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An experimental model of canine DNCB-induced allergic contact dermatitis: clinical and hematological features

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Abstract

Allergic contact dermatitis is an inflammatory skin reaction caused by direct contact with an offending substance. The aim of this study was to establish a suitable method for induction of allergic contact dermatitis in dogs for future studies. For this purpose, 1% and 2% dinitrochlorobenzene (DNCB) were injected with and without ethanol and olive oil or acetone and olive oil subcutaneously in the back of 8 BALB/c mice (4 equal groups). Then, based on the types and severity of the symptoms, it was decided to sensitize the dogs with only 2% DNCB. Finally, in two stages, 2% DNCB with or without dimethyl sulfoxide (DMSO) was injected with ethanol or acetone and olive oil subcutaneously in thoracic and scapular regions of five dogs. Two percent DNCB challenge caused clinical findings including erythema, edema and skin scaling, as well as pruritus and scratching behavior in the mice. Clinical findings in dogs developed in mild severity including redness and swelling within a few days. There was a significant increase in total blood leukocytes, neutrophils, lymphocytes, eosinophils and monocytes counts at seven days in dogs. Also statistical analysis showed that one week after DNCB injection, IgE expression increased significantly and this increase continued until day twenty-one. Based on the result of this study, intradermal injection of 100 μL of 2% DNCB mixture with DMSO (dissolved in a 4:1 mixture of ethanol and olive oil) was a suitable method for induction of allergic contact dermatitis in dogs. Furthermore, the scapular region was also a convenient location to prevent self-induced lesions.

Key words: Allergic contact dermatitis, Atopic dermatitis-like, Dinitrochlorobenzene, Dimethyl sulfoxide, Dog

Introduction

Allergic contact dermatitis is an allergic skin disease whose described as a type IV hypersensitivity requiring sensitization and elicitation. Allergic contact reactions are characterized by immunologic reaction to a hapten, usually a small, chemically reactive, lipid-soluble molecule (Miller, et al., 2013). Probiotics are most frequently defined as live microorganisms, which when consumed in adequate amounts confer a health benefit on the host (Schmitz & Suchodolski 2016). Probiotics are proposed to exert their beneficial effects by several mechanisms, including displacement of

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intestinal pathogens (Lee *et al.,* 2003), production of antimicrobial substances (Jones & Versalovic 2009), enhancement of immune responses (Pagnini *et al.,* 2010), and/or up-regulation of various metabolites (Soo *et al.,* 2008). To study the effect of probiotics on allergic contact dermatitis in dogs, it was decided to use dinitrochlorobenzene (DNCB) to induce experimental dermatitis. A review of the literature showed that only two studies have used dinitrochlorobenzene to induce contact dermatitis in dogs. The method of intradermal injection of DNCB in present study led to allergic contact dermatitis much earlier than Kim *et al.,* (2012) and Krawiec & Gaafar (1975) studies, thus induction time was saved. Krawiec & Gaafar (1975) was dissolved DNCB in propylene glycol, and 95% ethanol. They were sensitized twelve pups by intradermal inoculation of 0.1 ml of 0.1 % DNCB in the skin of the scapular area every other day for a total of 10 injections. In addition, all pups were rechallenged on the skin of the ventral area 2 weeks after the last sensitizing application of DNCB. Six to eight patches were placed on each pup and left for 24 hr. In the second study, both inguinal regions of five dogs were sensitized by topical application of 1000 µl of 1% DNCB for first sensitization (Kim *et al.,* 2012). Seven days later, the dogs were rechallenged on four sites of the back with 200 µl of 0.5% DNCB. Due to the lack of a similar pattern in the use of DNCB for induction of contact dermatitis, murine similar studies were considered as a model. Unfortunately, despite the presence of many studies in this field, but there were great differences in terms of the types of solvents, DNCB concentration, frequency and repeat of challenge between them (Cho *et al.,* 2018, Li *et al.,* 2018, Wu *et al.,* 2019). Therefore, the aim of present study was to first inject different concentrations of DNCB and solvents on mice to prepare a suitable model for induction of allergic contact dermatitis in dogs. Then after examination of the safe and efficient DNCB concentration and solvent in dogs, IgE concentrations were measured to ensure its safety and efficacy in dogs.

Materials and Methods

The ethical approval for this study was obtained from Ethics Committee of Shahid Chamran University of Ahvaz (approval number: scu.ac.ir /1400.3.02.7810/ EE). This study consists of three Phases. In first phase, eight adult male BALB/c mice (weight, 20–22 g) were obtained from the Animal Center of Shahid Chamran University of Ahvaz. All mice were divided in four equal groups and were acclimatized to standard laboratory conditions for a week before experiments and procedures. The dorsal skin of BALB/c mice was sensitized with DNCB (Sigma-Aldrich, St. Louis, MO, USA) to induce atopic dermatitis symptoms. Briefly, following dorsal hair removal, 150 µL of 1% or 2% DNCB solutions (dissolved in a 4:1 mixture of ethanol and olive oil) were injected intradermally at 5 sites of groups 1 and 2, respectively. Also 150 µL of 1% or 2% DNCB solutions (dissolved in a 4:1 mixture of acetone and olive oil) were injected intradermally at 5 sites of groups 3 and 4, respectively. A 7-day clinical examination revealed that only 2% DNCB concentration was able to cause skin lesions. Therefore, in second phase of this study only 2% DNCB was applied for induction of allergic contact dermatitis in four dogs (2 equal groups). The used amount of DNCB, the solvents and route of injection were similar with mice. So that, on the left thoracic area, DNCB dissolved in ethanol and olive oil (group 1) or dissolved in acetone and olive oil (group 2) was injected while only solvents were injected on the right thoracic area, as control. During 7 days, necrotic and ulcerative lesions developed only on left side of the body of both dogs. Due to the incomplete solubility of DNCB in ethanol and olive oil, it was decided to use dimethyl sulfoxide (DMSO) as a better solvent for DNCB.

In third phase, 100 µL of 2% DNCB mixture with DMSO (dissolved in a 4:1 mixture of ethanol and olive oil) were injected intradermally at 5 sites on the left scapula of five dogs. The scapular region was chosen because in second phase, dogs licked their sensitization area. Also these dogs were received ethanol and olive oil on the right scapula as control group. As the clinical symptoms subsided, another injection was given in the same area after three weeks to re-stimulate and prolong the sensitivity to allergen. Therefore, the dogs were rechallenged intradermally with 20 µL of 2% DNCB mixture with DMSO (dissolved in a 4:1 mixture of ethanol and olive oil).

Hematological Analysis

One ml of blood was collected through the cephalic veins of dogs in the days before injection and 7, 21, 28 and 35 days after the injection of DNCB. A complete blood cell test was performed with a hematology analyzer.

IgE Evaluation

Whole bloods collected with Heparin were centrifuged 10 minutes at 800 g and the plasma supernatants were discarded; the buffy coat layers were collected carefully and transferred to the new sterilized tubes. The remained red blood cells (RBC) were lysed using sterilized RBC lysis buffer. Extracted leucocytes were refrigerated at -70 º C until use. RNA was extracted from WBC by using the TRIzol reagent (Applied Jena Bioscience, Germany) according to manufacturer's protocol with an additional DNAse I digestion step. Quality of the extracted RNA was defined on 1.5% agarose gel electrophoresis. Total RNA was reverse transcribed to first strand complementary DNA (cDNA) using the High Capacity cDNA Reverse Transcription (Applied Jena Bioscience, Germany). For cDNA synthesis using Random hexamer primers and Super-Script II reverse transcriptase (Invitrogen), 9 µL of total RNA was used and subsequently diluted with nuclease-free water.

The expression of IgE was analyzed using real-time PCR. The sequences of chosen genes including Canine glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Accession: NM_001003142) and IgE (Accession: L36872) genes were obtained from NCBI database. Primers were designed using Primer-Blast software (NCBI database) and verified using Oligo Calc: Oligonucleotide Properties Calculator (free software available online, provided by Northwestern University) to exclude sequences showing self-complementarity. To reduce chances of amplifying traces of genomic DNA, the primers were positioned in different exons. Gene expression was calculated with the comparative Ct method and normalized to the endogenous levels of GAPDH (Schlotter *et al.*, 2011, Schmitz *et al.*, 2012, Brinkhof *et al.*, 2006). The primer sequences are listed in Table 1. Real Time PCR analyses were performed on individual samples of total RNA using SYBR Select Master Mix (Applied Yekta Tajhiz azma, Iran) on Stratagene Mx3005P Quantitative PCR instrument for RT-PCR, following the manufacturer's protocol. cDNA was amplified with an initial denaturation at 94ºC for 3 minutes, followed by 40 cycles of 94ºC (30 seconds) and 61ºC (30 seconds); all experiments were performed in duplicate and mean Ct was calculated in each sample.

Table 1. Oligonucleotide primers for gene expression analysis by real time reverse transcription-polymerase chain reaction (RT-PCR).

Gene name	Accession number	Primer sequence (5'-3')	Product size (bp)	Reference
IgE(F)	L36872.1	CTCATGCAGCCTCTCACACA	97	blast.ncbi.nlm.nih.gov
IgE(R)	L36872.1	CGCCTTGTGGACATACAGGT	97	blast.ncbi.nlm.nih.gov
GAPDH(F)	NM_001003142.1	GGAGAAAGCTGCCAAATATG	100	blast.ncbi.nlm.nih.gov
GAPDH(R)	NM_001003142.1	ACCAGGAAATGAGCTTGACA	100	blast.ncbi.nlm.nih.gov

Statistical Analysis

SPSS 23.0 was used for testing the statistical significance of data (SPSS inc., USA). The quantitative variables were analyzed within and among groups by using analysis of variance (ANOVA) for repeated measures and the post hoc Tukey method were applied. For all measurements, means ± standard deviations were determined. For all comparisons, $p \leq 0.05$ was considered statistically significant.

Results

A 7-day clinical examination revealed that there were no clinical signs in the first phase of the study following 1% DNCB injection in mice (Fig.1). While, 2% DNCB challenge caused skin lesions with clinical findings including erythema, edema and skin scaling, as well as pruritus and scratching behavior in the mice (Fig.2). At this point, all of the mice survived, and after about 3 weeks, all symptoms disappeared completely.

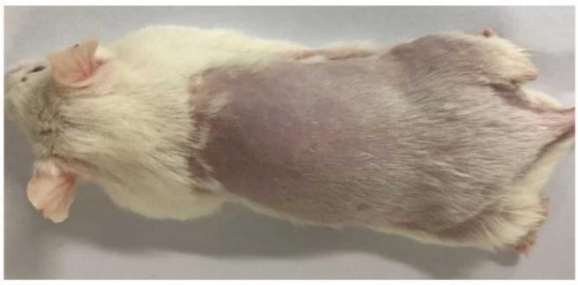

Fig 1: There were no clinical signs in mice received 1% DNCB.

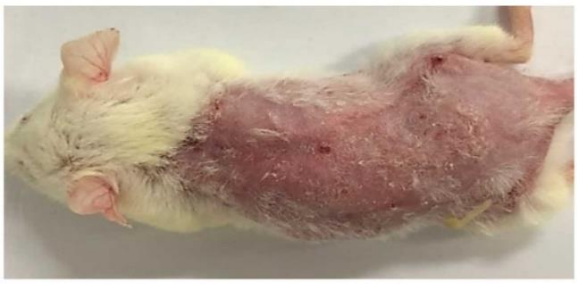

Fig 2: A variety of clinical signs ranging from redness and swelling to ulceration and necrosis occurred in mice that received 2% DNCB.

Therefore, in second phase of this study only 2% DNCB was applied for induction of irritant dermatitis in dogs. During 7 days, the clinical symptoms developed with erythema and edema and progressed toward necrotic and ulcerative lesions of the skin developed on left side of the body of both groups of dogs as well as on right side of dogs receiving acetone as the solvent (group 2) (Fig.3).

Due to the incomplete solubility of DNCB in ethanol and olive oil, it was decided to use dimethyl sulfoxide (DMSO) as a better solvent for DNCB in the third phase of the study. In this phase, clinical findings developed in mild severity including redness and swelling within a few days. Symptoms gradually subsided within 3 weeks after DNCB injection. The second DNCB injection resulted in a longer recurrence of clinical findings (Fig.4).

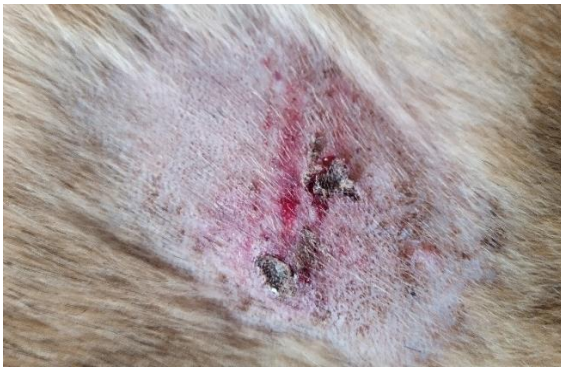

Fig 3: Redness, swelling, ulceration and necrosis of the skin developed in dogs receiving 2% DNCB dissolved in acetone and olive oil.

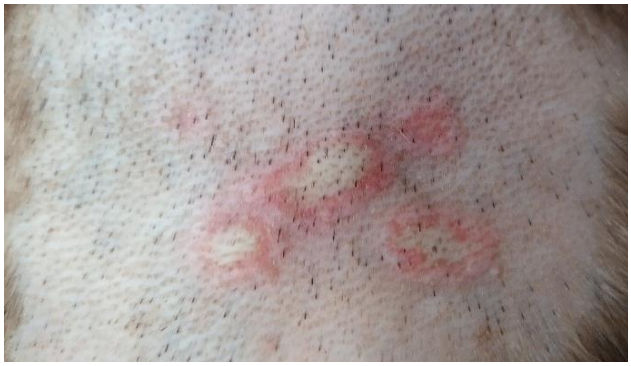

Fig 4: Redness and swelling occurred in dogs receiving 2% DNCB dissolved in DMSO plus ethanol and olive oil.

Results of hematological evaluation of this study are presented in Table 2. Statistical evaluation of these results showed that between the RBC count, hemoglobin level, hematocrit percentage, MCV, MCH, MCHC and RDW, no significant changes were observed after DNCB injection at different times ($p>0.05$). While there was a significant increase in total blood leukocytes, neutrophils, lymphocytes, eosinophils and monocytes counts at 7 days after DNCB injection in comparison with other times within its normal ranges ($p\leq0.05$).

Table 2. Mean ± Standard deviation of hematological parameters of dogs intradermally received 100 µL of 2% DNCB mixture with DMSO (dissolved in a 4:1 mixture of ethanol and olive oil).

Parameters	Time				
	Before injection (a)	7 days after injection (b)	21 days after injection (c)	28 days after injection (d)	35 days after injection (e)
WBC ($*10^3$/µl)	10.80±1.59 B	14.82±0.72 Cde	8.40±0.50 B	9.85±1.43 b	10.17±1.13 b
Lymph ($*10^3$/µl)	2.73±0.98	4.23±0.73	3.29±1.33	3.47±0.51	3.08±1.22
Mon ($*10^3$/µl)	0.34±0.05 Cd	0.43±0.02 Cd	0.20±0.06 Ab	0.06±0.02 abe	0.30±0.01 d
Neut ($*10^3$/µl)	5.75±1.05 B	9.60±0.33 acde	4.44±0.29 B	5.53±1.26 b	5.94±0.52 b
Band ($*10^3$/µl)	0.54±0.09	0.69±0.08	0.72±0.04	0.82±0.02	0.49±0.03
Eos ($*10^3$/µl)	1.62±0.85 C	0.50±0.15	0.35±0.13 A	1.04±0.25	0.82±0.13
RBC ($*10^6$/µl)	6.73±0.3	6.87±0.7	6.76±0.63	6.79±0.41	6.98±0.88
Hb (g/dl)	16.18±0.79	16.54±0.65	16.12±0.46	15.92±0.67	16.76±1.08
HCT (%)	48.16±1.51	47.16±4.24	45.53±3.71	44.20±1.40	46.06±3.70
MCV (fl)	66.62±4.43	67.30±3.93 E	66.54±3.64 E	66.42±4.62	65.40±4.42 bc
MCH (pg)	23.02±2.49	23.52±1.64	23.24±0.97	23.38±1.52	23.36±2.03
MCHC (%)	34.56±1.80	35.02±1.33	35.02±0.97	35.26±0.36	35.72±1.03
RDW (%)	12.00±0.80	11.78±1.26	11.60±1.24	11.76±1.37	12.06±0.85
PLT ($*10^3$/µl)	230.00±18.58 Ce	242.00±17.00 D	167.00±8.14 A	161.66±5.89 b	174.00±15.14 a

Serum IgE concentrations of dogs in this study are presented in Table 3. Accordingly, the serum IgE level of dogs on days 28 and 35 after DNCB injection was significantly reduced compared with days 7 and 21 ($p\leq0.009$). It was also significantly increased on day 35 compared with day 28 ($p=0.003$).

Table 3. Mean ± Standard deviation of fold changes of IgE concentrations of dogs intradermally received 100 µL of 2% DNCB mixture with DMSO (dissolved in a 4:1 mixture of ethanol and olive oil) by RT-PCR.

Time (Days after DNCB injection)			
7	21	28	35
72.24±9.25 Acdef	88.78±7.16 Abcef	4.74±1.72 Bde	17.19±0.81 bdf

Statistical analysis showed that one week after DNCB injection (day 7) IgE expression increased significantly and this increase continued until day 21. Four weeks after DNCB injection, IgE level (day 28) decreased significantly and due to DNCB re-injection on day 35, it increased significantly but at a lower level than the initial injection.

Discussion

Contact dermatitis is an inflammatory skin reaction caused by direct contact with an offending substance. The disease is classically divided into two types: primary irritant contact dermatitis and contact hypersensitivity (Miller, *et al.*, 2013). Most of the work on pathogenesis and immunology of contact allergy has been done in laboratory animals and humans. On the other hand, dogs have been considered as a species that generally considered poorly responsive to experimental allergic contact dermatitis. For this reason, there is the need to study contact dermatitis in dogs today. Dinitrochlorobenzene is one of the substances which used to induce this disease in animals. The present study suggested that intradermal injection of 100 µL of 2% DNCB mixture with DMSO (dissolved in a 4:1 mixture of ethanol and olive oil) was a suitable method for induction of contact dermatitis in dogs. Experimental attempts to induce contact hypersensitivity in dogs have been shown they could be successfully sensitized to dinitrochlorobenzene. Krawiec & Gaafar (1975) was dissolved DNCB in propylene glycol, and 95% ethanol. In Kim *et al.*, (2012) study, DNCB was diluted in mixture of acetone and olive oil (4:1 v/v). Subcutaneous injection of acetone alone as a control resulted in injuries such as erythema, excoriations and eventual ulceration in both mice and dogs. As a result, in this study acetone is not recommended as a DNCB solvent especially in dogs.

DNCB did not fully dissolve in ethanol and olive oil, so DMSO was used as the vehicle in this study. Also based on the clinical results of present study, it was shown that intradermal injections of DNCB, DMSO, ethanol and olive oil are safe in dogs. DMSO as a DNCB solvent has been used in many relevant experimental studies (Heo. *et al.*, 2010; Mori, *et al.*, 2012; Wu. *et al.*, 2019). They did not report any side effects in control groups (vehicle only). It is necessary to mention that DMSO is freely miscible with lipids, organic solvents, and water, it is an excellent vehicle. It tends to cross the skin barrier, penetrates skin (within 5 minutes), mucous membranes, and the blood-brain barrier. Unlike most solvents, DMSO achieves penetration without membrane damage. It facilitates absorption of many other substances across membranes, especially corticosteroids (Galvao, *et al.*, 2014).

Based on the results of the current study, application of DNCB onto the skin of dogs caused to swell accompanied by the rise of serum specific IgE levels. Kim *et al.*, (2012), reported that acute contact dermatitis induced by DNCB and related apoptosis in epidermis as well as downregulation of pro-inflammatory cytokine (tumor necrosis factor-α and interferon-γ) immunoreactivities in the dermis of dogs were significantly inhibited by treatment of poly-γ-glutamic acid (a mucous polymer produced from the genus Bacillus strain). IgE is reported to play a key role in the pathogenesis of canine atopic dermatitis, including the functions of macrophages and eosinophils, dermal infiltration of T helper cells, imbalances of Th1 and Th2 cells (Jegal *et al.*, 2017). Excessive production of cytokines by

activated Th2 cells is also known to contribute to disease developments. Hence, the suppressions of these cytokines in the skin cells might be an attractive target for the treatment of this disease by reducing the serum IgE levels of patients (Fang *et al.*, 2015; Deo *et al.*, 2010). Hwang *et al.* (2021) demonstrated that Rosa davurica pall (a traditional Chinese plant) decreased skin inflammation by suppressing the levels of serum IgE and pro-inflammatory cytokine IL-6 in DNCB-induced atopic dermatitis mice. Ku *et al.* (2018) found that topical application of Jawoongo (a traditional herbal medicine composed of Lithospermum root and Angelica gigas Nakai) strongly suppressed DNCB-induced AD-like lesions and reduced skin thickness by downregulating serum IgE levels, CD4 levels and mast cell infiltration in sensitized skin of BALB/c mice.

In current study, all of the sites of DNCB injection were showed positive reaction (erythema and edema) after one day and became more intensive on day 2. There was a sharp demarcation line between bright red reactive area and adjacent normal skin. In Kim *et al.*, study (2012), after application with DNCB, a significant increase in the erythema index, skin hydration, transepidermal water loss and skin thickness were observed at each site tested as compared with the baseline values. For DNCB application, the maximum increase in erythema index was reached on day 2. In present study, the dermatitis was compatible with irritant or allergic contact dermatitis based on clinical findings, and skin biopsies may be needed to completely characterize the condition. The present results were inconsistent with those reported by Kimura (1994) and Krawiec & Gaafar (1975).

Upon the application of DNCB, the total numbers of WBCs and each subtype of WBCs including neutrophils, basophils, eosinophils, monocytes, and lymphocytes were increased significantly in this study which indicates the inflammatory responses of dogs to DNCB application. Although the hematological changes resulting from DNCB administration have not been studied in dogs, but there are several studies in this field in mice. For example, Yousif & Abu-Raghif (2020), Hwang *et al.* (2021) studies have evaluated the blood changes of mice to prove the anti-inflammatory effect of topical dapsone and Tacrolimus, and Rosa davurica Pall respectively. They also reported increased total WBCs, neutrophils, eosinophils and basophils counts in agreement with the current study.

In conclusion, this study demonstrated that intradermal injection of DNCB in dogs can increase serum IgE level and total WBC counts accompanied by clinical signs consistent with allergic contact dermatitis.

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Conflict of interest

The authors declare that there is no conflict of interest.

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مدل تجربی ایجاد درماتیت تماسی آلرژیک در سگ با استفاده از دی‌نیتروکلروبنزن: یافته‌های بالینی و هماتولوژیک

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چکیده

درماتیت تماسی آلرژیک یک واکنش التهابی پوستی است که در اثر تماس مستقیم با یک ماده‌ی آزاردهنده ایجاد می‌شود. هدف از انجام این مطالعه ایجاد روشی مناسب برای القای درماتیت تماسی آلرژیک در سگ‌ها، برای مطالعات آتی بود. بدین منظور، محلول ۱ و ۲ درصد دی‌نیتروکلروبنزن (DNCB) با و بدون اتانول و روغن زیتون، یا استون و روغن زیتون، به صورت زیر جلدی در ناحیه‌ی پشت ۸ سر موش BALB/c (در ۴ گروه مساوی) تزریق شد. سپس بر اساس نوع و شدت علائم، تصمیم بر این شد که سگ‌ها تنها با محلول ۲ درصد DNCB حساس شوند. در نهایت، در دو مرحله، این محلول با یا بدون دی‌متیل‌سولفوکسید (DMSO) با اتانول یا استون و روغن زیتون به صورت زیر جلدی در ناحیه‌ی قفسه‌ی سینه و کتف پنج قلاده سگ تزریق شد. تزریق محلول دو درصد DNCB باعث ایجاد یافته‌های بالینی از جمله اریتم، ادم، پوسته پوسته شدن پوست و همچنین خارش و رفتار خاراندن در موش‌ها شد. یافته‌های بالینی در سگ‌ها به صورت خفیف شامل قرمزی و تورم در عرض چند روز ایجاد شد. افزایش قابل توجهی در تعداد کل لکوسیت‌های خون، نوتروفیل‌ها، لنفوسیت‌ها، ائوزینوفیل‌ها و مونوسیت‌ها در روز ۷ مطالعه در سگ‌ها مشاهده شد. همچنین تجزیه و تحلیل آماری نشان داد که یک هفته پس از تزریق DNCB، بیان IgE به طور معنی‌داری افزایش یافت و این افزایش تا روز ۲۱ ادامه داشت. بر اساس نتایج این مطالعه، تزریق داخل جلدی ۱۰۰ میکرولیتر محلول ۲ درصد DNCB با DMSO در مخلوط ۴:۱ اتانول و روغن زیتون، روشی مناسب برای القای درماتیت تماسی در سگ‌ها بود. علاوه بر این ناحیه‌ی کتف نیز مکان مناسبی برای جلوگیری از ایجاد جراحات توسط خود حیوان بود.

کلمات کلیدی: درماتیت تماسی آلرژیک، درماتیت شبه آتوپیک، دی‌نیتروکلروبنزن، دی متیل سولفوکسید، سگ

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