Research article

An experimental model of contact dermatitis: Evaluation of the oxidative profile of Wistar rats treated with free and nanoencapsulated clobetasol

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Objective: An experimental animal model of contact dermatitis (CD) was used to investigate the effects of free and nanoencapsulated clobetasol propionate on the skin and on the oxidative profile of liver tissue.

Methods: Female Wistar rats were divided into six groups, each containing eight rats. The first group, control (C), was sensitized with solid vaseline. Group 2, (CD), was sensitized with 5% NiSO4. Groups 3 and 4 were sensitized with 5% NiSO4 and treated with free (FC) and nanoencapsulated (NC) clobetasol (0.42 mg/g), respectively, daily for 5 days. Group 5 was treated with nanoencapsulated clobetasol (0.42 mg/g) on days 1, 3, and 5 (C135) and group 6 received a hydrogel containing empty nanoparticles (NP) daily for 5 days. Thiobarbituric acid reactive substances (TBARS), carbonyl levels, non-protein sulfhydryl groups (NPSH) and catalase activity were measured in liver homogenates.

Results: A significant increase was observed in the levels of TBARS, NPSH, and catalase activity for the groups CD and NP.

Discussion: Our results suggest that both NiSO4 sensitization and NP administration induced oxidation of cellular lipids and activated the antioxidant enzyme catalase to protect from this damage. These results also indicated that daily treatment with the free and nanoencapsulated clobetasol, as well as treatment with the nanoencapsulated clobetasol every other day, were able to prevent these redox alterations and protect against histological damage.

Keywords: Clobetasol, Contact dermatitis, Nanostructured, Oxidative stress

Introduction

Contact dermatitis (CD) is a disorder that affects the skin, which is the body’s first barrier against the physical and chemical agents present in the environment. According to the physiopathological mechanisms involved, there are two types of CD: irritative and allergic.1,2 Irritative CD is caused by the pro-inflammatory and noxious effects of xenobiotics (e.g. strong acid or alkali, soaps, detergents, solvents) that are able to activate skin immunity. Allergic contact dermatitis (ACD) requires the activation of
antigen-specific acquired immunity, leading to the development of effector T cells that mediate cutaneous inflammation.\(^1\)\(^-\)\(^3\)

ACD, also known as contact hypersensitivity (CHS), is an inflammatory reaction of the skin that is mediated by T cells and results from repeated contact with non-protein chemical substances known as haptens.\(^4\) This is different from classical delayed hypersensitivity, which requires an intradermal shot of exogenous proteins.\(^1\) In CHS, the dermatitis is triggered by the topical application of haptens, such as nickel, chrome, dinitrofluorobenzene (DNFB), trinitrochlorobenzene (TNCB), and oxazoline, which in turn sensitizes the epidermis.\(^4\)\(^-\)\(^8\)

Clobetasol propionate is a synthetic glucocorticoid. It is widely used due to its non-specific anti-inflammatory and immunosuppressive effects, which leads to vasodilation, constriction, and decreased collagen synthesis.\(^9\) It is administrated topically for the treatment of inflammatory disease, such as psoriasis, discoid lupus erythematosus, serious atopic dermatitis, neurodermatitis, scalp dermatoses, and other disorders that do not respond satisfactorily to less potent steroids.\(^10\)\(^-\)\(^11\) The minimization of systemic absorption by topical application of clobetasol should decrease the incidence of side effects, such as skin thinning, cellular atrophy, the appearance of veins, adrenal suppression, growth delay in children, weight gain, and glaucoma.\(^12\)\(^-\)\(^13\)

Recently, there has been increased interest in the use of topical drug delivery systems (TDDSs), such as liposomes,\(^14\)\(^-\)\(^15\) solid lipid microparticles,\(^16\) solid lipid nanoparticles,\(^17\) and polymeric nanoparticles.\(^18\)\(^-\)\(^19\) Among these TDDSs, the polymeric nanoparticles have demonstrated many advantages, such as higher physicochemical stability compared with liposomes. This enhances the protective effect and diminishes the direct contact of the drug with the skin.\(^20\)\(^-\)\(^22\) These polymeric nanoparticles can modulate the release of the drug through the skin and its permeation into the different skin layers.\(^23\)\(^-\)\(^24\)

Furthermore, polymeric nanoparticles may allow for the prolonged release of the drug.\(^25\)\(^-\)\(^26\) This characteristic is important in those cases where a high concentration of the drug may cause irritation. Therefore, polymeric nanoparticles can supply the skin with the drug and decrease the systemic absorption.\(^27\) As result of their gradual drug release, polymeric nanocapsules have been extensively studied.\(^28\)\(^-\)\(^29\) Many researchers have administered topical medicines containing nanocapsules and found that the release of drug was reinforced by the gradual release of the drug into the skin.\(^18\)\(^-\)\(^20\)\(^,\)\(^30\)

Researchers investigated whether the drug could pass through the skin and determined that the majority of the applied drug was restricted to the stratum corneum upper layer and was therefore unable to cross the skin and reach the systemic circulation.\(^24\)\(^-\)\(^31\)

Recently, our group developed a hydrogel nanomedicine containing clobetasol-loaded nanocapsules for topical administration. Our results showed that drug release from this hydrogel was prolonged when administered in this formulation. In addition, it was possible to improve the efficacy of the treatment of CD in an in vivo rat model.\(^12\)

As previously mentioned, clobetasol propionate induces epithelial changes and can also be absorbed and carried to the bloodstream. Once the drug has been absorbed, the liver is the major organ involved in its metabolism. The aim of this study is to establish an experimental model for CD in Wistar rats by sensitizing with nickel sulfate (NiSO\(_4\)) and to determine the effect of treatment with free and nanoe encapsulated clobetasol in this established model.

**Materials and methods**

**Reagents**

Nickel sulphate (Vetec, Brazil), solid vaseline (Embacaps, Brazil), Coomassie Brilliant Blue G-250 (Sigma-Aldrich, St Louis, MO, USA), clobetasol propionate was a gift from Neo Quimica (Goiás, Brazil). All other reagents used in the experiments were of analytical grade and of highest purity.

**Animals**

Female Wistar rats (280–300 g), with a mean age of 12 weeks, were obtained from the Central Animal House of the Federal University of Santa Maria (UFSM) for use in this study. They were housed five to a cage with a natural day/night cycle at a temperature of 22–24°C. They had access to water and standard chow ad libitum. All animal procedures were approved by the Animal Ethics Committee at UFSM.

**Groups**

The animals were divided into six groups of eight rats each. Group I: control (C), sensitized only with solid vaseline. Group II: CD, induced by sensitization with 5% NiSO\(_4\) dissolved in vaseline. Group III: CD treated with free clobetasol (FC; 0.42 mg/g) daily for 5 days. Group IV: CD treated with nanoe encapsulated clobetasol (NC; 0.42 mg/g) daily for 5 days. Group V: CD treated with nanoe encapsulated clobetasol (0.42 mg/g) on days 1, 3, and 5 (C135). Group VI: CD treated daily with the hydrogel containing empty nanoparticles (NP).

**Induction of contact dermatitis and treatment with clobetasol**

First, all the animals were trichotomized in the abdominal region. The control group was sensitized in the abdomen with vaseline only. Groups CD, FC, and NC were sensitized with 4 g of solid vaseline containing 5% NiSO\(_4\). Six days after sensitization, 6 g of solid vaseline containing 5% NiSO\(_4\) was applied to each ear that had already been trichotomized. Five
applications of vaseline containing 5% NiSO₄ were performed at 72 hours intervals. The CD group was anesthetized with isoflurane and euthanized 72 hours after the last application. The FC group was treated with 6 g of free clobetasol (0.42 mg/g) and the NC group was treated with 6 g of nanoencapsulated clobetasol at the same dose, daily for 5 days. Three days after treatment, the FC and NC groups were euthanized. The C135 group was treated with 6 g of nanoencapsulated clobetasol on days 1, 3, and 5. The NP group was treated daily with hydrogel containing empty nanoparticles. Three days after treatment, the C135 and NP groups were euthanized. After the animals were euthanized, the ears and liver were dissected and histological analyses and oxidative stress assays were performed. During the application of the 5% NiSO₄, the dermal lesions were monitored daily by observation to evaluate any possible allergic reactions characteristic of CD.

**Nanoparticles**

Nanocapsules (NP) were prepared by interfacial deposition according to the preformed polymer method. First, an acetone solution containing 12.5 mg of drug, 250 mg of the polymer poly (ε-caprolactone), a mix of medium chain triglycerides (0.825 ml) and sorbitan monostearate (191.5 mg) was poured into an aqueous solution (134 ml) containing polysorbate 80 (191.5 mg). The solution was magnetically stirred for 10 minutes. Subsequently, the formulation was evaporated in a rotatory evaporator at 40°C and the final volume was adjusted to 25 ml.

**Hydrogels**

Carbopol Ultrez® (Lubrizol Corporation, Lakeland Boulevard Wickliffe, Ohio, USA) 10 NF was used as the polymer to prepare the hydrogels (0.5% acrylic acid polymer). It was dispersed in the nanoparticle suspensions containing clobetasol propionate, resulting in a drug concentration of 0.05%. The dispersion was neutralized with 0.2% triethanolamine to obtain an adequate semisolid formulation for skin application. Imidazolidinyl urea (0.6%) was added as a preservative. Following the same methodology, we also prepared hydrogels containing empty nanoparticles. The hydrogel containing free clobetasol propionate was prepared using a hydroethanolic solution of the drug (0.5 mg/ml) instead of the nanoparticle suspension. These hydrogels had physiochemical and rheological characteristics that were optimized for skin administration. The drug formulation was verified by HPLC before use in the animal model.

**Histopathological analysis of ear tissue**

To validate this exposure model, a histopathological analysis of the ear tissue was performed. Samples of ex vivo ear tissue were collected and fixed in a 10% formalin solution and then dehydrated and embedded in paraffin. This was followed by sectioning and histological staining with hematoxylin and eosin (H&E). The slides were observed in an optical microscope (×400) to check for possible changes in the ear tissue that were indicative of CD induction.

**Liver homogenization**

Liver tissue was homogenized (1:10 weight/volume) with 50 mM Tris–hydrochloride buffer pH 7.5 and centrifuged at 2000 rpm for 10 minutes. The supernatant was used in the subsequent biochemical assays.

**Thiobarbituric reactive substances**

As an index of lipid peroxidation, we used thiobarbituric acid reactive substances (TBARS) formation during an acid-heating reaction as described previously with some modifications. In brief, 200 μl of homogenized tissue supernatant (1:10 w/v) samples were mixed with 500 μl of 2.5 M acetic acid pH 3.4, 500 μl of 0.8% thiobiarbituric acid, 200 μl of 8.1% sodium dodecyl sulfate (SDS) and 100 μl of distilled water. This mixture was then heated in a boiling water bath for 120 minutes. TBARS were determined by the absorbance at 532 nm and were expressed as malondialdehyde equivalents (nmol MDA/ml).

**Carbonyl proteins**

Carbonyl proteins were measured by reaction with 2,4-dinitrophenylhydrazine (DNPH) following the method previously described. Assays were performed in duplicate for both the DNPH-treated samples and the blanks. A 1-ml aliquot of the homogenized tissue supernatant (1:10 w/v) sample containing approximately 6 mg of protein was placed in each of four tubes. The sample tubes received 200 μl of 10 mM 2,4-DNPH while the blanks received the same volume of 2 N HCl. The reaction was left in the dark at room temperature for 60 minutes. The samples were vortexed every 15 minutes and then 500 μl of 3% SDS, 2 ml of ethanol, and 2 ml of heptane were added to each tube. After vortexing, the tubes were centrifuged at 3000 rpm for 15 minutes. The pellets were washed in ethanol/ethyl acetate (1:1). Following this wash, 1 ml of 3% SDS was added to all the tubes and the absorbance was read at 370 nm. To calculate the concentration of carbonyl proteins (nmol carbonyl/mg of protein), a delta value was obtained by subtracting the blanks from the samples, dividing by 0.022 (extinction coefficient) and multiplying by the quantified proteins (mg/ml).

**Tissue non-protein thiols**

Tissue non-protein thiols were determined as described previously. In brief, an aliquot of the homogenized tissue supernatant (1:10 w/v) was diluted (1:1) with 10% trichloroacetic acid, vortexed, and centrifuged at
2000 rpm for 10 minutes. Subsequently, the supernatant was reacted with 250 μM DTNB in a final volume of 2 ml and the absorbance was read at 412 nm. A standard curve was constructed with 0.5 mM cysteine to calculate the total sulfhydryl groups in the samples.

**Catalase activity**
The homogenized tissue supernatant (1:10 w/v) was further diluted by 1:60 and used to determine the catalase (CAT) activity. In brief, enzymatic activity was determined by the method of Aebi38 by measuring the rate of catalysis of 30 mM hydrogen peroxide (H₂O₂) at 240 nm in 50 mM potassium phosphate buffer at pH 7.0.

**Protein determination**
Protein was measured by the Coomassie blue method39 using serum albumin as standard.

**Statistical analysis**
The statistical analysis was performed using one-way analysis of variance (ANOVA), followed by the Newman–Keuls multiple comparison test. P < 0.05 was considered to represent a significant difference among the analyses. All data were expressed as mean ± standard error of the mean (SEM).

**Results**

**Histopathological analysis of ear tissue**
The H&E histopathological analyses of the experimental groups are described in Fig. 1. The tissue in the control group was preserved, with no sign of lesions. The Malpighian extract presented three or four layers of cells (arrow no.1), absence of lymphocytes and four well-developed hair follicles per high power field (HPF) (Fig. 1A). Dermal edema (arrow no. 2), acanthotic Malpighian layers (up to five layers of cells – arrow no. 3) and moderate lymphocytic infiltrates (up to six lymphocytes per HPF – arrow no. 4) were observed in the CD group (Fig. 1B). The FC group had a shortening of the cornea (one or two layers of keratinocytes – arrow no. 5) and granulomatous layers, a decrease of lymphocytic infiltrates (up to three lymphocytes per HPF), reduction in the number and thickness of hair follicles (two per HPF) and Malpighian layer atrophy (Fig. 1C). Both the NC and C135 groups showed Malpighian layer atrophy; however, in a lesser degree (two to three layers of keratinocytes), an increased number of hair follicles (three per HPF), and muscular layer with increased thickness when compared with group FC (Fig. 1D and E). The empty nanoparticles (NP) group showed normal epidermal thickness (three or four layers of cells), absence of dermal edema, and usual number and thickness of hair follicles (four per HPF) (Fig. 1F).

**TBARS levels**
TBARS content was measured as an index of lipid peroxidation in liver homogenates. One-way ANOVA showed a statistically significant difference among the groups [F(5, 42) = 7.62, P < 0.001] (Fig. 2). Subsequently, post hoc analysis showed an increased lipid peroxidation in the liver of groups CD and NP compared with C, FC, NC, and C135 (P < 0.001).

**Carbonyl levels**
Carbonyl groups were measured as an index of protein oxidation products in liver homogenates. Statistical analysis did not reveal any difference in the protein oxidation products in the liver tissue among the groups [F (5, 42) = 0.14, P > 0.05] (Fig. 3).

**Non-protein sulfhydryl group levels**
Non-protein sulfhydryl group (NPSH) is represented predominantly by reduced glutathione (GSH) and serve as an index of non-enzymatic antioxidant defense. One-way ANOVA showed a statistically significant difference among the groups [F (5, 42) = 5.57, P < 0.001] (Fig. 4). Post hoc analysis demonstrated an increased level of NPSH in the liver tissue of group CD compared with all the groups (P < 0.001).

**Catalase activity**
CAT belongs to the enzymatic antioxidant defense system and is one of the most outstanding endogenous antioxidants. One-way ANOVA indicated significant difference among the groups [F (5, 42) = 17.32, P < 0.001]. Subsequently, post hoc analysis showed an increased CAT activity in the liver of groups CD and NP compared with C, FC, NC and C135 (P < 0.001) (Fig. 5).

**Discussion**
In this study, we induced CD in Wistar rats by sensitization with nickel sulfate (NiSO₄) and then investigated the effects of free and nanoencapsulated clobetasol propionate on histological and oxidative stress parameters in the liver.

The stratum corneum is the outermost layer of the skin and is an obstacle to the absorption of many drugs used for dermatological therapy. To overcome this obstacle, nanostructured systems have been developed that can act over a large surface area and provide homogeneous release of drug into the skin surface.18 In addition, topical application of these systems favors the retention of nanoparticles on both the skin40 and the hair follicle. Studies have shown that the hair follicle plays an important role in the penetration of substances through the skin when the substances are associated with nanostructures.41

First, to verify this experimental model of CD in rats, we proceeded with a histopathological analysis.
of ear specimens from control and CD groups to confirm the diagnosis of a skin inflammatory reaction. In the control group, no pathological alterations were detected in the tissue, while in the sensitized group, pathological alterations were observed. This verified the efficacy of this experimental model. We also observed, via histology, that treatment with both free (FC) and nanoencapsulated drug (NC, C135) reversed the damage caused by NiSO₄. It is important to note that the NC group showed more promising results than the FC group. This was most likely due to the gradual release of the drug. Furthermore, the NP group presented results similar to the CD group, which may be an inherent property of the nanoparticles because CD was also induced in this group.

Clobetasol propionate is a synthetic topical glucocorticoid; however, it may have several adverse side effects when used for an extended period of time. It can also be absorbed through the skin into the circulatory system. After this experimental procedure was proven to be effective in inducing CD, several oxidative stress parameters were evaluated in the liver tissue. These parameters include measuring the levels of malondialdehyde (MDA), carbonyl and NPSH content and the antioxidant activity of the enzymes superoxide dismutase and catalase.

Levels of MDA are positively related to lipid peroxidation and it is a commonly used biomarker. Sensitization with NiSO₄ resulted in damage to the
Liver tissue as indicated by an increased level of MDA. This also resulted in an increase in NPSH and in the antioxidant activity of catalase. These results indicate that the NiSO₄ effects were not only restricted to the ear tissue but also affected the liver tissue, as indicated by alterations in several of the oxidative stress parameters. Increased MDA levels are known to be related to plasma membrane instability in the face of reactive oxygen (ROS) and reactive nitrogen species generation. The increased catalase activity is in agreement with the lipid peroxidation data. This increase in catalase activity may be an adaptive mechanism to scavenge the ROS.

As many authors have described, there are two distinct phases in cutaneous hypersensitivity. First, in the sensitization phase, there is an increase in the number of T cells in the lymph nodes. Second, in the induction phase, chemokines are synthesized, endothelial cells and mast cells are activated, and there is an infiltration of polymorphonuclear cells. The T cells interact with cutaneous antigen-presenting cells. Therefore, activated CD8⁺ cytotoxic T cells produce type 1 cytokines, such as IFN-γ and chemokines that induce the activation of cutaneous cells. They also induce the apoptosis of keratinocytes and allow for the recruitment of a cellular infiltrate that is characteristic of cutaneous hypersensitivity.

In this study, an increase in oxidative damage markers in livers from the CD group was observed. These animals had a partial recovery when they were treated with free and nanoencapsulated clobetasol.
According to these results, it is hypothesized that cutaneous lesions may be related to the redox imbalance observed in the hepatic tissue and may possibly be mediated by the immune response. Furthermore, the administration of clobetasol propionate, both in the free and nanostructured formulations, was protective as observed by histopathology and oxidative stress parameters. This indicates that this drug has both topical and systemic effects.

**Conclusion**

In conclusion, our findings reveal that the sensitization of rats with NiSO₄ was able to break the redox balance in the liver tissue and cause tissue damage. We also showed that treatment with both free and nanoencapsulated clobetasol propionate formulations was effective in preventing this damage, as measured by oxidative stress and histological observation.

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**References**

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