Use of infrared thermography in an animal model as a complementary tool for monitoring the inflammatory process: a preliminary study

Agnes Batista Meireles

Federal University of Jequitinhonha and Mucury Valleys (UFVJM)

Timilly Martins Cruz

Federal University of Jequitinhonha and Mucury Valleys (UFVJM)

Izabela Cristina Brandão Moreira

Federal University of Jequitinhonha and Mucury Valleys (UFVJM)

Valéria Gomes de Almeida

Federal University of Jequitinhonha and Mucury Valleys (UFVJM)

Bethânia Avelar-Freitas

Federal University of Jequitinhonha and Mucury Valleys (UFVJM)

Marcelo Henrique Fernandes Ottoni

Federal University of Jequitinhonha and Mucury Valleys (UFVJM)

Cíntia Pimenta Araújo

Federal University of Jequitinhonha and Mucury Valleys (UFVJM)

Gustavo Eustáquio Brito Alvim de Melo

Federal University of Jequitinhonha and Mucury Valleys (UFVJM)

Patrícia Furtado Gonçalves

Federal University of Jequitinhonha and Mucury Valleys (UFVJM)

Wagner de Fátima Pereira (✉ wagnerufvm@gmail.com)

Federal University of Jequitinhonha and Mucury Valleys (UFVJM)

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Abstract

Purpose

Temperature changes on a surface can be measured by infrared thermographic cameras. Thus, the images obtained by these cameras can be useful for a wide range of biological *in vivo* studies, including animal models of inflammation. In this preliminary study, the use of thermography in rat paw inflammation was evaluated.

Methods

CFA-induced paw edema on rats (n=5) was performed and discrepancies between animals treated or not with anti-inflammatory drugs as triamcinolone acetonide and diclofenac sodium were analyzed. Experimental times were: T0, before chemical induction of inflammatory process (control); and times after injection: T1 (30 min); T2 (24 hours); T3 (48 hours); T4 (72 hours); T5 (96 hours); T6 (7 days); T7 (14 days); T8 (21 days); T9 (28 days). The measured parameters were temperature, the edema (thickness and volume) of each animal paw (left and right), histological analysis, and blood leukocyte count.

Results

The results demonstrated that the profile of local temperature changes was similar to the volume and thickness of the paws, with an increase at 24 hours. Such increase (the 24 hours peak) is expected for this specific type of inflammation model. From T7 onwards, the temperature values, in all groups, returned to baseline values (T0).

Conclusions

This preliminary study shows a possible use of quantitative high-resolution infrared as a complementary tool for monitoring the inflammatory process.

Introduction

Animal models are important to evaluate the biological effects of substances, drugs, treatments, and new biomaterials on living organisms. Paw edema is a classical inflammation model [1]. In this model, an inflammatory agent is administered subcutaneously in the plantar region of rodent paws to generate an inflammatory response. Inducing agents such as carrageenan and Complete Freund's Adjuvant injection (CFA) are commonly used to trigger an acute and chronic inflammatory response, respectively. The CFA is an oily solution containing lyophilized *Mycobacterium tuberculosis* strain that enables to prolonging the presence of antigens at the site of injection, thus generating a chronic inflammation [2] which could be analyzed by radiographic features, histological analysis, and presence of edema.

The temperature of a surface can be obtained by thermographic profiles from images obtained by specialized radiation-sensitive cameras [3]. Therefore, it can be a low-cost tool for a wider range of uses. For medical applications, it is attractive by dispensing invasive procedure as venous access or ionizing radiation, traditionally used [4]. Infrared (IR) thermography was used to measure temperature profiles of fingers to detect inflammation in patients with rheumatoid arthritis and also to improve the diagnostic accuracy of the cold provocation test for such disease [5].

This technology has been employed in animal studies [6, 7], like animal models of cancer being reported as a useful approach for superficial vascularization [8]. Other animal applications measured temperatures in different parts of animals, using IR technology and related it to feed efficiency, average daily gain and methane emission [9], evaluation of mastitis in cattle [10], and fever investigation in pigs [11].

Local temperature is one of the cardinal signs of inflammation [12], although it is not commonly analyzed after the chemical induction of inflammatory processes. Therefore, the present study evaluated the IR technique as a

complementary tool for the analysis of inflammation in an animal model. For this purpose, a preliminary study was performed. We tested the technique in CFA-induced paw edema on rats and observed the discrepancies between animals treated or not with anti-inflammatory drugs.

Materials And Methods

2.1 Animals

Fifteen male Holtzman rats from the UFVJM (Federal University of Jequitinhonha and Mucuri Valleys, Diamantina, MG/ Brazil) with 8 weeks old and an average weight of 150-250 grams were used in this study. Experiments occurred during the light phase between 07:00 a.m. and 10:00 a.m. This study was previously approved by the Animal Ethics Committee of UFVJM regarding the Guiding Principles in the Care and Use of Animals, with a protocol number of 050/2016.

2.2 Inflammation induction

The inflammatory process was induced by injecting 200 μ L of Complete Freund's Adjuvant (CFA) (lyophilized Mycobacterium powder, Santa Cruz Biotechnology, Inc., Dallas, Texas, USA) into the right hind paw at the plantar region of each animal at a concentration of 5% (m v-1). In the left paw of the animals, 200 μ L of saline solution was injected. The animals were divided into 3 groups: G1 (n=5) with animals that received CFA injection and no treatment; G2 (n=5) with animals that received CFA and treated with 0,3 g the topic anti-inflammatory drug, triamcinolone acetonide (1mg g-1; daily application for 1 minute), and G3 (n=5) with animals that received CFA and treated with topic anti-inflammatory drug diclofenac sodium (10mg g-1; daily application for 1 minute). Experimental times were: T0, before chemical induction of inflammatory process (used as a control time for comparison); and times after injection, T1 (30 minutes); T2 (24 hours); T3 (48 hours); T4 (72 hours); T5 (96 hours); T6 (7 days); T7 (14 days); T8 (21 days); T9 (28 days). The measured parameters were temperature, edema of animal's paw, histological and leukometry analysis.

2.3 Euthanasia

At the end of the experiment, all the animals were anesthetized with ketamine (60 mg kg-1) and xylazine (8 mg kg-1) intraperitoneally and the animals were euthanized by the exsanguination process [13].

2.4 Volume and thickness of paws (Oedema)

The thickness (in mm) of the hind paws was obtained using a digital caliper (0,01mm/0.005" resolution, 500 series, Mitutoyo, São Paulo, Brazil) positioned in the middle region of the plantar surface. The volume (mL) of the paws was obtained with a plethysmometer (SLFC 008, ScienLabor, Ribeirão Preto, Brazil), using standardized anatomical reference regions (tibiotarsal joint). Measurements were performed in triplicate, by trained researchers. Mean values were used to calculate the difference (Δ) between values for thickness or volume of the right paw (RP) and the left paw (LP) as follows:
$$\Delta = RP - LP.$$

2.5 Histological analysis

After euthanasia, tissue fragments from the CFA injection site, of each animal, were surgically removed and immersed in (10% v v-1) buffered formalin solution for 72 hours, washed with saline transferred to cassettes, and stored in (10% v v-1) formaldehyde buffer solution. Sections (3-4 μ m) were obtained using a microtome (HM 430, Thermo Scientific™ Massachusetts, EUA) and then stained with hematoxylin and eosin (HE). Histological analysis was performed with light microscopy (Opton®, Guiyang, China), for a qualitative description.

2.6 Total and differential blood cells counting

A blood volume of 4 ml (milliliters) was collected from the animals by cardiac puncture and stored in heparinized tubes. The profile of the different leukocyte populations (differential leukogram) was performed by blood smear analysis. Leukocytes were counted on a Neubauer chamber, after blood dilution on Turk's erythrocytes lysis solution [14].

2.7 Thermography analysis

A thermographic camera (FLIR i7®, Flir Systems, Portland, United States) was used for recording images at different experimental times of the right and left hind paws of all animals. The camera was positioned perpendicularly at a distance of 0.6 meters from the plantar surface of the hind paws and the images were obtained in triplicate by a trained researcher, before the administration of CFA (T0) and then at the other experimental times (T1 to T9). Thermographic profiles were analyzed using FLIR® Tools software (FLIR® Systems, Portland, OR, United States) where the experimental parameters and emissivity $\epsilon = 0.95$ were assumed. After processing, maximum, minimum, and average temperature values of the plantar region of each of the animal's hind paws were obtained.

2.8 Statistical analysis

Data were analyzed using Minitab and GraphPad Statistical Software, version 3.0 (GraphPad, La Jolla, CA, USA). Results were expressed as the mean and standard error of the mean (SEM) from triplicates to the independent experiments, with a significance level of 95% ($P < 0.05$). One-way ANOVA, with Tukey posthoc, was used for multiple comparisons.

Results

3.1 Temperature profiles

Figure 1 exemplifies 3 images taken at each time for each animal and group. There was the maintenance of low values with standard deviations (SD) that ranged from 0.00 to 0.54 ° C, thus demonstrating the good repeatability of the method.

The values of temperature for the left paw (injected with 200 µL of saline solution) are presented in Fig.2 and showed no difference between groups for all evaluated times.

The mean temperature values for the right hind paws are presented in Fig. 3. It is possible to notice that, after the induction, temperatures increased for all groups and at 24 hours presented the maximum values. In general, after 24 hours temperatures tended to decrease towards the values of the baseline (T0), 21 days after CFA injection. The results for ANOVA were also observed, comparing the initial time (T0) with other experimental times. There was a difference between temperature means for the time before induction (T0) compared to times of 24h (T2), 48h (T3), 72h (T4), and 96h (T5). The other experimental times did not present a difference for the means when compared with baseline. There were differences in temperature at T2 for all groups. At T3, there were differences for groups G1 (control) and G3 (diclofenac). At T4, there were differences for groups G1 (control) and G2 (triamcinolone) and in T5 the difference was achieved only for group G1 (control).

It is possible to notice (table 1) that at T2 (24 hours) the mean values for paw temperature were different for groups G2 (triamcinolone). At T3 (48 hours), the means for all groups were different. From T4 (72 hours) to T7 (14 days) there were no differences. Nevertheless, for T8 (21 days) the means for all groups were again different and at T9 (28 days) there was a difference only for group G3 (diclofenac).

GROUPS	MEAN TEMPERATURE IN °C (±SD)									
	T0	T1	T2	T3	T4	T5	T6	T7	T8	T9
Control	23,6 ^a (±0,21)	26,8 ^a (±0,89)	33,24 ^a (±2,94)	31,26 ^a (±2,86)	31,22 ^a (±3,79)	31,24 ^a (±3,8)	27,78 ^a (±1,88)	27,48 ^a (±3,21)	26,86 ^a (±0,86)	28,7 ^a (±2,80)
Triamcinolone	24,04 ^a (±0,36)	26,62 ^a (±1,26)	33,32 ^b (±0,92)	27,32 ^b (±2,05)	30,92 ^a (±3,53)	27,22 ^a (±1,41)	26,54 ^a (±1,45)	25,90 ^a (±2,22)	24,38 ^b (±1,05)	26,68 ^a (±2,49)
Diclofenac	24,38 ^a (±0,98)	28,10 ^a (±1,21)	32,24 ^a (±2,44)	29,58 ^c (±3,45)	29,00 ^a (±2,31)	28,59 ^a (±3,58)	27,74 ^a (±3,14)	25,50 ^a (±2,51)	24,02 ^c (±1,05)	26,60 ^c (±2,86)

Tab. 1 Variation in rat paws temperature, after CFA injection, (n = 5), values represented as mean ± standard deviation, ^{a-c} different letters in the same column indicate significant statistical differences (p<0,05; One-way ANOVA with Tukey *post hoc*). CFA (Complete Freund Adjuvant)

The room temperature during the experiment did not change significantly, presenting a mean value of 19.12 °C (± 1.05°C).

3.2 Volume and thickness of paws (Oedema)

The paws volume and thickness values are presented in Fig. 4 and 5, respectively. It is possible to notice an increase of the parameter's values, with maximum values at 24 hours followed by a decrease, in both Graphs.

The values of the paws volume were different compared to values at baseline (T0) for groups G1 (control) and G2 (triamcinolone) for all times. In group G3 (diclofenac), this difference was achieved for times from T2 (24 hours) to T7 (14 days). The paws thickness measured presented a difference for all times and groups when compared to T0.

3.3 Histological analyses

Figure 6 shows the histological sections obtained for all experimental groups at the end of the experiment (T9). The three histological sections showed areas of inflammatory cell infiltration in all groups. A granuloma formation was revealed in groups G1 (control) e G2 (triamcinolone).

3.4 Total and differential blood cells counting

Table 2 presents the results for total and differential blood cell counting for all the groups. There was no difference between the groups.

Groups	Total Leucocytes (%)	Neutrophils (%)	Monocytes (%)	Lymphocytes (%)	Eosinophils (%)	Basophils (%)
Control	5880 (100)	3132 (53,3)	791 (13,4)	1936 (33)	7 (0,1)	14 (0,2)
Triamcinolone	5180 (100)	3227(62,3)	528 (10,2)	1376 (26,6)	33(0,6)	16 (0,3)
Diclofenac	7510 (100)	4713(62,8)	1177 (15,7)	1588 (21,1)	14 (0,2)	18 (0,2)

(n = 5), Values represented as mean (p<0,05; One-way ANOVA with Tukey's test)

Tab. 2 Total and differential blood cells counting for all groups, (n = 5), values represented as mean (p<0,05; One-way ANOVA with Tukey *post hoc*)

Discussion

Thermography is a method of imaging using an infrared radiation detection sensor to measure radiation emitted from a surface. After the acquisition, such images are organized as a distribution diagram with temperature information [15] so it is a non-invasive method. High sensitivity [8; 16; 17] is reported for such method and it allows the registration of the trophic conditions of the tissues, in areas with increased tissue metabolism or with an inflammatory response [18; 19]. By this method, the temperature is represented graphically (thermogram), with different colors for each temperature interval [7]. Each pixel in the thermogram represents a measured temperature of the surface of an object. Variations in the color pattern indicate thermal differences due to changes in surface temperature, which can be quantified by heat transfer principles [20; 21].

In the present study, using the thermographic camera, it was possible to observe an increase in tissue temperature in 24 hours and a further slow decrease until 21 days. From T7 onwards, the temperature values, in all groups, returned to baseline values (T0). To verify if detected modifications in temperature occurred simultaneously with other inflammatory signals, the paw's thickness and volume were also evaluated. A similar increase at 24 hours observed in the temperature through the thermography method, also were noticed in the thickness and volume paws. Such finding is stimulating since, for this specific type of inflammation model, this biological behavior is expected (the 24 hours peak).

The infrared technique has also been shown to be useful to differentiate the effectiveness of treatments with anti-inflammatory drugs with different mechanisms of action. Considering the animal's paw thickness, differences were demonstrated between the initial time and all subsequent experimental times, in all groups. Considering data from the paw volume analysis for animals treated with topic diclofenac there was no difference at 30 minutes or 21 and 28 days, which demonstrated that in this group and times volume changes reached values similar to the baseline. This result could suggest that, for this group, the diclofenac topic treatment was more effective in volume change than in thickness. The formation of granuloma, as observed in the present study, could explain why the volume of the animals' paws did not return to the initial values with exception of the animals treated with topic diclofenac.

The graphics curves demonstrated that temperature behavior followed edema (thickness and volume) behavior with an increase at 24 hours followed by a decrease reaching values similar to those of the baseline in a shorter experimental time, compared to volume and thickness parameters. A hypothesis considered for these outcomes is that temperature decrease could be solved faster in the inflammatory response than edema, however other studies must be performed with different animal models of inflammation.

The induction of chronic inflammation in rodents was achieved with injection of a suspension of inactive strains of *Mycobacterium tuberculosis* in Freund's adjuvant and it is expected a larger sensibilization period by the presence of non-metabolizable oils, such as paraffin that promotes the continuous release of antigens. With this, chronic inflammation is triggered inducing a strong and persistent inflammatory response that could achieve 35 days of duration [1; 22-24]. Some musculoskeletal disorders, related to chronic inflammation lack objective diagnostic and gold standards, then it is a challenge to effectively validate the present technique.

In histological sections, it was possible to qualitatively determine the presence of cellular infiltrate, consistent with chronic inflammation. Leukocyte differential counting informed the relative amount of different leukocyte types in blood cells (neutrophils, lymphocytes, basophils, eosinophils, and monocytes) according to their morphological characteristics. There is no reduction in the percentage of lymphocytes in the blood of groups that received the CFA injection and were treated with triamcinolone (26,6%) and diclofenac (21,1%) when compared to animals in the control group (33%), which could be related to the anti-inflammatory effect of the drugs used.

Inflammation is the process of recruitment of leukocytes and plasma proteins from the blood, their accumulation in tissues, and their activation to destroy the microorganisms. Many of these reactions involve cytokines, which are produced by dendritic cells, macrophages, and other types of cells during innate immune reactions. The major leukocytes that are recruited in inflammation are phagocytes neutrophils (which have a short life span in tissues) and monocytes (which develop into tissue macrophages) [12]. Therefore, a possible cause for the reduction in the percentage of lymphocytes in the blood cell counting could be due to their accumulation in tissues. In the present study, we also noticed a reduction, although not significant, of blood neutrophils in the control group (without treatment). However, we cannot say that these neutrophils would be more concentrated in the inflamed tissue since the characterization of the tissue cells was not carried out.

Drugs used were selected since topical treatments for inflammation disorders are frequently well-tolerated and preferred by many patients [25]. For these reasons, a topical corticosteroid was used. Another anti-inflammatory drug was used due to the current evidence that indicates that topical non-steroidal anti-inflammatory drugs may be effective for pain relief in osteoarthritis [26]. Diclofenac sodium is a potent inhibitor of cyclooxygenase-2 with analgesic and anti-inflammatory properties; however, it has little antipyretic action. It is recommended for the treatment of chronic inflammatory conditions such as rheumatoid arthritis and osteoarthritis [27]. Triamcinolone acetonide is a synthetic corticosteroid that has anti-inflammatory, antipruritic, and antiallergic action [27]. Components of the formula act as an adhesive vehicle to the active medication [28].

Our results suggest that thermography may also be useful to differentiate the anti-inflammatory efficacy of different drugs. When compared to the diclofenac sodium animal group (96 hours), the animals treated with triamcinolone acetonide returned faster (48 hours) to the initial temperature values. The pharmacology of triamcinolone as a corticosteroid drug could explain anti-inflammatory effects and also its vehicle since adhesive vehicles could improve drug substantively by prolonging the supply of the drug in the site as a result of the ability to adhere to the substrate and persist at effective drug concentration [29].

The right paws temperatures (injected with saline solution) were not different, as expected, since they are regions that did not receive pro-inflammatory stimulation.

The temperature of the extremities and skin depends on the blood flow dynamics and temperature. Additionally, individual variations at different times of the day can occur [19]. For this reason, all images were recorded at the same time, early in the morning in a controlled environment to prevent such aspects.

Thermography does not provide specific details of disease however it may be useful in defining the area affected by inflammation and also can assist the progression of the lesion. The temperature patterns can be associated with healthy or pathological situations [30].

Conclusions

This preliminary study shows a possible use of quantitative high-resolution infrared as a complementary tool for monitoring the inflammatory process in this animal model of inflammation.

Declarations

Funding

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Technological Development (CNPq - Process 439373 / 2018-2), and Coordination for the Improvement of Research Personnel Higher Education (CAPES).

Conflicts of interest

The authors declare that they have no conflict of interest.

Availability of data and material

The datasets generated during the current study are available from the corresponding author on reasonable request.

Code availability

Not applicable.

Author contributions

Conception and design were performed by Agnes Batista Meireles, Cíntia Pimenta Araújo, Gustavo Eustáquio Brito Alvim de Melo, Patrícia Furtado and Wagner de Fátima Pereira. Material preparation, data collection were performed by Agnes Batista Meireles, Timilly Martins Cruz, Izabela Cristina Brandão Moreira, Marcelo Henrique Fernandes Ottoni. Data analysis was performed by all authors. The first draft of the manuscript was written by Agnes Batista Meireles and Izabela Cristina Brandão Moreira. All authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

Ethics approval

This study was previously approved by the Animal Ethics Committee of Federal University of Jequitinhonha and Mucury Valleys (UFVJM) regarding the Guiding Principles in the Care and Use of Animals, with an approved protocol number of 050/2016.

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Figures

Figure 1

Data on the repeatability of the evaluated method. In A, B and C have represented three thermographic records at different times of the same hind paw (within the black circle) of the same animal. SD values of 0.00 to 0.54 ° C

Figure 2

Variation in rat paws temperature, after saline injection. Values are represented as mean $\pm$ standard error of the mean at different experimental times. Temperature for the left paw (control paw) after 200 μ L of saline solution injection (n = 5), One-way ANOVA with Tukey post hoc (P < 0.05)

Figure 3

Variation in rat paws temperature, after CFA injection. Values are represented as mean $\pm$ standard error of the mean at different experimental times. Temperature for the induced right paw (n = 5), one-way ANOVA with Tukey post hoc (P < 0.05). a statistical difference when compared to T0 for the control group; b statistical difference when compared to T0 for the group treated with triamcinolone; c statistical difference when compared to T0 for the group treated with diclofenac

Figure 4

Variation in the volume of the paw of rats, after injection of CFA. Value in Δ (Δ = RP - LP) represented as mean $\pm$ standard error of the mean, in different experimental times; (n = 5). One-way Anova with Tukey post hoc (P < 0.05). a statistical difference between T0 and the time evaluated on the (x) axis for the control group; b statistical difference between T0 and the time evaluated on the (x) axis for the group treated with triamcinolone; c statistical difference between T0 and the time evaluated on the (x) axis for the group treated with diclofenac

Figure 5

Variation in the thickness of the paw of rats after injection of CFA. Value in Δ ($\Delta = RP - LP$) represented as mean ($\pm$ standard error of the mean), in different experimental times, ($n = 5$). ANOVA with Tukey post hoc ($P < 0.05$). a Statistical difference between T0 and the time evaluated on the (x) axis for the control group; b statistical difference between T0 and the time evaluated on the (x) axis for the group treated with triamcinolone; c statistical difference between T0 and the time evaluated on the (x) axis for the group treated with diclofenac

Figure 6

Histological aspects of rat paws after CFA injection. Presence of inflammatory cell infiltration in the hind right paws, after 28 days of CFA injection. HE staining and 400 magnitudes. G1 (CON): Histological aspect of rat paw of the control group (no treatment): Sites with intense inflammatory cell infiltration (If) with lymphocytes, foreign body oily substance (Ce), necrosis area (Ne), granuloma (Gr), and part of a blood vessel site with red blood cells inside (Vs). G2 (TRI): Histological aspect of rat paw treated with triamcinolone. Sites with the presence of foamy macrophages (Me), granuloma (Gr), and foreign body oily substances (Ce) were observed. G3 (DIC): Histological aspects of the rat paw treated with diclofenac potassium. It was observed sites with intense inflammatory cell infiltration (If) and foreign body oily substance (Ce)