Microcirculatory effect of topical vapocoolants

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BACKGROUND: Vapocoolant sprays are commonly used to minimize pain following minor interventions such as venipuncture, shave biopsy or needle insertion. Although these sprays have been widely used in clinical practice, little is known about their effect on microcirculation or cutaneous blood flow.

OBJECTIVE: To evaluate the real-time effect of a topical vapocoolant using a well-established, rat cremaster muscle microcirculatory model, allowing direct measurement of changes in vessel diameter, capillary density and leukocyte behaviour.

METHODS: Fifty rats were divided into a control and four experimental groups: group 1: 4 s spray with vapocoolant at 18 cm distance; group 2: 10 s spray at 18 cm distance; group 3: 4 s spray at 8 cm distance; and group 4: 10 s spray at 8 cm distance. Vessel diameters, capillary density and leukocyte behaviour were monitored for 1 h thereafter. Muscle was harvested for immunohistochemistry analysis of proangiogenic markers (vascular endothelial growth factor and von Willebrand factor), leukocyte behaviour markers (E-selectin, vascular cell adhesion molecule, intercellular adhesion molecule), pimonidazole-hypoxia staining and ApopTag (Millipore, USA) staining for apoptosis. Gene expression for inflammatory markers (interleukin [IL]-1 β , IL-2, IL-4, IL-6, IL-10, tumour necrosis factor- α and interferon- γ) was evaluated using polymerase chain reaction and myeloperoxidase assay for inflammation was performed.

RESULTS: The use of refrigerant spray decreased vessel diameter and capillary density initially, although none of these decreases were statistically significant. Polymerase chain reaction showed no significant changes. The myeloperoxidase assay showed statistically significant increase in myeloperoxidase activity in groups 2, 3 and 4. Immunohistochemistry was negative for angiogenic and proinflammatory markers.

CONCLUSIONS: The lack of statistically significant changes in vessel diameter and inflammatory markers corroborated the safety on microcirculation.

Key Words: Cremaster muscle; Microcirculation; Refrigerant; Vapocoolant

Vapocoolant spray (Pain Ease, Gebauer, USA) has been used clinically to minimize pain following minor interventions such as venipuncture, shave biopsy or needle insertion (1). Other studies have shown that the use of cryoanalgesic sprays can be helpful in assuring adherence to proper vaccination scheduling in the pediatric population (2,3). Studies document that the use of refrigerant spray can significantly reduce injection pain, making the office experience less objectionable and compliance more likely (2-4). Vapocoolant sprays have also been used to manage myofascial trigger points and to lessen the pain of analgesic injection for dental procedures (5).

Although refrigerant sprays, such as Pain Ease, have been widely used in practice, little is known about their effects on the microcirculation or cutaneous blood flow. The mechanism of action for reducing pain is unknown, but believed to be related to decreased nerve conduction velocity of pain fibres, as observed with decreased temperature (1,6). While several studies have shown that hypothermia on its own can cause damage to tissues, none have been specifically focused on

L'effet des pulvérisateurs topiques à froid sur la microcirculation

HISTORIQUE : Les pulvérisateurs à froid sont couramment utilisés pour réduire la douleur après des interventions mineures comme les ponctions veineuses, les biopsies par rasage ou l'insertion d'aiguilles. Même si ces vaporisateurs sont largement utilisés en pratique clinique, on ne sait pas grand-chose de leur effet sur la microcirculation ou la circulation cutanée.

OBJECTIF : Évaluer l'effet en temps réel d'un pulvérisateur à froid topique au moyen d'un modèle microcirculatoire bien établi du muscle cremaster de rats, afin de mesurer directement les modifications au diamètre, à la densité capillaire et au comportement leucocytaire des vaisseaux.

MÉTHODOLOGIE : Les chercheurs ont réparti 50 rats en un groupe témoin et quatre groupes expérimentaux (groupe 1 : pulvérisation à froid de quatre secondes à une distance de 18 cm; groupe 2 : pulvérisation à froid de dix secondes à une distance de 18 cm; groupe 3 : pulvérisation à froid de quatre secondes à une distance de 8 cm; groupe 4 : pulvérisation à froid de dix secondes à une distance de 8 cm). Ils ont ensuite surveillé le diamètre, la densité capillaire et le comportement leucocytaire des vaisseaux pendant une heure. Ils ont prélevé le muscle pour effectuer une analyse immunohistochimique des marqueurs proangiogéniques (facteur de croissance de l'endothélium vasculaire et facteur de von Willebrand), des marqueurs de comportement leucocytaire (E-sélectine, molécule d'adhésion des cellules vasculaires, molécule d'adhésion intercellulaire), du pimonidazole, marqueur d'hypoxie, et de la trousse ApopTag (Millipore, États-Unis) pour déceler l'apoptose. Ils ont évalué l'expression génique des marqueurs inflammatoires (interleukine [IL]-1 β , IL-2, IL-4, IL-6, IL-10, facteur de nécrose tumorale α et interféron γ) au moyen de la réaction en chaîne de la polymérase et du dosage de myéloperoxydase pour déterminer l'inflammation.

RÉSULTATS : Au départ, l'utilisation d'un pulvérisateur à froid a réduit le diamètre et la densité capillaire des vaisseaux, mais pas de manière statistiquement significative. La réaction en chaîne de la polymérase n'a pas changé de manière significative. Le dosage de myéloperoxydase a révélé une augmentation statistiquement significative de l'activité de la myéloperoxydase dans les groupes 2, 3 et 4. L'immunohistochimie était négative aux marqueurs angiogéniques et pro-inflammatoires.

CONCLUSIONS : L'absence de changements statistiquement significatifs du diamètre des vaisseaux et des marqueurs inflammatoires en ont corroboré l'innocuité sur la microcirculation

cryoanalgesic sprays (7-11). Our study was designed to evaluate the effects of the refrigerant spray (ie, Pain Ease) on the microcirculation in real time using the rat cremaster model.

The rat cremaster muscle model is well established in our laboratory over the past 20 years, and it is a unique model for both evaluating and experimentally manipulating microcirculation in the experimental design pertinent to surgical cases. It has been extensively used to demonstrate real-time microcirculatory changes in response to muscle denervation, ischemia-reperfusion injury, tumour implantation, isograft and allograft transplantation, surgical trauma, hypothermia, anesthesia and thrombosis (9-15).

METHODS

The Animal Research Committee of the Cleveland Clinic (Ohio, USA), accredited by the American Association for the Accreditation of Laboratory Animal Care, approved the present study. All animals were cared for in accordance to the Guide for the Care and Use of

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TABLE 1
Control and four experimental groups decribing duration and distance of Pain Ease* spray application

Group	Animals, n	Spray		Comments
		Duration, s	Distance, cm	
Control (water)	10	4	18	
1	10	4	18	Manufacturer's recommended time and distance
2	10	10	18	Longer time, same distance
3	10	4	8	Same time, shorter distance
4	10	10	8	Longer time, shorter distance

*Gebauer, USA

Figure 1 Scheme depicting first-order (A1), second-order (A2) and third-order (A3) arterioles and first-order venules (V1). A1 represents the major pedicle; A2 the first branch and A3 branches off A2

Laboratory Animals as published by the National Institutes of Health. Animals were housed in cages at room temperature, kept on a 12 h light/dark cycle, and had ad libitum access to standard laboratory food and water.

Experimental groups

Fifty male Lewis rats weighing between 100 g and 150 g were divided into a control group and four experimental groups. The observation period for all groups was from time 0 to 1 h in 15 min measurement intervals.

In the control group (n=10), the cremaster muscle was sprayed with room temperature water for 4 s at 18 cm distance. In the first experimental group (group 1 [n=10]), the refrigerant spray Pain Ease (95% 1,1,1,3,3-pentafluoropropane and 5% 1,1,1,2-tetrafluoropropane) was sprayed for 4 s at a distance of 18 cm. This coincides with the manufacturer's recommendations with regard to time and distance. In group 2 (n=10), Pain Ease was sprayed for a longer duration (10 s) at a distance of 18 cm. In group 3 (n=10), Pain Ease was sprayed for 4 s from a shorter distance than recommended by the manufacturer (8 cm). In group 4 (n=10), Pain Ease was sprayed for a longer time period (10 s) and at a shorter distance (8 cm) (Table 1).

Surgical technique

Rats were anesthetized intramuscularly with an anesthetic containing xylazine (20 mg/mL), acepromazine (10 mg/mL) and ketamine (100 g/mL). Supplemental doses of the combination were given as needed. After the microcirculatory observations had been completed, the rats were euthanized with a 2 mL intravenous injection of Beuthanasia-D (Intervet/Schering-Plough, USA). All animals were kept on heating pads with body temperature maintained between 35°C and 38°C throughout the experiment to counteract hypothermic effects of anesthesia.

A well-established technique for cremaster muscle flap isolation was used (8,9,11). Using 40× magnification and an operating microscope

Figure 2 Microscopic view of a first-order arteriole (A1) (above) and first-order venule (V1) (below). Vessel diameters were measured at all observation times (5 min, 15 min, 30 min, 45 min and 60 min) using a digital imaging measurement device

(Carl Zeiss OPMI 6-SD; Carl Zeiss, Germany), the skin was incised on the right from the anterior iliac spine to the midline tip of the scrotum. The spermatic cord and testes were extracted and the pudic-epigastric vascular pedicle of the muscle was left intact. The muscle was opened along the anterior wall to create an oval flap. The vasculature of the cremaster muscle consists of a series of arteriolar and venular branches with each successive branch being a different order of vein or artery (Figure 1). With the rat placed in a dorsal position on a plexiglass tissue bath, the cremaster muscle was spread out to showcase the axial pattern of blood vessels, secured using 6/0 silk sutures and irrigated with Ringer's solution. It was then covered with an oxygen-impermeable plastic film that had been presoaked in distilled water for 24 h to keep the muscle moist and protect it from the external environment. The muscle flap was then allowed to equilibrate for 30 min to prevent any surgical trauma from affecting the microcirculation measurements. Rats were injected with Hydroxyprobe-1 (pimonidazole HCl; Natural Pharmacia International, Inc, USA) 60 mg/kg body weight just before the beginning of the observation period.

In vivo microcirculatory observations

The rat and plexiglass chamber were placed on the stage of an intra-vital microscope (Nikon Optiphot-2, Nikon, Japan) equipped with a colour digital camera (Carl Zeiss Axiocam MR and Carl Zeiss AxioVision Rel4.8; Carl Zeiss, Germany). The final magnification on the computer was 1800× and the data were stored on a hard drive (Hewlett Packard HPL1940T and HP xw8400 Workstation, Hewlett Packard, USA).

Measurements were taken just before spraying, at the time of the initial spray, and 5 min, 15 min, 30 min, 45 min and 60 min following spray with either the Pain Ease refrigerant spray or with room temperature water.

Vessel diameters: Images of the four vessels (first-order arteriole, A1; first-order venule, V1; second-order arteriole, A2; third-order arteriole, A3) were taken at all observation points using a digital image measurement device that was used to calculate vessel diameter in μm (Carl Zeiss Axiocam MR and Carl Zeiss AxioVision Rel.4.6) (Figure 2).

Functional capillary density: Three areas with clear visualization and constant and clearly visualized capillary flow were chosen in the proximal, middle and distal zones of the flap. In these three zones, the number of capillaries was counted in nine high-powered fields, for a total of 27 fields per cremaster muscle being used for total area of 0.18 mm² at a magnification of 450×.

Rolling and sticking leukocytes: Three regions of the muscle were observed for 2 min each and the number of rolling and sticking leukocytes (remaining stationary for ≥20 s) were counted (Figure 3).

Following euthanasia, the cremaster muscles were harvested from each of the animals. One-half of the muscle was taken for immunostaining

Figure 3) Microscopic view of (A) rolling and (B) sticking leukocytes. Three regions of the cremaster muscle were observed for 2 min each and the number of rolling and sticking leukocytes were counted. Sticking leukocytes remained stationary for ≥ 20 s

and the other half was taken for polymerase chain reaction (PCR) and the myeloperoxidase assay.

Real-time PCR proinflammatory gene expression in cremaster muscle tissue

RNA isolation from cremaster muscle tissue: Immediately following the cremaster harvest, muscle was placed in RNAlater solution (Ambion, USA) and kept overnight at 4°C. It was then frozen at -80°C until processed. The sample was then homogenized in TRIZOL reagent (Invitrogen, USA). The isolation was performed using an RNeasy Mini Kit (Qiagen, USA) according to the manufacturer's instructions. Quality and concentration of the total RNA extraction was measured spectrophotometrically using NanoDrop ND-1000 (Thermo Scientific, USA). RNA was determined to be pure when the ratio of sample absorbance A260 nm/230 nm was 1.8 to 2.2, and the ratio of A260 nm/280 nm was < 2.0 .

Complementary DNA synthesis: For reverse transcription, 1 µg of RNA in a total of 20 µL was transcribed to complementary (c)DNA using High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, USA). All reverse transcription reactions were performed according to the manufacturer's instructions.

Relative quantification real-time PCR: Local cytokine gene expression was investigated after Pain Ease application, focusing on interferon-gamma, tumour necrosis factor-alpha, interleukin (IL)-1 β , IL-2, IL-4, IL-6 and IL-10. Amplification was achieved via real-time PCR using the 7300 Real-Time PCR detection system with 7300 SDS software (version 1.3.1.21; Applied Biosystems 2011-2005). Amplification was performed using 1 µL of cDNA undiluted product reverse transcribed from 1 µg of isolate RNA, TaqMan Universal PCR Master Mix (2X), and Gene Expression Assay Mix (20X) to obtain a total volume of 25 µL. Reactions were cycled 40 times using the following parameters: 50°C for 2 min, 95°C for 10 min, 95°C for 30 s and 60°C for 1 min. Nontemplate controls were also run with every assay to ascertain whether any PCR contamination had occurred. All runs were performed in triplicate to ensure reproducible results. Expression of all genes was then compared with GAPDH as an endogenous control.

Myeloperoxidase assay

The cremaster muscle was harvested, immersed in 500 µL phosphate-buffered saline (PBS) and then homogenized. It was then stored at -80°C until ready for assay. Once thawed, the cells were centrifuged for 30 min at 4°C and $> 10,000$ g. Of note, only supernatant was taken for the assay. The assay was performed using the Myeloperoxidase Activity Assay (Life Science Specialties, LLC, USA) according to manufacturer's instruction and analyzed for colour change at 460 nm using MPM6 (Bio-Rad, Co, USA). As such, the myeloperoxidase assay measures the activity of myeloperoxidase, which is an enzyme unique to neutrophils. This enzyme catalyzes the reaction between

chloride and hydrogen peroxide to form hypochlorous acid, and is indicative of inflammation.

Immunostaining

A section of the cremaster muscle was rolled into a cylinder and attached to gum tragacanth (Sigma-Aldrich, Co, USA) on a small circular cork board. It was then immersed in 2-methyl-butane (Sigma-Aldrich, Co, USA) chilled in liquid nitrogen. All samples were stored at -80°C until stained. There was a total of 2 h between initial Pain Ease spraying and muscle harvesting. Staining was performed for angiogenic markers (vascular endothelial growth factor [VEGF], von Willebrand factor [vWF]) and markers of leukocyte transmigration (endothelial leukocyte adhesion molecule/E-selectin, intercellular adhesion molecule [ICAM], vascular cell adhesion molecule [VCAM]). Frozen sections were cut into 4 µm sections and fixed for 10 min in cold acetone. Sections were then blocked using fixation buffer containing fetal bovine serum (BD Pharmingen, USA), then incubated with Image Signal Enhancer (BD Pharmingen), washed in PBS and incubated with Stain Buffer (BD Pharmingen). Following this process, the slides were incubated with monoclonal antibodies for mouse-derived VEGF (C-1) (Santa Cruz Biotechnology, USA), mouse-derived ICAM/CD106 clone 1A29 (BD Pharmingen), mouse-derived VCAM/CD54 clone MR106 (BD Pharmingen), rabbit-derived vWF Ab-2 (clone F8/86) (LabVision, USA) or goat-derived E-selectin (R&D Systems, USA) diluted 1:50 in fetal bovine serum for 1 h. Binding of primary antibodies was detected using goat anti-mouse, goat anti-rabbit or rabbit anti-goat FITC-conjugated antibodies (diluted 1:200) during a 1 h incubation period (BD Pharmingen). Sections were then rinsed in PBS and cover-slipped using DAPI.

Pimonidazole hypoxia staining: Staining was also performed to determine tissue hypoxia. Frozen sections were cut into 4 µm sections and fixed in cold acetone for 10 min. They were then rinsed in PBS and incubated for 5 min with permeability buffer, washed in PBS for another 5 min, and then incubated with Image Signal Enhancer for 30 min. After another rinse, sections were incubated for 30 min with stain buffer then again rinsed in PBS. Sections were then incubated for 1 h with FITC-conjugated monoclonal antibody to pimonidazole. Sections were rinsed in PBS one final time and then cover-slipped using DAPI.

Fluorescein in situ apoptosis detection: Frozen samples were sectioned into 4 µm slices and stained for apoptosis using the ApopTag Fluorescein In Situ Apoptosis Detection Kit as per the manufacturer's instructions (Millipore, USA).

Statistical analysis

Vessel diameters, capillary numbers and leukocyte numbers were summarized using means and standard deviations or standard errors. ANOVA models followed by Dunnett's multiple comparison procedure for IL-1 β and myeloperoxidase were used to evaluate differences

Figure 4 Vessel diameters for first-order (A1), second-order (A2) and third-order (A3) arterioles and first-order venules (V1) over time for group 1 animals (4 s spray, 18 cm distance [ie, recommended time and distance])

Figure 5 Leukocyte counts over experimental time period. Initial increases were observed in all groups including controls followed by a gradual decline toward pretreatment levels by 1 h. Plots depict rolling leukocyte measurements only. Control group: 4 s water spray at 18 cm distance; group 1: 4 s Pain Ease (Gebauer, USA) spray at 18 cm distance; group 2, 10 s Pain Ease spray at 18 cm distance; group 3, 4 s Pain Ease spray at 8 cm distance; group 4, 10 s Pain Ease spray at 8 cm distance

between the experimental groups as compared with the controls. Repeated measure linear models were used to evaluate changes in microcirculation measures (vessel diameters, capillary numbers and leukocyte behaviour) over time. Equivalency was evaluated between postspray responses and baseline measures using an equivalency region equal to $\pm 10\%$ of the baseline measure. A two one-sided test approach was used with an overall significance level of 0.05 for all tests. Analyses were performed using SAS version 9.2 (SAS Institute, USA).

RESULTS

Microcirculatory monitoring of vessel diameters

The diameters of most vessels in the control group and all four experimental groups decreased on initial spraying. However, the most significant drop in diameter was seen in group 4 (increased time [10 s] and decreased distance [4 cm]). Group 4 showed the greatest decrease in the first-order arterioles (A1) and groups 2 and 4 showed almost comparable decreases in the diameter of the first-order venules (V1). In the second-order arterioles (A2), the drop in diameter was again most pronounced in group 4 while, in the third-order arterioles (A3), the biggest decrease in diameter was comparable between groups 3 and 4. At the end of the 1 h observation period, all vessels in all groups had come close to recovering their original diameter, or had recovered

Figure 6 Myeloperoxidase activity was significantly increased in groups 2, 3 and 4, but not in group 1. * $P=0.004$, $P=0.047$ and $P=0.012$, respectively (ANOVA). Control group: 4 s water spray at 18 cm distance; group 1: 4 s Pain Ease (Gebauer, USA) spray at 18 cm distance; group 2, 10 s Pain Ease spray at 18 cm distance; group 3, 4 s Pain Ease spray at 8 cm distance; group 4, 10 s Pain Ease spray at 8 cm distance

completely. Some had even overcompensated and exceeded their original diameters.

Data showed equivalence between all time points for the first-order arteriole for both the control group and group 1, meaning that any changes in diameter were not, in fact, statistically significant (Figure 4). Equivalence was also observed in the A2 in group 1 only. There was no observable equivalence in the third-order arterioles in any of the groups. First-order venule diameters were equivalent in all groups except group 3.

Functional capillary density: As expected, the functional capillary density decreased upon initial spraying in all groups, including controls. However, the most significant drop was seen in groups 3 and 4 across all three zones of observation (proximal, middle and distal muscle flap).

Capillary density showed equivalence in the control group uniformly in all zones. Group 1 showed widespread equivalence in capillary number and group 2 showed equivalence at later time points (after 30 min of observation) among all three zones.

Leukocyte behaviour: The number of leukocytes increased in the first 30 min in both categories (rolling and sticking leukocytes). After 30 min, the number of leukocytes declined gradually towards original numbers in both categories (Figure 5).

Real-time PCR gene expression evaluation

IL-1 β : Gene expression of all factors except IL-1 β was similar across all groups, including controls. IL-1 β was found to be upregulated in group 2 and slightly upregulated in group 4; however, the values were not found to be significantly increased when compared with controls ($P=0.60$ to $P=0.99$ [ANOVA]).

Myeloperoxidase assay

Myeloperoxidase activity increase in groups 2, 3 and 4 was shown to be statistically significant when compared with controls ($P=0.004$, $P=0.047$ and $P=0.012$, respectively [ANOVA]). However, there was no significant increase in myeloperoxidase activity when group 1 was compared with the control group (Figure 6).

Immunohistochemistry

Immunostaining for VEGF, vWF, E-selectin, ICAM and VCAM showed no differences when compared with controls. ApopTag-staining for signs of apoptosis and pimonidazole-staining for hypoxia were both negative in the control groups and all four experimental groups (Figure 7).

Hematoxylin and eosin staining

There were no significant changes found on routine histology using hematoxylin and eosin.

DISCUSSION

Pain Ease, a topical refrigerant spray, has been approved for use on nasal passages, lips and skin. Refrigerant sprays have been shown to lessen needle injection pain in both the pediatric and adult age groups in a variety of clinical settings (1-4,16). In the pediatric population, it reduces immunization discomfort, which may, in turn, improve pediatric patient compliance regarding immunization (2-4). In the adult population, the refrigerants have been effective as preinjection anesthetics (16). In the cosmetic surgery arena, it has also been shown to significantly reduce pain associated with botulinum toxin injections (1).

While this spray's physiological mechanism of action is believed to be related to decreased pain fibre conduction velocity induced by reduced temperature, there is little information regarding the immediate and short-term effects on the microcirculation (1,6). Furthermore, there is no literature addressing longer spray times or closer spray distances than what is currently recommended by the manufacturer.

To address these questions, we used the rat cremaster muscle flap. This model is a well-established experimental, small-animal model that allows real-time evaluation of microcirculation (9-15).

As such, our study showed that there was mild, immediate vasoconstriction of all arterioles and the first-order venules in the rat cremaster muscle following refrigerant spray application. Interestingly, vessels returned to pretreatment baseline size within 30 min to 45 min. Furthermore, none of these changes were statistically significant when evaluated according to equivalence, a statistical analysis comparing pre- to post-treatment values within the experimental group.

In summary, the widespread statistical equivalence of arteriolar and venular diameters seen throughout our observation is consistent with the transient effects of the spray. Our study demonstrated capillary density equivalence predominantly in group 1 animals, supporting and confirming the manufacturer's guidelines of spraying from an 18 cm distance for 4 s. These parameters resulted in the least amount of change in the hemodynamic parameters, and our histological evaluation of inflammation suggests that this combination of spray time and distance results in reduced tissue damage. This provides the greatest margin of safety.

The significant increase in myeloperoxidase activity and neutrophils found within the cremaster muscle suggests some type of short-lived inflammatory response (17). However, the lack of ICAM immunohistochemistry evidence and the failure to identify any statistically significant increase in proinflammatory markers (via PCR) indicate that the inflammation is both transient and mild. In contrast, if we had found increased gene expression inflammation (ie, indicating a longer-lasting inflammatory response) there would be greater cause for concern of more significant cremaster muscle damage. Additionally, there was no evidence of proangiogenic or proinflammatory marker concentration with immunohistochemical analysis, thereby confirming preserved tissue viability. Finally, the lack of inflammatory evidence found via gene expression analysis also suggests minimal levels of ischemia (18).

Alternatives to cryoanalgesic sprays include injection of local anesthetics such as lidocaine, which can also have mild constricting effects on precapillary vessels much like this refrigerant spray (19,20). Clinically, lidocaine mixed with epinephrine activates α , β 1 and β 2 receptors in blood vessels, leading to significant vasoconstriction of at least 1 h. As such, this vasoconstriction is greater than that seen with any spray (21,22). Another topical anesthetic commonly used is EMLA cream (eutectic mixture of local anesthetics). This cream also causes vasoconstriction, which peaks at approximately 90 min. Although an effective anesthetic for medicinal purposes, it is limited by the duration of onset, which makes it less ideal for office use. In addition, the cost per treatment with EMLA is significantly higher than with vapocoolants. Finally, there are rare side effects associated with EMLA, including irritation to mucous membranes, hemorrhages and tissue necrosis for those patients with small vessel disease related to hypertension, diabetes mellitus and/or sepsis (23,24).

Figure 7) Immunostaining was negative for all antibodies to inflammatory markers. Had antibodies been present, green fluorescence would have been visible. Control group: 4 s water spray at 18 cm distance; group 1: 4 s Pain Ease (Gebauer, USA) spray at 18 cm distance; group 2, 10 s Pain Ease spray at 18 cm distance; group 3, 4 s Pain Ease spray at 8 cm distance; group 4, 10 s Pain Ease spray at 8 cm distance

Limitations of the present study

While the cremaster model is ideal for real-time evaluation of the microcirculation, it is a muscle flap that lacks a skin component. Therefore, it is not completely analogous to a clinical situation. However, the cremaster flap represents a unique model with neurovascular pedicle independent of surrounding tissues and is significantly better than random pattern flaps, which represent models not directly applicable to the field of reconstructive surgery. Also, the cremaster muscle is thin – only 25 μ m to 300 μ m in thickness – and, as such, can be transilluminated, allowing for direct monitoring of microvascular hemodynamics and cellular interactions. The ideal experiment would have investigated cutaneous microcirculation to mirror clinical applications. However, skin microcirculation models such as, for example, the ear chamber model cannot be transilluminated to the extent that they are transparent enough and, thus, do not provide adequate visibility for direct monitoring of leukocyte endothelial interaction and capillary perfusion. In addition, skin microcirculation models such as the ear chamber model allow for microcirculatory evaluation only within the chamber lumen and do not represent axial pattern of vessels, which is pertinent to models mimicking reconstructive surgery. Additionally, the ear chamber model represents an 'end-organ' model and does not reflect surgical trauma, which is an important factor for plastic and reconstructive surgery. In contrast, cremaster muscle isolation on a neurovascular pedicle requires hemostasis on the flap borders and reflects true surgical flap isolation where the effect of surgical trauma can be tested. Thus, this makes the cremaster flap a unique model for microcirculatory monitoring. Also, it would have been informative to compare each experimental group to a corresponding control of the same time and distance. However, that these

microcirculatory effects were found to be transient in all groups with no significant gene expression supports our reasoning that supplementary control groups would have added minimal value to this study. Additionally, short-interval, surface temperature measurements before and after cooling would have allowed for better interpretation of the minute differences observed among the groups. However, with the transient effects identified here, we believe that temperature data would not have impacted our conclusions, especially because the hypothermic effects of anesthesia were counteracted with heating pads. The data of the current study confirm our previously published data on the effect of hypothermia as well as anesthesia on the cremaster muscle microcirculatory responses (11,12). Therefore, we believe these observations are attributed directly to the vapocoolant without any confounding input from systemic anti-inflammatory effects surrounding reduced core body temperature. Furthermore, there are several other markers of inflammation that could have also been investigated; however, testing all available markers is cost-prohibitive and may serve to complicate the picture. As such, we decided to focus on those most extensively studied and noted in the literature. Finally, evaluation of red blood cell velocity would have been yet another

method for evaluating immediate changes in blood flow caused by the topical vapocoolants.

CONCLUSION

We have demonstrated that when tissue is sprayed with vapocoolant, regardless of manufacturer guidelines, vascular diameters show minimal transient decreases followed by rapid recovery. Furthermore, these changes were not statistically significant when measured using equivalency calculations. Leukocyte counts and myeloperoxidase assays were statistically significant, but these changes were mild. Furthermore, the lack of significant increases in proinflammatory and/or proangiogenic markers (ie, gene expression) emphasizes the lack of significant or long-lasting vascular changes. Finally, histological evaluation of specimens, treated according to manufacturer's recommendations, showed no permanent muscular changes and are, therefore, safe in all age populations.

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