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% $NiSO_4$. Groups 3 and 4 were sensitized with 5% $NiSO_4$ 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 $NiSO_4$ 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

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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 its non-specific anti-inflammatory and immunosuppressive effects, which leads to vasoconstriction 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.[32]

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 nanoencapsulated 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 Química (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 nanoencapsulated clobetasol (NC; 0.42 mg/g) daily for 5 days. Group V: CD treated with nanoencapsulated 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% $NiSO_4$ 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% $NiSO_4$, 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.[26,33] 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.[32] These hydrogels had physicochemical and rheological characteristics that were optimized for skin administration. The drug formulation was verified by HPLC before use in the animal model.[32]

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).[34] 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[35] 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% thiobarituric 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.[36] 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.[37] 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 Aebi[38] by measuring the rate of catalysis of 30 mM hydrogen peroxide (H_2O_2) at 240 nm in 50 mM potassium phosphate buffer at pH 7.0.

Protein determination

Protein was measured by the Coomassie blue method[39] 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 ($NiSO_4$) 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 skin[40] 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

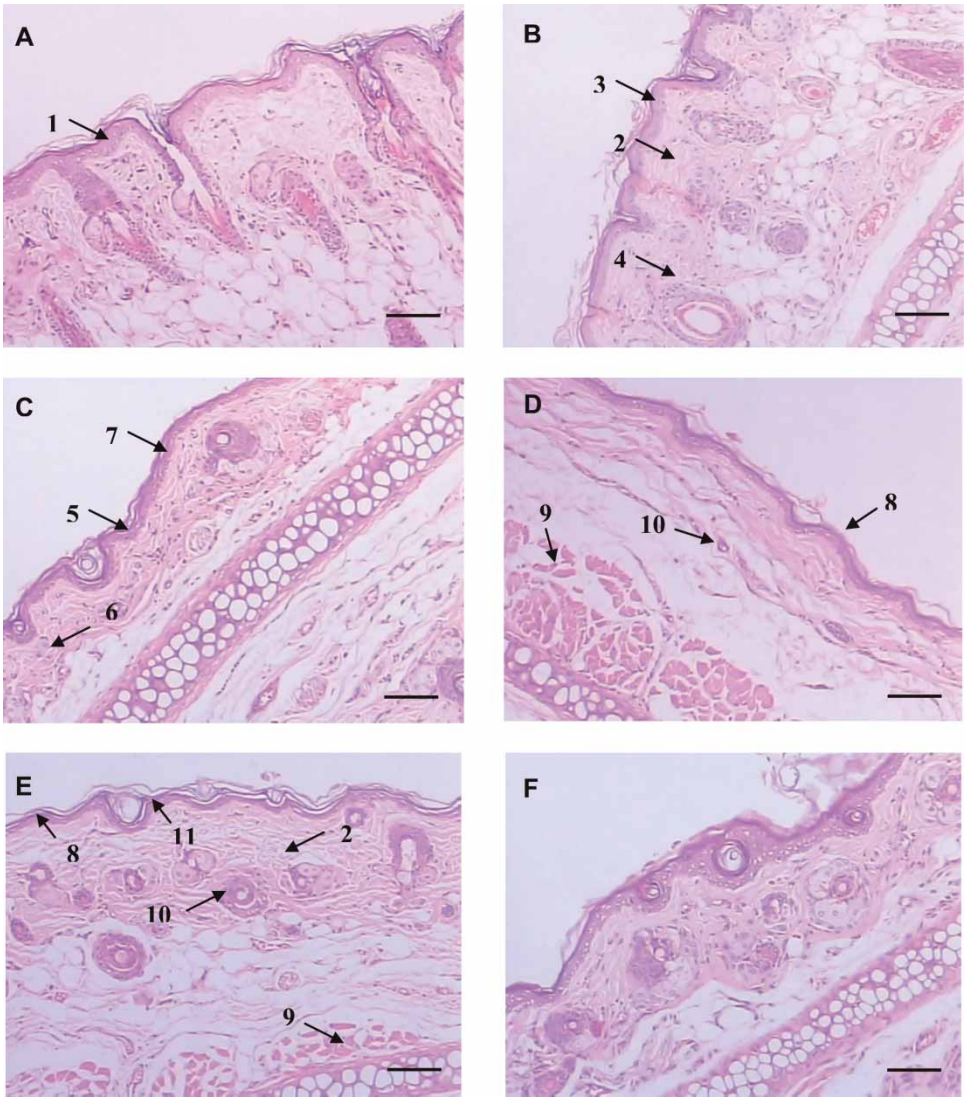

Figure 1 Histopathological analysis of ear tissue, H&E. Scale bars: 100 μm. (A) Control: normal histology of rat ear tissue with the absence of inflammatory infiltrate and edema. Basal layer and epidermal cell maturation preserved (1). (B) $NiSO_4$ sensitized; contact dermatitis characteristics such as dermal edema (2), acanthotic Malpighian layer (3) and moderate lymphocytic infiltrate (4). (C) $NiSO_4$ sensitized and treatment with free clobetasol; shortening of the cornea and thickness of the granulomatous layer (5), decrease of lymphocytic infiltrate (6), and Malpighian layer atrophy (7). (D) $NiSO_4$ sensitized and daily treatment with nanostructured clobetasol; Malpighian layer (8), muscular (9) and fur atrophy (10). (E) $NiSO_4$ sensitized and alternately treated with clobetasol (days 1, 3, and 5); Malpighian layer (8), muscular (9) and fur atrophy (10), desquamation of the cornea layer (11) and edema (2). (F) Nanoparticles treatment: absence of atrophy.

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 $NiSO_4$. 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.[42]

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.[43,44] Sensitization with $NiSO_4$ resulted in damage to the

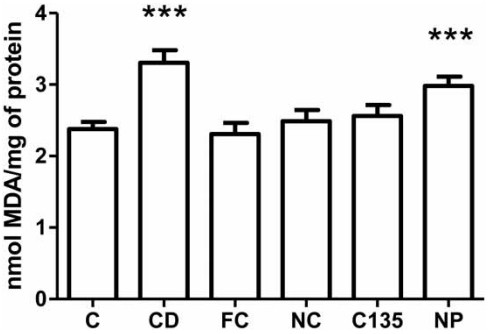

Figure 2 TBARS levels in livers from rats with $NiSO_4$-induced contact dermatitis treated with free and nanostructured clobetasol. C, control group; CD, contact dermatitis; FC, CD treated with free clobetasol; NC, CD treated with nanostructured clobetasol; C135, CD treated with nanostructured clobetasol on days 1, 3, and 5; NP, empty nanoparticles. Bars represent mean ± SEM for eight animals in each group. ANOVA Newman–Keuls multiple comparison test. ***$P < 0.001$ compared with C, FC, NC, and C135.

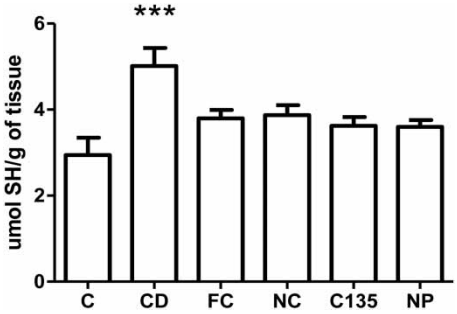

Figure 4 Non-protein sulfhydryl group levels in livers from rats with $NiSO_4$-induced contact treated with free and nanostructured clobetasol. C, control group; CD, contact dermatitis; FC, CD treated with free clobetasol; NC, CD treated with nanostructured clobetasol; C135, CD treated with nanostructured clobetasol on days 1, 3, and 5; NP, empty nanoparticles. Bars represent mean ± SEM for eight animals in each group. ANOVA Newman—Keuls multiple comparison test. ***$P < 0.001$ compared with all the groups.

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 $NiSO_4$ 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.[3,45] Second, in the induction phase, chemokines are synthesized, endothelial cells and mast cells are activated, and there is an infiltration of polymorphonuclear cells.[46] 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.[47,48] A number of skin diseases are thought to be associated with oxidative stress, including psoriasis, cutaneous vasculitis, and CD, both during the early pre-immunological phase following exposure to contact allergens that readily auto-oxidize and also during the later stages of inflammatory cell infiltration.[49–51]

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.

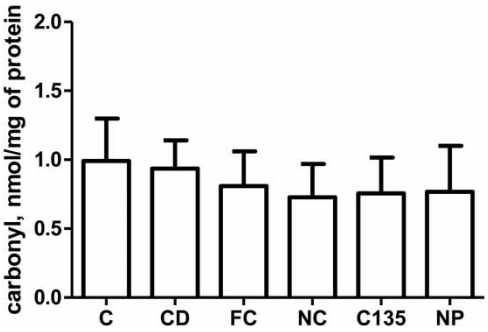

Figure 3 Carbonyl levels in livers from rats with $NiSO_4$-induced contact dermatitis treated with free and nanostructured clobetasol. C, control group; CD, contact dermatitis; FC, CD treated with free clobetasol; NC, CD treated with nanostructured clobetasol; C135, CD treated with nanostructured clobetasol on days 1, 3, and 5; NP, empty nanoparticles. Bars represent mean ± SEM for eight animals in each group. ANOVA Newman–Keuls multiple comparison test.

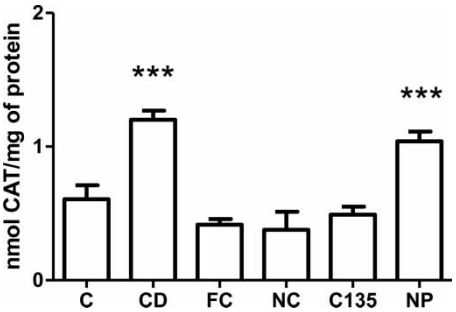

Figure 5 Catalase activity in livers from rats with $NiSO_4$-induced contact dermatitis treated with free and nanostructured clobetasol. C, control group; CD, contact dermatitis; FC, CD treated with free clobetasol; NC, CD treated with nanostructured clobetasol; C135, CD treated with nanostructured clobetasol on days 1, 3, and 5; NP, empty nanoparticles. Bars represent mean ± SEM for eight animals in each group. ANOVA Newman–Keuls multiple comparison test. ***$P < 0.001$ compared with C, FC, NC, and C135.

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 $NiSO_4$ 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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