 1 SD; a P value less than 0.05 was considered statistically significant.

Results

A total of 20 mice underwent either isoflurane anesthesia or local anesthesia by using the ethylene chloride spray for the tail biopsy procedure. The skin of the tail turned white after application of the ethylene chloride spray (Figure 1).Tail-tip

biopsy was performed in 18 mice; 2 mice of the ethylene chloride spray group had to be excluded from the study because of respiratory distress.

All mice gained weight in the 2-wk time period. Those in the ethylene chloride spray group gained weight rapidly, with a mean body weight of 16.8 ± 1.8 g on day 4 compared with 15.5 ± 1.6 g on day 1 ($P > 0.05$). The weight of the mice receiving isoflurane was 16.5 ± 1.7 g on day 5 compared with 15.1 ± 1.7 g on day 1 and was 16.2 ± 1.2 g on day 6 compared with 15.2 ± 0.5 g on day 1 of the combined (no-biopsy) control group ($P > 0.05$; Figure 2).

Tail-pinch assay results (latency to response [s]; mean ± 1 SD) at 2 min after introduction of isoflurane was compared with those at 0, 2, and 20 min after application of ethylene chloride spray. In the ethylene chloride spray group, the latency to response was 0.68 ± 0.35 s at 0 min, 7.08 ± 4.46 s at 2 min, and 2.20 ± 1.51 s at 20 min. Tail-pinch latency at 2 min was shorter ($P \leq 0.05$) for mice that received the ethylene chloride spray (7.08 ± 4.46 s) than for the isoflurane group (12.4 ± 3.53 s).

There was a difference in the distribution of sexes among the groups. Whereas the isoflurane and ethylene chloride spray groups had equal numbers of male and female mice, the no-treatment control group had more ($P < 0.05$) female mice. There was no difference in food and water consumption during and after the 2-wk period (repeated-measures ANOVA, $P > 0.05$).

No difference in mentation score was observed between groups (Figure 3; Mann–Whitney *U* test, $P > 0.05$). The ethylene chloride spray group was more active and had higher attention to their tails after the application of the ethylene chloride spray; their mentation score for the first 15 min (2.8 ± 0.3 min) was higher (Dunn multiple comparison test, $P < 0.05$) than that 2 h later (2.5 ± 0.2 min). More licking and rebleeding were observed in these mice compared with the isoflurane mice after 15 and 30 min (Fisher exact test, $P < 0.05$).

Two mice were depressed and in respiratory distress after application of the ethylene chloride spray. These mice were treated and recovered successfully but were excluded from analysis. No difference in breathing score occurred between groups at any time point (Mann–Whitney *U* test, $P > 0.05$).

The blood glucose level was recorded at 2, 15, and 30 min after the induction of anesthesia in the mice that underwent tail biopsy. At 2 min, the blood glucose level was 253 ± 43 mg/dL for the isoflurane group compared with 222 ± 34 mg/dL for the ethylene chloride spray group. Blood glucose increased in both groups after 15 min but was significantly (*t* test, $P < 0.05$) lower in the ethylene chloride spray group (262 ± 59 mg/dL) than in the isoflurane group (331 ± 66 mg/dL). After 30 min, the blood glucose decreased to 258 ± 59 mg/dL for the isoflurane group and to 243 ± 29 mg/dL for the ethylene chloride spray group (Table 1).

Among mice that underwent tail biopsy, the tail-pinch latency at 2 min was longer ($P < 0.05$) for the isoflurane group (12.4 ± 3.5 s) than for the ethylene chloride spray group (7.1 ± 4.5 s; Figure 2). Eight mice that received ethylene chloride spray did not show any response before the cutoff time of 15 s. The latency to respond in the ethylene chloride spray group was 0.7 ± 0.3 s at 0 min, 7.1 ± 4.5 s at 2 min, and 2.2 ± 1.5 s at 20 min (Figure 2).

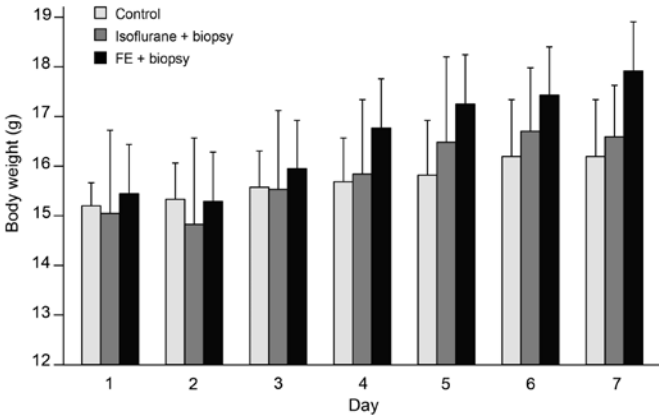

Figure 2. Daily body weight (g; mean ± 1 SD) during the first 7-d period in mice that received isoflurane with biopsy, ethylene chloride spray with biopsy, ethylene chloride spray without biopsy, and no treatment.

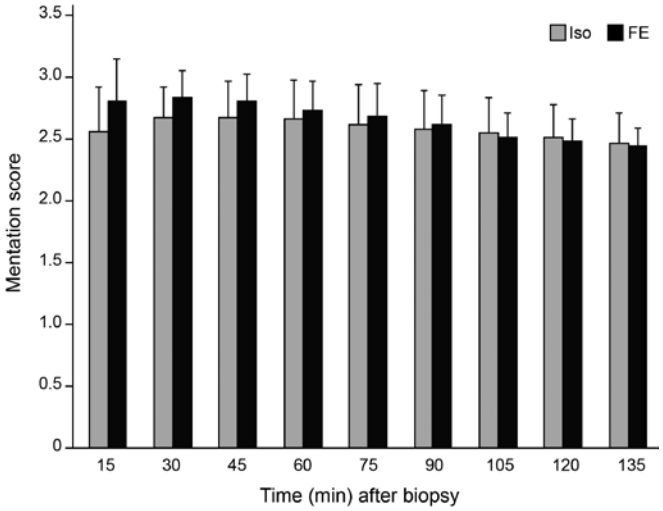

Figure 3. Mentation score (mean ± 1 SD) did not differ between groups. In mice that received the ethylene chloride spray, the mentation score was higher ($P < 0.05$) during the first 15 min (2.81 ± 0.35) than at 2 h (2.52 ± 0.20).

Discussion

Our results demonstrate that ethylene chloride spray is a feasible, efficient, and effective local anesthetic. All mice gained weight as expected during the 2 wk after the procedure; no decrease in weight or food and water consumption was noted. Moreover, the ethylene chloride spray mice gained weight more rapidly, showing a significant increase from their initial weight at day 4. The mice in the isoflurane and control groups reached a significant increase from their initial weight on days 6 and 7, respectively. This difference from the ethylene chloride spray group may be explained by several factors. First, there was a difference in the distribution of sexes among the groups. Whereas the isoflurane and ethylene chloride spray groups had equal numbers of male and female mice, the combined control group had more female mice. Male mice grow faster than do female mice, and this characteristic may be reflected in the difference in weight gain between the groups.[4] However, no difference in weight was noted between the isoflurane and combined control groups.

Another factor influencing weight gain may be changes in behavior. The ethylene chloride spray mice were very active during the observation period. They had a higher mentation score and presented with eating, nesting, and licking behaviors in the first 30 min. Similar behavior was observed in a previous study,[1] in which rats reacted to a tail pinch with eating, gnawing, and licking behavior. Such behavior has been noted as a 'consummatory' response and is induced by dopamine as a by-product of the stress response.[1] In the current study, we

Table 1. Blood glucose (mg/dL; mean ± 1 SD) at various times after provision of isoflurane or ethylene chloride spray

	2 min	15 min	30 min
Isoflurane	252.83 ± 43.42	331.42 ± 65.51[a]	258.42 ± 59.43
Ethylene chloride	221.87 ± 33.63	262.00 ± 59.15	242.75 ± 28.83

[a]Blood glucose at 15 min was significantly higher ($P < 0.05$) in the isoflurane group than the ethylene chloride group.

observed a similar phenomenon in the ethylene chloride spray mice, and this behavior may explain their rapid body weight gain compared with that of the isoflurane mice. However, whether this active behavior persisted is unknown, because we did not monitor behavior beyond the 2-h observation period.

In addition to eating and nesting, mice reacted immediately after application of the ethylene chloride spray with high attention to and licking of their tails. This reaction may be, beyond the consummatory response, explained by changes of sensation. The rapid vaporization results first in numbness and decreased circulation and sensation,[13,21] which cause an increased attention and licking response that is indicated as a supraspinal, cognitive response. After 15 to 20 min, when the vaporization effect has waned, the vasoconstriction of the blood vessel reverses, explaining both the mice's attention to their tails and the recurrence of bleeding after biopsy, which was observed only in the ethylene chloride group. This recurrence of bleeding was minor and can be prevented in the future by increasing the duration of cautery. Another key factor in increased bleeding recurrence is blood pressure, which might be increased due to physical stress from handling;[2,7] such stress would be prevented by general anesthesia. The assessment of blood pressure and heart rate require special equipment; these parameters were not assessed in the study, which focused on behavior and safety. However, assessment of these vital functions gives insight into stress and should be considered for further studies.

After the 2-h observation period, all mice had returned to normal behavior, giving almost no attention to their tails. No bleeding occurred in the ethylene chloride spray mice after 2 h, and the isoflurane mice became more active after 2 h than they had been at the beginning of the observation period. No difference in behavior was noted between groups after 2 h.

Blood glucose is another key factor for determining the stress response. Mice demonstrated a high blood glucose level under isoflurane anesthesia.[10,19] This finding was expected and consistent with previous data[8] regarding the influence of isoflurane on blood glucose in C57BL/6 mice. Blood glucose levels at 15 min were elevated in the mice receiving the ethylene chloride spray, although the effect was less than that of mice under isoflurane anesthesia. This finding may be explained by the fact that the mice were restrained and conscious during the procedure, which causes stress.[7,23] Physical and emotional stress lead to elevated blood glucose levels in mice.[23] Stress raises the production of catecholamines, such as adrenaline and noradrenaline, that stimulate processes of glyconeogenesis, glycogenolysis, and lipolysis.[6,17] In the cited studies,[6,17] exposure to stress resulted in an increased blood glucose level after 30 min, which returned to normal after 120 min. In our study, the glucose level normalized after 30 min, perhaps indicating that the stress was less intense than that in previous studies.

In clinical studies, vapocoolant spray was effective in children[9,15] yet ineffective in adults.[5] In the current study, we noted that to be effective, a narrow stream should be focused on the tip of the tail until the skin blanches. If the application time is too short, ethylene chloride spray is not effective, and if the application time exceeds 10 s, skin damage may occur. In addition, caution needs to be taken during application. Accidental inhalation can lead to respiratory complications, as occurred in 2 mice in the beginning of the study. However, this drawback can be eliminated by using a narrow cone that is focused on the tail tip, covering the face of the mouse, and directing the spray away from the mouse's head.

Our findings from the current study suggest that the use of ethylene chloride spray may be useful for mice as an alternative to isoflurane. The vapocoolant that we used is nontoxic, nonflammable, eco-friendly, and easy to apply. However, additional studies are warranted to reveal more information about the stress experienced by the mice. Assessment of heart rate, blood pressure, and fecal cortisol would be useful to evaluate the intensity of both pain and stress reactions. Despite this limitation of our study, we conclude that ethylene chloride spray is an acceptable alternative to isoflurane in mice, particularly when the strain is susceptible to side effects caused by general anesthesia.

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