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Antinociceptive Effect of Vapocoolant Medium Stream Spray onHotplate LatencyinRat Pups

Short Running Title: Vapocoolant has antinociptive effect on glabrous skin.

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Abstract

Background: Heel sticks account formostblood tests performed in neonateswithout analgesia because topical localanestheticsare ineffective on heelglabrous skin. We investigated the antinociceptive effect of an alternative topical analgesic, avapocoolant spray, on hindpaws glabrous skin of rat pups. The spray was applied by two methods: method-1 for 4 seconds at a distance of 8 cm and method-2 for 10 seconds at a distance of 18 cm.

Methods: The rat pups were randomized to either method-1 (n = 32) or method-2 (n = 31). Vapocoolant spray was applied to one hind paw randomly and saline spray was applied to the contralateral paw. The paws were exposed to a hotplate test to measure withdrawal latency timebefore and 30 seconds after the spray applications. Additionally, rat pups were tested for tissue toxicity in method-1 (n = 20) and method-2 (n = 20)after application of the vapocoolant spray before heel sticksthree times a day for two consecutive days.

Analyses of spray andmethod effects on hotplate withdrawal latency time weredetermined by nonparametric Wilcoxon tests to assess paired difference between vapocoolant spray and saline spray and to compare difference in medians between the two methods.

Results: Method-1 and method-2vapocoolant spray applications significantly prolonged the withdrawal latency time compared to saline, a median difference of0.6 seconds (IQR 0.1-1.2)for method-1 and 9.5 seconds (IQR 5.5-10.7)for method-2 (a 15-fold longer latency time with method-2). Method-2 produced significantly longer withdrawal latency time than method-1 with a difference in median time of 8.9 seconds (CI:95% 7.3-10.4 seconds, $P< 0.0001$). No histopathological changes were detected.

Conclusions: Compared to method-1, the vapocoolant spray in method-2 produced significantly longer withdrawal latency time that is clinically applicable to collecting blood samples after a heel stick.

Keywords: rat pups, topical anesthetic, heel stick, efficacy, toxicity.

Clinical Implication

What is already known

- Heel stick is more commonly performed than venipuncture on newborn infants because it provides greater success for testing frequent and adequate blood samples. It is usually performed without analgesia because topical local anesthetics are ineffective on newborn glabrous heel skin. Repeated painful skin breaking procedures in NICU including the heel sticks without analgesia can negatively affect neurodevelopmental outcomes later in life.

What this study adds

- A single application of the medium stream vapocoolant spray is effective in raising the withdrawal latency time to noxious heat on glabrous heel skin of rat pups; this may provide its potential clinical utility for testing in human.

Introduction

Infants in neonatal intensive care units (NICU) are exposed to frequent painful skin-breaking procedures such as heel sticksfor capillary blood sampling and venous and arterial blood sampling.[1]These procedures could negatively affect pain sensitivity and neurological outcomes later in life.[2,3, 4]Although the total number of NICU procedures has declined in recent years to minimizing the harm of neonatal stress, further reduction of number of medically necessary procedures for diagnosis and treatment is limited particularly insevere illnesses.[5]In newborn infants,heel stick is more commonly performed than venipuncture because the highly vascularized heel skin lends itselftotesting frequentand adequateblood samples. Infant veins are difficult to access and too small to provideadequate blood volumes and often require a trained phlebotomistto limit theunsuccessful attempts.[6]

Topical local anesthetics are preferable for anesthetizingskin before venipuncture because most lack systemic sideeffects,although in newborn infants they lackefficacy on heel glabrous skin.[7]Several anatomical and physiologicalcharacteristicsof the glabrous skin may account for theirineffectiveness. A controlled trial in neonates showeda high microvascular blood flow of heel skin comparedto non-glabrous skin and speculated that rapid vascularuptake might be responsible for the high clearance of topical localanesthetics before they can reach subcutaneous nociceptors.[8]It is this limitation of topical local anesthetics that prompted exploration of an alternative, a fast vaporizing volatile liquid vapocoolant agent that can produceskin hypoesthesia by rapid lowering of the skin surface temperature and suppressing the velocity of nociception transmission.[9]Vapocoolant spraysare commercially available skin-coolant andareFDA approvedfor anesthetizing non-glabrous skin. For an aerosol medium stream spray (Pain Ease®) (chemical name 1,1,1,3,3-Pentafluoropropane 95% and 1,1,1,2-Tetrafluoroethane 5% and formula $CHF_2CH_2CF_3/CH_2FCF_3$; Gebauer Company, Cleveland, OH 44128, USA. www.GebauersPainEase.com)two methods of clinical application are recommended for non-glabrous skin.The dosing parameters in this study were chosen based on manufacturer's package insert recommendations and based on efficacy and safety clinical studies of venipuncture in children and adults.[10,11,12,13].

In method-1the sprayis applied for 4 seconds at an 8 cmdistance from the skin and in method-2it isapplied for 10seconds at an 18 cm distance.[13]A randomized controlled trial in children demonstrated

effectiveness of both these methods in anesthetizing the non-glabrous skin during venipuncture without significant adverse effects.[11]

A preliminary rat pup modelshowed that a single application of the medium stream spray on glabrous hind paw produced antinociceptive effect, as determined by prolongation of nociceptive flexor withdrawal latency time (WLT) in response to heat stimulus.[14]We therefore, hypothesized that the antinociceptive effect of the medium vapocoolant spray applied by method-1 and method-2, as determined by WLT in response to heat stimulus, is similar when applied on glabrous hind paw of rat pups.Alternatively, the antinociceptive effect in one method is longer than the other. We also hypothesized that application of the vapocoolant spray before repeated heel sticksdoes not producetissue toxicity.

Material and Methods

Test Materials: Vapocoolant and saline spray canisters were identical. The canisters were stored at room temperature between 21.4°C and 23.5°C.

A modified hotplate test was used to testing the effectiveness of the vapocoolant spray by measuring the WLTof a hind pawin response to noxious heat stimulus. This test is a behavioral model for nociception that is commonly employed for screening analgesic drug effects.[15]The spray was directed to the hind paw using a plastic straw extension attached to the nozzle of aerosol vapocoolant and saline cannisters.We used BD Quikheel™ Infant Lance (BD Vacutainer Systems, Franklin Lakes, NJ)for performing allhind paw heel sticks. This device is used routinely in NICU for collecting heel blood in term infants. It is an automated lancing device, applied at 90^0 angles to the length of lateral plantar surface with a mild pressure. When activated it automatically thrusts out and rapidly retracts a very thin surgical blade that pierces the skin at a depth of 1 mm and width of 2.5 mm. Pressure is applied to the incision site until bleeding stops.

Animals: After approval from IRB (Boston Children's Hospital protocol # P00017631) and Biomere's Institutional Animal Care and Use Committee (IACUC protocol # 16-30).[16]This study was conducted at the Biomere-Biomedical Research Models laboratory (57 Union Street Worcester, MA 01608. Ph. 508-459-7544, info@Biomere.com).The study was performed on awake Sprague-Dawley rat pups aged 7 days old, both male and female (Charles River Laboratories Wilmington, MA) a total of 64 rat pups were included in efficacy test and 40 rat pups in tissue toxicitytest. These pups were housed in a room on a 12-hour light/dark cycle with free access to water and food. They were kept in cages with their littermates and dams.

Study Design

Vapocoolant Efficacy Test: The vapocoolant and saline sprayswere applied randomly to either left or right hind paw of 32 rat pups in method-1 and 31 rat pups in method-2(Figure 1). The sprays were applied continuously for4 seconds at an 8-cm distance from the paw in method-1 and for 10 seconds at an 18-cm distance from the paw in method-2 and the paws were subjected to hotplate test before and 30 seconds after the spray application.Both methods of vapocoolant spray applicationproduce adequate analgesia in human non-glabrous skin lasting approximately 30 secondswhich is adequatefor performinga heel stick and collecting blood samples.[11]Heat pain sensitivity to the spray applications

was measured by changes in WLTin contact with a hotplate using a modified hotplate test that has been used in our and others' previous infant rat pup studies.[17,18]A rat pup was positioned so that its hind paw was placed on a52°C (accuracy is ±0.1°C) hotplate (model 39D hotplate analgesia meter; IITC Inc., Woodland Hills, CA). Hindpaw withdrawal latencyin response to nociception was determined as time in seconds between contact and withdrawal of the paw away from the hotplate. If there was no withdrawal response after 12 seconds, the experimenter removed the paw to avoid tissue injury.[14]The hotplate test was repeated 3 times at 10-second intervals at baseline and 3 more times after application of the sprays with a 30-60 second interval between trials.The median WLT was calculated from the 3 responses to hotplate testin each trial.A research staff whoapplied the sprays was unaware of the type of spray i.e., vapocoolant or salinein the efficacy test.After completion of the test, rat pups were returned to their dams for breastfeeding until euthanasia on day 7.

Vapocoolant Safety Test: Histologicalanalysis was performed in 20 rat pups in each of the method-1 and method-2 (Figure 1).An unblinded research investigator applied the vapocoolant spray randomly to one hind paw and the contralateral paw was used asa control.Thirty seconds after the vapocoolant application in each methoda heel stick was performed usinga BD Quikheel™ Stick device. This procedure was performedon the same hind paw3 times a day at 08:00 hrs., 12:00 hrs., and 16:00 hrs. for 2 consecutive days.To avoid contact with the heel bone and perform repeated heel sticks at previously un-lanced skin, the heel sticks were performed along the posterior curvature of the hind pawand some were performed interior to the curvature depending on availability of previously un-lanced skin.

Euthanasia: Seven days after the completion of all the experiments, the rat pups were euthanized in an induction chamber with medical grade inhaled compressed 100% carbon dioxide gas. In the safety test, after the rat pups were unconscious and the respiration ceased both hind paws were collected, and specimens were preserved in formaldehyde for histopathological analysis.

Histopathology: Eighty rat pup hind paw specimens, 40 in method-1 and 40 in method-2, were collected in separate containers and each labeled with an identification number and right or left. All the specimens were fixed in 4 % neutral buffered formalin. The tissue was examined and a representative cross section of the paw was submitted for routine processing and paraffin embedding. Five micron sections were cut from each of the 80 samples and stainedwith hematoxylin and eosin

stain(H&E)using a fully automatic Roche HE600 Stainerand two serial sections were cut from each paraffin block. A board certified dermato-pathologist (B.S)who was "blinded" to both the methods allocation and the hind paw spray treatment assignment examined the H&E slides.

Statistical Analysis

Power analysis indicated that a total sample size of 64 rat pups (32 randomized to each method) would provide 90% statistical power to test for equivalence in hotplate withdrawal latency to within a margin of 0.6 second (assuming a standard deviation of 0.8 seconds; effect size = 0.6/0.8 = 0.75) and to compare the difference between the two methods(nQuery Advisor version 7.0, Statistical Solutions, Cork, Ireland).[19]Therefore, the study design provided excellent statistical power to determine whether the single application spray-based method-1 compared to method-2 are equivalent to within 0.6 seconds (margin of equivalence) regarding withdrawal latency. All rats received vapocoolant spray and randomization determined the side of either vapocoolant or saline as well as whether a rat pup was randomly assigned to method-1 or method-2 for treatment. Analysis of vapocoolant versus saline spray and method effects on hotplate withdrawal latencyweredetermined bythe nonparametric Wilcoxon signed-ranks testand Wilcoxon rank-sum test, respectively. Quantile (median) regression was used to determine the 95% confidence interval for the difference in median WLT between method-1 and method-2.[20]Analysis of the data and randomization was performed using IBM SPSS Statistics software (version 23.0, IBM Corporation, Armonk, NY). Stata 12 was used for quantile regression (StataCorp LLC, College Station, Texas).

Results

One hundredand four rat pups aged postnatal day 7 were included for the experiments. Of the 64 equally randomized to the two methods, one rat pup in method-2was excluded because of lack of response to hotplate stimulus at baseline. Therefore, for efficacy testing, n=32 rat pups were included in method-1 and n=31 in method-2. All experiments were performed on the same day in each method. Rat pups in both methods responded with increasedWLTto vapocoolant compared to saline spray.The difference in medians for the paired deltas (vapocoolant spray - saline spray) in the WLT between the two delivery methods was 8.9 seconds (longer with method-2). Quantile regression indicated that the 95% confidence interval around this observed difference in medians for WLT is 7.3 - 10.4 seconds longer for method-2, $P< 0.0001$. (Figure 2). Application of room temperature saline spray is expected to produce mild skin cooling and elevation of WLTin response to hotplate nociception and reduction in the differences between the vapocoolant and saline WLT.[9]

In the safety study, H&E sections demonstrated a representative cross section with clear visible epidermis, appendageal structures, dermis, subcutis, nerve bundles, muscle, fibro-connective tissue, cartilage and bone with bone marrow elements. The epidermis, dermis with appendageal structures and nerve bundles were all carefully examined. The epidermis was intact, the appendageal structures and nerve bundles were within normal limitsand no differences noted between specimens treated with vapocoolant and untreated.There were samplesthat demonstrated mild perivascular lymphocytic infiltrates~~observed~~ in the superficial dermis. These changes were observed in both vapocoolant and untreated samples of the hind paw tissues.There were no areas in the papillary dermis or reticular dermis where neutrophilic infiltrate were seen. In addition, there were no areas of fibrosis or increased density of fibroblasts observed. All the examined tissues appear to be within normal limits and no significant pathologic changes were identified in any of the analyzed H&E stained slides (Figure 3).

Discussion

The primary finding of this study is that a singleapplication of vapocoolant sprayby two different methods significantly increased the nociceptive reflex ofWLTin response to heat nociceptioncompared to saline spray (Figure 2). The method of vapocoolant application produced significantly longer WLTinmethod-2 than method-1, a 15-fold longer.The WLTof a median of 9.5 seconds in method-2although seeminglyshortispracticallysuitablefor performing a heel stick and collecting the usual blood sampleof <1 mL in capillary tubes or drops of blood on filter papers for analyses. This WLT of vapocoolant antinociceptionon glabrous skin of rat pups is much shorter than reported analgesic duration of30-60 seconds on non-glabrous skin during pediatric venipuncture.[11] The increase in WLT in this study reflects a reduction in heat noxious stimulus-evoked behaviorafter application of vapocoolant spray regardless of the method of application. While the method-1 application of the vapocoolant spray significantly increased WLT relative to saline, the latency duration is too shortfor collecting blood samples after a heel stick. Application of the vapocoolant spray by either method repeatedly beforeheel stickson the same paw did not produce visibletissue pathology(Figure3).

Therelevance of thispreclinical model to the human newborn remains to be tested i.e., does the decrease in sensitivity to surface heat nociceptionin rat pups translate to decreased pain sensitivity to a heel stickin newborns.Theanimal studiessuggestthat neurodevelopment and nociception detection pathways in 7 to 10-day-old rat pupsapproachthat of preterm human infantsaged 28 to 29 weeks post-conception.[21]And the evidence confirms that untreatedrepeated procedural pain in human newborns andrat pups lead to adverse neurodevelopmental changes later in life.[22]

Blood sampling for diagnostic tests in NICU expose infants to substantial number of painful procedures that cause discomfort, physiological stress and long-term neurological consequences.[2]Most commonly performed procedures for blood sampling are heel stick and venipuncture. Heel stick is performed more often in neonates because it is easy to withdraw capillary blood samples rapidlywith a high success rate. Venipuncture is less painful than heel stick but requires special training and oftenmultiple sticks to obtainingadequate blood samples.[23]Compared to manual heel stick lancing, the use of an automatic lancing device lessens the pain as it punctures the superficial dermal blood vessels reliably for collection of blood for diagnostic screening and capillary

blood gas analysis.[24]

Neonatal exposure to frequent untreated or ineffectively managed skin-breaking procedural pain such as the heel stick at a crucial time of nervous system development may trigger short- and long-term adverse behavioral and neurodevelopmental outcomes.[25]A recent neuroimaging study showed that cumulative procedural pain in early infancy produced pathological changes at term-equivalent age of former premature neonates brain white and grey matter morphology that positively correlated with the number of skin breaking procedures.[2]A study of school age children born very premature demonstrated that greater numbers of invasive procedures (adjusted for confounders) during NICU care was associated with lower intellectual functioning.[26]

In addition to alleviating procedural pain in infants, several studies have shown that pain-evoked distress can be lessened with integration of non-pharmacological interventions such as positioning, sucrose administration, nonnutritive sucking, breastfeeding, multisensory stimulation and skin contact between infant and mother.[27]While these approaches decrease acute behavioral responses to procedural painthey do notreduce nociception andtheir impact on neurodevelopment outcomes has yet to be investigated.[28]

Although infants as young as 25 weeks gestational age are capable of mounting cortical responses to painful heel sticks, oral and systemic analgesics are rarely used because of safety concerns with opioid-induced respiratory depression, ineffectiveness of NSAIDs and lack of analgesia from topical local anesthetics on heel skin.[29] Intravenous acetaminophen is an effective and safe analgesic in infants but many infants may not have intravenous access at the point-of-carefor blood testing.[30]It is also important to note that repeated heel stickswithout the benefit of analgesia cause nociception-inducedneuroendocrine stress responses that may potentially result inlong-term maladaptiveneurodevelopmental plasticity later in life.[31]Both human and animal studies show that early life exposure to unalleviated pain and nociception present substantial biopsychosocial health risksduring development.[32]Although there are no studies performed yet to show whether effective analgesia for heel stickswould prevent neurodegenerative changesin human newborns, a neonatal rat model of repeated 5-day saline injections into paws to produce mild pain demonstrated that morphine analgesiacan protect against brain cell degeneration.[33]

Collectively, these data may have implications for the unmet need ofexploring ways to alleviate heel

stick pain in human infants. Assuming this study model is relevant to human infants, further investigation of the vapocoolant spray'seffectiveness on heel stick pain may be worth pursuing as an alternative to ineffective current topical local anesthetics.

Vapocoolant spray has been used safely in children and adults in various clinical settings such as the emergency departments for venous cannulation, pediatrician offices for vaccination in school-age children, venous and arterial cannulation before surgery and for facial cosmetic surgery in outpatient clinics. A recentCochrane review reported minor and infrequent side effects with the use of pressurized topical analgesic sprays including vapocoolant spraysuch as cold sensation, transient reactions of erythema, and burning sensation.[34]

There are several limitations to this study. First,the hind paw region is very small and the vapocoolant spray likely spread beyond the hind paw.Because we did not identify heel stick areas specificallyfor histology we cannot confirm that histological analyses included all regions of heel sticks in the hind paw and therefore cannot assess the effect of heel sticks. Second, we did not weigh or identify the pups' sex. Hotplate withdrawal latencytests in Sprague Dawley ratshave showna small significant inverse correlation with body weight but there was no difference in WLT between male and female rats on the first test.[35]The effect of weighton WLTmight have been negligible in this study because all the pups were of same ageatpostnatal day 7. Third, we measured the WLT to hotplate test only once because repeated testing produces anticipatory heat nociception or habituation leading to potentially shortening of WLT over time.[15]And we conducted repeat heel stick tests on one hind paw for two days only because of the limited heel spots available that were not previously lanced. Repeated heel sticks on the same spots result in persistent inflammation and cutaneous hypersensitivity.[36]Finally, we did not test the potential local and/or systemic neurotoxicity biochemical markers as these testingare cost-prohibitive. In vitro cyto-toxicology of the vapocoolant spray as applied in this study did not produce human skin cellular toxicity.[37]

In conclusion, the findings from this study demonstrate that brief cooling of glabrous skin of rat pups after a single application of the medium stream vapocoolant spray by method-2 is more clinically

relevant than method-1 to increasethe withdrawal latency time to noxious heat and provides adequate time for collection of blood samples after a heel stick.

Neither method-1 nor method-2produceddetectabletissue pathology after repeated applicationsof vapocoolant spraybefore performing heel sticks for a couple of days.

We plan to extend this investigation to determine whether this approach of vapocoolant spray applications before repeated hind paw sticks over several days in a rat pups model would effectively minimize the negative alternations in brain neuroimaging similar to that is observed in NICU infants who were exposed to repeated skin-breaking procedures including heel sticks when no or ineffective analgesia was used.[2,33]

Conflicts of Interest: The co-authors declare no conflicts of interest.

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Legends

Figure 1. Flow diagram illustrating experimental design of rat pups randomization to either method-1 or method-2 delivery of the vapocoolant spray in hotplate test and histological analyses. In the hotplate testing the vapocoolant spray was randomly applied to left or right hind paw and saline spray to the contralateral paw. One rat pup was excluded in method-2 because of the lack of response to hotplate stimulus at baseline. In the histological analyses vapocoolant spray was randomly applied to left or right hind paw and the contralateral hind paw was untreated. The vapocoolant spray was applied before repeated heel sticks and both treated and untreated hind paws were subjected to analyses.

Figure 2. Comparison of two methods of application of the topical vapocoolant sprays on the rat pup hind paws. The figure shows individual rat pup values and differences in withdrawal latency time between vapocoolant and saline sprays in method-1 and method-2. Both methods produced longer Within paired (vapocoolant- saline) sprays differences relative to saline spray, but method-2 produced a much longer response (in seconds) compared to saline control. The red line for each method shows the median difference between vapocoolant and saline (0.6 second for method-1 and 9.5 seconds for method-2), with a significant method effect ($P< 0.0001$). IQR = interquartile range of 25-75th percentile.

Figure 3. A hind paw histology after application of vapocoolant spray at an 18 cm distance from the paw for 10-second (A, B, C) and vapocoolant spray at an 8 cm distance from the paw for 4 seconds (D, E, F). Representative views showing A. Epidermis, dermis and eccrine gland 20x. B. Dermis and nerves 20x. C. Dermis, vessels and nerves 20x. D. Epidermis 20x. E. Dermis and nerves 40x. F. Cross section of epidermis, papillary dermis with hair follicles and deep cartilage 40x. These sections may or may not include heel stick areas of the hind paw.

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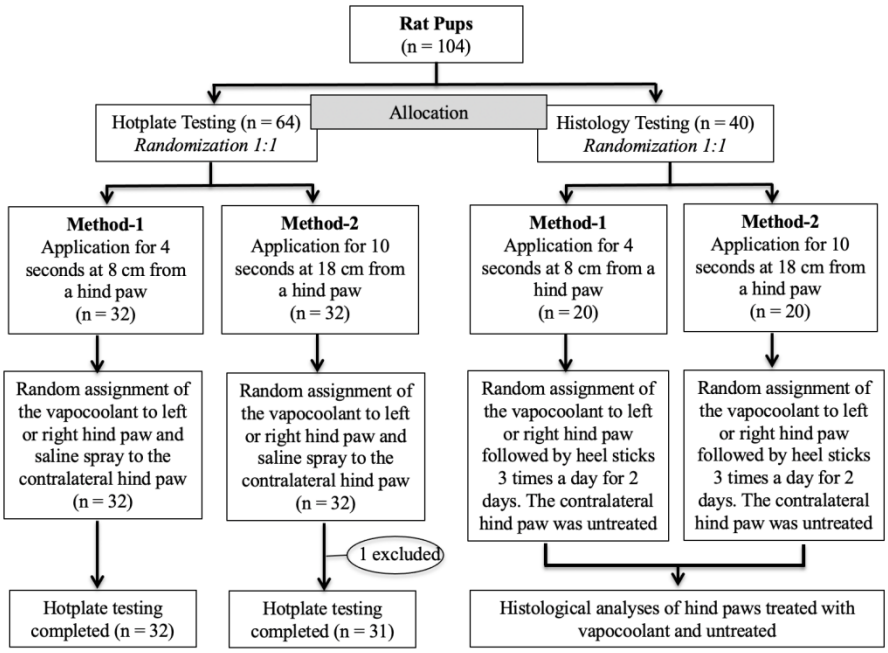

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Figure 2. Comparison of two methods of application of the topical vapocoolant sprays on the rat pup hind paws. The figure shows individual rat pup values and differences in withdrawal latency time between vapocoolant and saline sprays in method-1 and method-2. Both methods produced longer within paired (vapocoolant- saline) sprays differences relative to saline spray, but method-2 produced a much longer response (in seconds) compared to saline control. The red line for each method shows the median difference between vapocoolant and saline (0.6 second for method-1 and 9.5 seconds for method-2), with a significant method effect ($P < 0.0001$). IQR = interquartile range of 25-75th percentile.

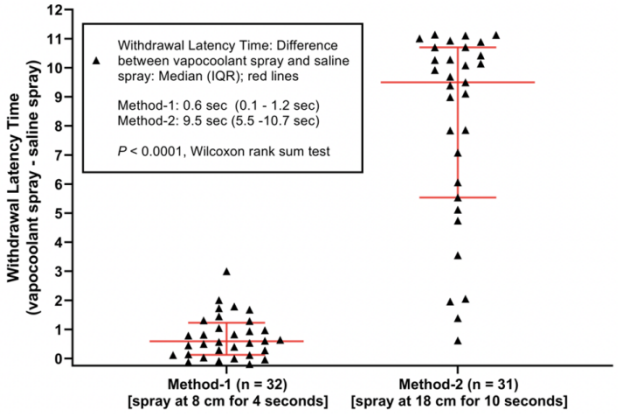

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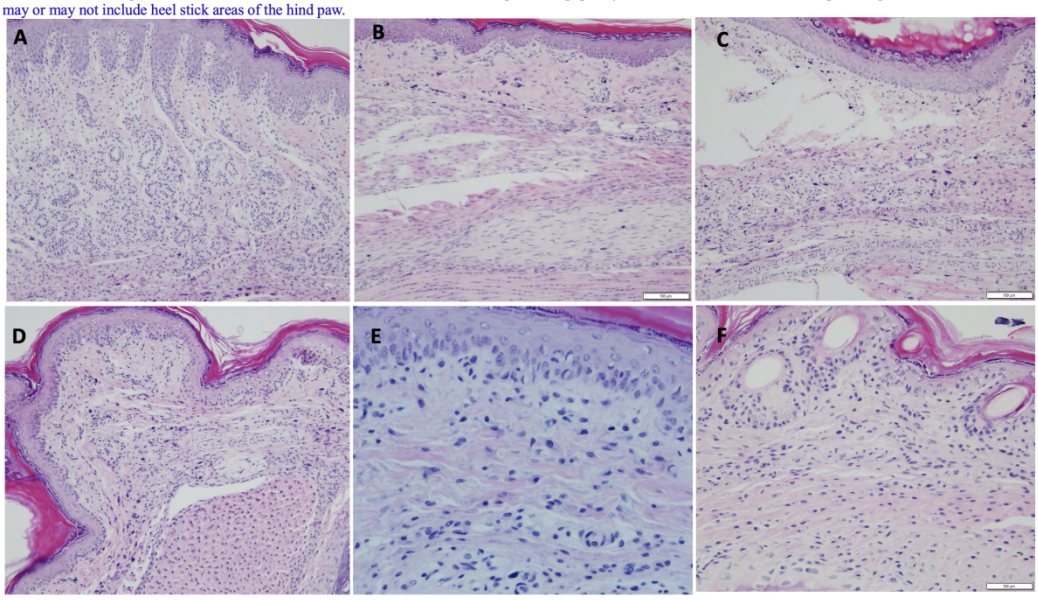
Figure 3. A hind paw histology after application of vapocoolant spray at an 18 cm distance from the paw for 10-second (A, B, C) and vapocoolant spray at an 8 cm distance from the paw for 4 seconds (D, E, F). Representative views showing A. Epidermis, dermis and eccrine gland 20x. B. Dermis and nerves 20x. C. Dermis, vessels and nerves 20x. D. Epidermis 20x. E. Dermis and nerves 40x. F. Cross section of epidermis, papillary dermis with hair follicles and deep cartilage 40x. These sections may or may not include heel stick areas of the hind paw.



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