Infrared thermography is applicable in differentiating the effectiveness of anti-inflammatory drugs: A complementary test

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ABSTRACT

Aims: Images obtained by infrared thermography (IT) have potential to become a useful and low-cost tool for a wide range of biological *in vivo* studies, including topical inflammation models. Local temperature is one of the cardinal signs of inflammation, although it is not commonly analyzed in experimental model of inflammation. In the present study IT was used to evaluate the variation in tissue temperature, as well as the temperature response to treatment with different anti-inflammatory drugs, in an experimental model of inflammation.

Study design: Temperature, volume and thickness of paws, histological analyses, total and differential blood cells counting were the parameters analyzed. CFA-induced paw edema was performed in rats and discrepancies between animals treated or not with anti-inflammatory drugs were analyzed**.**

Place and Duration of Study: Holtzman male rats from Federal University of Jequitinhonha and Mucuri Valleys (Diamantina, Brazil) were tested during 28 days**.**

Methodology: CFA-induced paw edema was performed in rats and discrepancies between animals treated or not with triamcinolone acetonide and diclofenac sodium were analyzed. Experimental times were: T0, before chemical induction of inflammatory process (control); and several times after induction: 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, paw volume, histological and leukometric analysis.

Results: Standard deviations (SD) presented low values (0.00 to 0.54 °C), thus demonstrating the good repeatability of the infrared thermography method. Temperature values in the paws injected with saline showed no significant difference between groups ($p < 0.05$). There was a significant difference between the mean temperatures before induction (T0) compared to 24h (T2), 48h (T3), 72h (T4) and 96h (T5) ($n=5$; $P<0.05$). Paw volume values were different ($p<0.05$) in relation to initial values (T0) for groups G1 (control) and G2 (triamcinolone). For group G3 (diclofenac) there was a statistical difference in the times from T2 (24 hours) to T7 (14 days). The thickness of the paws measured showed a statistical difference ($p<0.05$) for all moments when compared to T0. Histological sections showed areas of inflammatory cell infiltration in all groups.

Conclusion: In the present study, the temperature variation was similar to the variation in the volume and thickness of the rats paws, and the changes in tissue temperature reinforced the findings regarding the characteristics of inflammation. Furthermore, the infrared technique was useful to demonstrate different responses to anti-inflammatory tests in this animal model of inflammation.

Keywords: Thermography, Inflammatory response, Temperature, Anti-inflammatory drugs, Animal model.

1. INTRODUCTION

Paw edema is a classical inflammation model where a phloglogen agent is administered subcutaneously in the plantar region of rodent paws to generate an inflammatory response [1]. Inducing agents such as carrageenan and Complete Freund's Adjuvant injection (CFA) are commonly used to trigger acute and chronic inflammatory response, respectively [2] that could be analyzed by radiographic techniques, histological analysis and also the presence of edema. Local temperature is one of the cardinal signs of inflammation, although it is not commonly analyzed in experimental model of inflammation.

The temperature of a surface can be obtained by thermographic profiles from images obtained by radiation sensitive camera [3]. Therefore, Infrared thermography (IT) has potential to be a low-cost tool for a wider range of uses [4]. For medical applications it is attractive by dispensing invasive procedure traditionally used [5] in fact, clinical trials using thermal images were recently reported [6]. Infrared thermography was used to measure temperature profiles in order to detect inflammation in patients with rheumatoid arthritis [7] and acute appendicitis [8]. In animal studies, this technology has been employed [9, 10] including animal models of cancer [11]. In this case, it was reported as a useful approach for the analysis of superficial vascularization. Others animal applications measured temperatures different parts of animals using IT and related it to feed efficiency, average daily gain and methane emission [12], evaluation of mastitis in cattle [13] and for fever investigation in pigs [14].

Therefore, the present study evaluated the thermography technique as a complementary tool for analyses involving animal models, specifically, for the study of the inflammation process. We performed CFA-induced paw edema in rats and observed the differences between animals treated or not with topic anti-inflammatory drugs (triamcinolone

acetonide and diclofenac sodium) on the following parameters: local temperature, volume and thickness of paws, histological analysis, total and differential blood cells counting.

2. MATERIAL 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 average weight of 150-250 grams were used in this study. Experiments were performed 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 protocol number of 050/2016.

2.2 Inflammation induction

The inflammation was induced by injecting 200 µL of 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 µL of saline solution was injected. The animals were divided in 3 groups: Control (n=5) - animals that received CFA injection and no treatment; Triamcinolone (n=5) - 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 Diclofenac (n=5) - 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 [15].

2.4 Volume and thickness of paws (Edema)

The thickness (in mm) of the hind paws was obtained by means of 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 measured with a plethysmometer (SLFC 008, ScienLabor, Ribeirão Preto, Brazil), using standarized anatomical reference regions (tibio-tarsal articulation). 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 follow: Δ = RP - LP.

2.5 Histological analysis

After euthanasia, tissue fragment 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 μm) 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 was collected from the animals by cardiac puncture and stored in heparinized tubes. The profile of the different leukocyte populations (differential leukogram) was performed and also leukocytes were counted on a Neubauer chamber [16].

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, prior to the administration of CFA (T0) and then at the other experimental times (T1 to T9). Thermographics profiles were analyzed using FLIR® Tools software (FLIR® Systems, Portland, OR, United States) where the experimental parameters and emissivity ε = 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 was analyzed using Minitab and GraphPad Statistical Software, version 3.0 (GraphPad, La Jolla, CA, USA). Results were expressed as 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 post-hoc were used for multiple comparisons.

3. RESULTS AND DISCUSSION

3.1 Temperature profiles

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

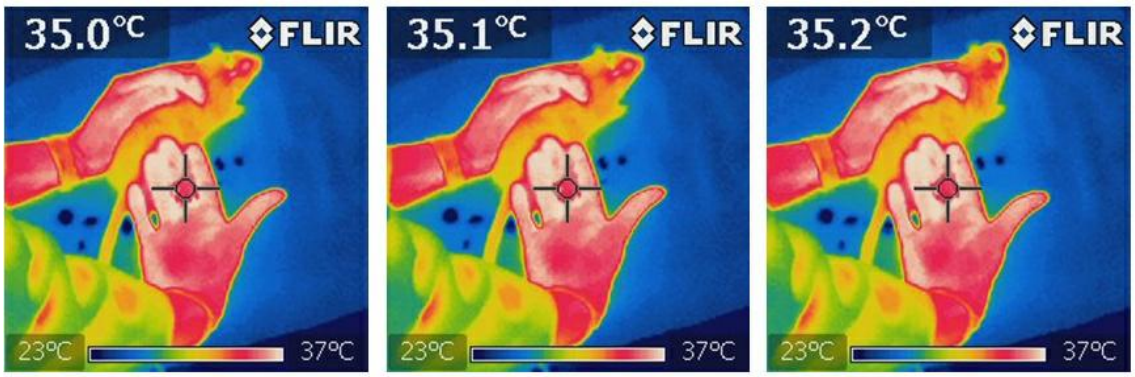


Fig.1 Repeatability of the evaluated method. In A, B and C are represented three thermographic records at different times of the same hind paw (within the black circle) of the same animal. SD values of 0.00 to 0.54 °C

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.

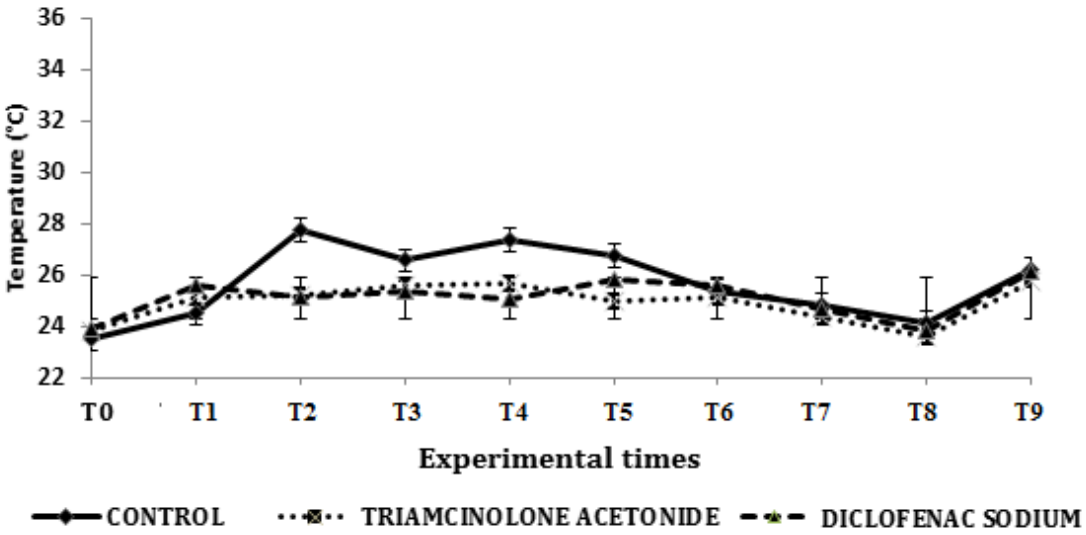
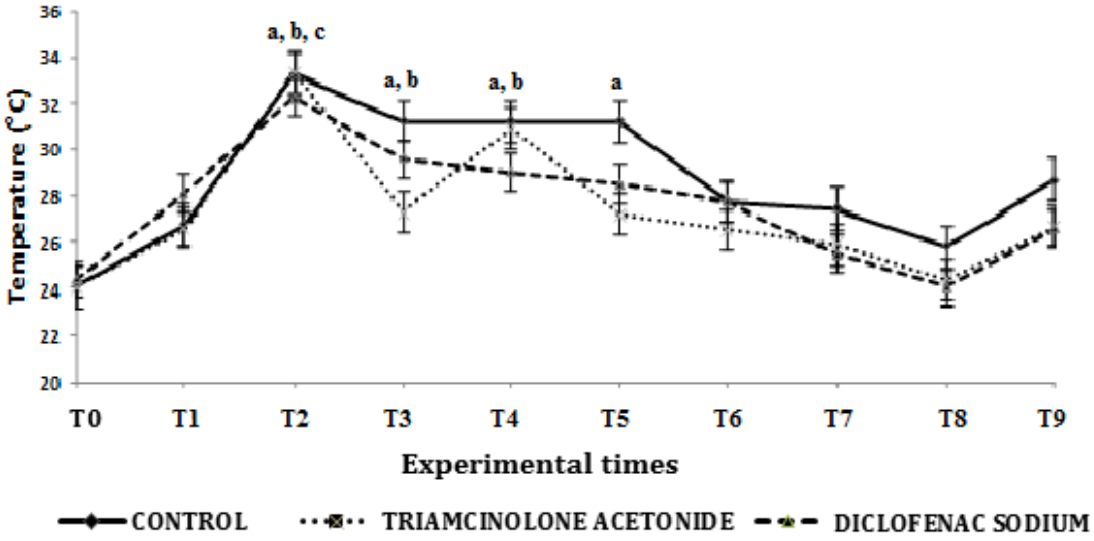


Fig.2 Variation in rat paws temperature, after saline injection at different experimental times. Values were represented as mean ± SEM.

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 to 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 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 difference for the means when compared with baseline. There were differences in temperature at T2 for all groups. At T3, there were differences for control and diclofenac groups. At T4, there were differences for control and triamcinolone groups and in T5 the difference was achieved only for control group.



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136 **Fig. 3** Variation in rat paws temperature, after CFA injection at different experimental times.
137 Values were represented as mean ± SEM. [a] statistical difference when compared to: T0 for
138 control group; [b] T0 for the group treated with triamcinolone and [c] T0 for the group treated
139 with diclofenac.

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

145 **Table. 1** Variation in rat paws temperature, after CFA injection. Values represented as mean
146 ± SEM, *(p<0,05).

Groups	**Mean temperature (°C)**									
	T0	**T1**	**T2**	**T3**	**T4**	**T5**	**T6**	**T7**	**T8**	**T9**

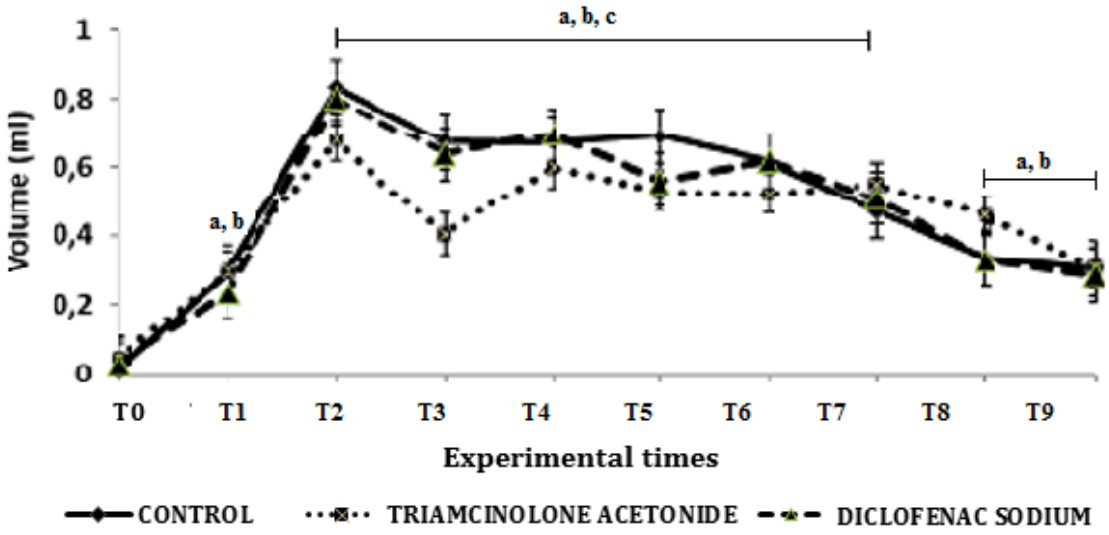
Control	23,6 (±0,21)	26,8 (±0,89)	33,24 (±2,94)	31,26 (±2,86)	31,22 (±3,79)	31,24 (±3,8)	27,78 (±1,88)	27,48 (±3,21)	26,86 (±0,86)	28,70 (±2,80)
Triamcinolone	24,04 (±0,36)	26,62 (±1,26)	33,32* (±0,92)	27,32* (±2,05)	30,92 (±3,53)	27,22 (±1,41)	26,54 (±1,45)	25,90 (±2,22)	24,38* (±1,05)	26,68 (±2,49)
Diclofenac	24,38 (±0,98)	28,10 (±1,21)	32,24 (±2,44)	29,58* (±3,45)	29,00 (±2,31)	28,59 (±3,58)	27,74 (±3,14)	25,50 (±2,51)	24,02* (±1,05)	26,60* (±2,86)

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 (Edema)

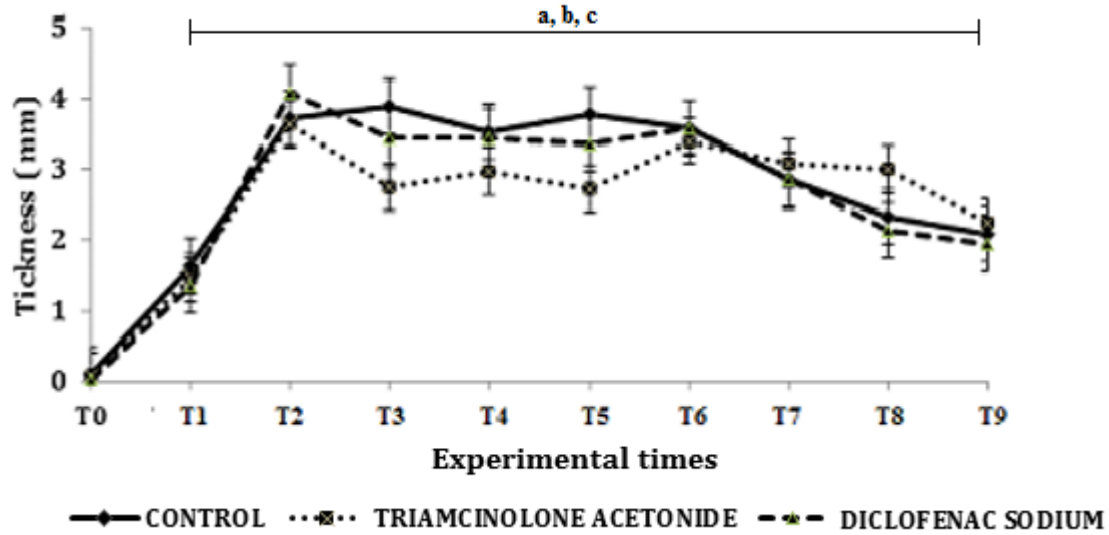
The paws volume and thickness values are presented in Fig. 4 and 5, respectively. There was an increase of the parameter's values, with maximum values at 24 hours followed by a decrease, in both Graphs.

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



157

158 **Fig. 4** Variation in the volume of the paw of rats, after injection of CFA in different
159 experimental times. Value in Δ (Δ = RP - LP) represented as mean ± SEM. [a,b,c] (P <0.05)
160 statistical difference between T0 and the time evaluated on the (x) axis for the groups:
161 control, treated with triamcinolone and treated with diclofenac respectively.



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163 **Fig. 5** Variation in the thickness of the paw of rats after injection of CFA in different
164 experimental times. Value in Δ (Δ = RP - LP) represented as mean (± SEM). [a,b,c] (P <0.05)

statistical difference between T0 and the time evaluated on the (x) axis for the groups: control, treated with triamcinolone and treated with diclofenac respectively. CFA (Complete Freund Adjuvant). RP and LP (Right and Left paw respectively).

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 control group and in animals treated with triamcinolone.

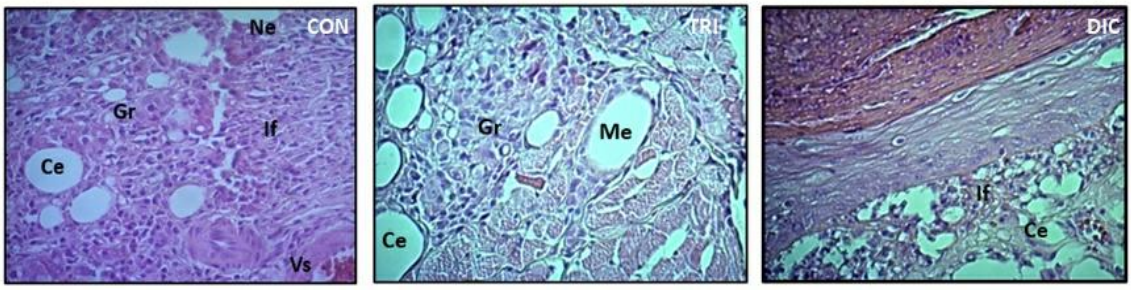

Fig. 6 Histological aspects of rat paws after CFA injection. Presence of inflammatory cell infiltration in the hind right paws, after 28 days of CFA injection. HE staining – 400x. CON: Histological aspect of rat paw of the control group (no treatment) - Sites with intense inflammatory cell infiltration (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). TRI: Histological aspect of rat paw treated with triamcinolone - Sites with the presence of foamy macrophages (Me), granuloma (Gr) and foreign body oily substance (Ce) were observed. 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).

3.4 Total and differential blood cells counting

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

Table. 2 Total and differential blood cells counting for all groups. Values represented as mean ± standard error of mean, (p<0,05).

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)

Thermography is a method of imaging using an infrared radiation detection sensor to measure radiation emitted from a surface. After acquisition, such images are organized as a distribution diagram with temperature information [18] so it is a non-invasive method. High sensitivity [11; 18; 19] 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 [20; 21]. By this method, 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. In fact, variations in the color

pattern indicate thermal differences due to changes of surface temperature, which can be quantified by heat transfer principles [22; 23].

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 paws thickness and volume were also evaluated. A similar increase at 24 hours observed in the temperature trough the thermography method were also noticed for the thickness and volume paws parameters. Such finding is stimulating since, for this specific type of inflammation model, this biological behavior is expected (the 24 hours peak).

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 to 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 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, a chronic inflammation is trigged inducing a strong and persistent inflammatory response that could achieve 35 days of duration [1; 24-26]. Some of musculoskeletal disorders, related to chronic inflammation lack in objective diagnostic and gold standards, then it is a challenge to effectively validate the present technique.

In the histological sections it was possible to qualitatively determine the presence of cellular infiltrate, consistent with a 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 change in the percentage of lymphocytes in the blood of groups that received the CFA injection and treated with triamcinolone (26,6%) and diclofenac (21,1%) when compared to animals in the control group (33%). This could be related to the anti-inflammatory effect of the drugs used.

Drugs used were selected since topical treatments for inflammation disorders are frequently well-tolerated and preferred by many patients [27]. 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 [28]. 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 [29]. 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 [30].

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 corticosteroid drug could explain anti-inflammatory effects and also its vehicle, since adhesive vehicles could improve drug substantively by prolonging the supply of drug in the site as result of the ability to adhere to the substrate and persist at effective drug concentration [31].

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

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 [21]. For this reason, all images were recorded at the same time, early in the morning in a controlled environment to prevent such aspects.

The temperature patterns can be associated to healthy or pathological situations [32]. Thermography does not provide specific details of a disease however it may be useful in defining the area affected by inflammation, assist progression of the lesion and has the potential to support studies testing effectiveness of different types of treatments. The effectiveness of this technique for this purpose should be further tested [32], since the preliminary results in the present study are compatible with the inflammation model and drugs there were used.

4. CONCLUSION

The paws temperature behavior was similar to those of volume and thickness. In this inflammation model, tissue temperature changes were observed and reinforced the findings about the characteristics of inflammation. The infrared technique can be helpful to demonstrate differences in features of drugs formulations. Further investigations, including

others animal models of inflammation should be performed to enhance the present findings. The infrared thermography presented as a reliable reproducible tool with lower standard deviations values and is a potential analysis in animal models.

ETHICAL 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 approved protocol number of 050/2016.

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COMPETING INTERESTS

Authors have declared that no competing interests exist

AUTHORS' CONTRIBUTIONS

This work was carried out in collaboration among all authors. author WFP designed and coordinated the study. Authors ABM, TMMC, ICBM, VGA, BA-V, MHFO, GEBAM, PFG,

CPA collected the data. Authors ABM and WFP analyzed the data and drafted the article. All authors read and approved the final manuscript.

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