

EFFECTS OF ELEPHANT GARLIC VOLATILE OIL (*ALLIUM AMPELOPRASUM*) AND T-2 TOXIN ON MURINE SKIN

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Abstract. Effects of elephant garlic (*Allium ampeloprasum*) volatile oil (GVO) and trichothecene (T-2) toxin were studied in Swiss albino mice. The animals were 1) topically applied GVO, 2) topically applied T-2 toxin, 3) topically applied GVO followed by T-2 toxin (GVO/T-2), and 4) T-2 toxin application followed by GVO (T-2/GVO) on the right footpad. All animals were observed by Langerhans cell enumeration and pathological changes of the footpad on days 1, 3, 5 and 7. The number of Langerhans cells in the GVO treated group ($1,097 \pm 33/\text{mm}^2$ to $1,624 \pm 19/\text{mm}^2$) was not significantly different when compared with the corresponding control left footpad ($1,143 \pm 33/\text{mm}^2$ to $1,674 \pm 21/\text{mm}^2$). Langerhans cells density in T-2 toxin treated group ($629 \pm 29/\text{mm}^2$ to $1,090 \pm 31/\text{mm}^2$) was reduced by 20-35% of the opposite control footpad ($962 \pm 40/\text{mm}^2$ to $1,392 \pm 29/\text{mm}^2$). Furthermore, GVO/T-2 and T-2/GVO treated mice showed a decrease in Langerhans cell number than a single T-2 toxin treated group. While Langerhans cells in T-2 toxin, GVO/T-2 and T-2/GVO groups revealed a smaller cell size with shortening dendritic processes when compare to the normal control group. Histopathological findings of the footpad skin in T-2 toxin treated group revealed epidermal desquamation and necrosis with edema and inflammatory cells infiltration. While GVO/T-2 and T-2/GVO showed a similar sequence but a lesser severe degree. These findings suggested that GVO both in pre-and post-treatment could protect T-2 toxin induced epidermal damage in a mouse footpad.

INTRODUCTION

Garlic (*Allium sativum* Linn.) is a common plant used as a spice and folk remedy in many parts of the world. Its origin is most likely in central Asia and spread rapidly throughout many parts of the world. For thousands of years they have been a part of folk medicine. The Codex Ebers, an Egyptian medical papyrus dating to about 1550 BC, gives more than 800 therapeutic formulas of garlic as an effective remedy for a variety of ailments (Block, 1985). Garlic has been shown to display antibacterial, antifungal, hypoglycemia, hypolipidemic, anti-atherosclerotic, fibrinolytic, detoxification and carcinogenic effects (Fenwick and Hanley, 1985).

The elephant garlic (*Allium ampeloprasum* Linn.) or 'Kratiam-tone' in Thai is believed to have anti-hepatotoxic activities and its steroidal saponin antifungal activities (Morita *et al*, 1988). At the present time, garlic in various preparations is regarded by the US Food and Drug Administration (FDA) as a natural and common food substance in the US food supply. Currently, there are no data regarding possible garlic toxicity from the National Toxicology Program, the US Government Office that is responsible for federal evaluation of chronic toxicity of chemicals (Dausch and Nixon, 1990).

Trichothecene (T-2) toxin is one of the most toxic of the 12,13-epoxytrichothecene mycotoxins produced by the *Fusarium* species. T-2 toxin has been implicated in alimentary toxic aleukia (ATA) in Russia, a disease which has killed thousands of people (Ueno, 1977). It is a potent inhibitor of protein and DNA synthesis (Rosenstein and Lafarge-Frayssinet, 1983) and suppressed

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antibody synthesis against sheep red blood cells (SRBC) observed by a delayed footpad swelling response in the classic delayed type hypersensitivity reaction (Masuko *et al*, 1977). The mechanism of action of T-2 toxin in inhibiting protein synthesis may be result from defective epidermal antigen presentation.

Langerhans cells play a critical role in cutaneous immunological reactions (Weiss, 1988). The epidermal Langerhans cell is a potent antigen-presenting cell for eliciting the success of intradermal immunization and carries out the immunological activity *in vivo* and *in vitro*. (Taweechaisupapong *et al*, 1996). The mobilization of the Langerhans cell to the draining lymph node is important to initiate the immune response to an epidermally acquired virus infection (Johnston *et al*, 2000). Langerhans cells are also the targets of dengue virus infection (Marovich *et al*, 2001). It implicated for impaired skin immunologic functions in animals or humans exposed epidermally to T-2 toxin.

The present study deals with the effect of elephant garlic volatile oil (GVO) and T-2 toxin on mice foot pad skin, which is richly supplied with epidermal Langerhans cells. Topical application of GVO after T-2 toxin should have to beneficial effects on mice footpads both in the dermatological and epidermal Langerhans cell populations.

MATERIALS AND METHODS

Animals

Eighty outbred males Swiss albino mice obtained from the National Laboratory Animal Center, Mahidol University at Salaya, Nakhon Pathom, Thailand, were used in our experiment. The animals were about 8 weeks old. They were acclimatized to our animals facilities for 7 days before treatment. The animals were housed in stainless steel cages in an air conditioned room with average temperature of $25 \pm 2^\circ\text{C}$ and supplied freely with standard mouse pellet (KMP Feedmills PTE Ltd, Jurong, Singapore) and tap water *ad libitum* and cared for according to the guidelines of the National Laboratory Animal Center (NLAC) as previously reported (Angsubhakorn *et al*, 2002).

Chemicals

Elephant garlic volatile oil (GVO) was prepared and extracted by steam distillation with water cohobation of the material in a Dean and Stark apparatus modified to allow the aqueous phase to flow back to the distilling flask to give a lower phase return of water. The yellowish volatile oil which was heavier than distilled water was collected and stored in a brown glass container at (-72°C) temperature. T-2 toxin was purchased from Makor Chemicals Ltd, Jerusalem, Israel. Ethyl acetate (AR-grade) was purchased from JT Baker Chemical Co, Phillipsburg, NJ, USA.

Experimental design

Eighty animals were randomly divided into 4 groups as follows: 1) GVO treated group, 2) T-2 toxin treated group, 3) GVO pretreated followed by T-2 toxin (GVO/T-2 toxin), and 4) T-2 toxin treated group followed by GVO (T-2 toxin/GVO). Five animals in each group was assessed 1, 3, 5 and 7 days after initial application.

In the GVO treated group, all mice had 50 μl of GVO applied topically via a cotton bud to the footpad. In the T-2 toxin treated group, one mg of T-2 toxin was dissolved in 1 ml of ethyl acetate to make a 1 mg/ml solution. All mice had 10 μl of T-2 toxin in ethyl acetate (10 $\mu\text{l}/\text{fp}$) applied topically to the right footpad (fp) and 10 μl of ethyl acetate to the left footpad (control) by using a microsyringe (Robbins Scientific, Sunnyvale, CA, USA). In the GVO pretreated group followed by T-2 toxin, all mice had 50 ml of GVO applied topically via a cotton bud to the right foot for 15 minutes followed by T-2 toxin (10 $\mu\text{l}/\text{fp}$). Ethyl acetate was applied as control to the left footpad. In the T-2 toxin treated group followed by GVO, all mice had T-2 at a dose of 10 $\mu\text{l}/\text{fp}$ applied topically to the right foodpad for 15 minutes followed by 50 μl of GVO. The left footpad had 10 μl of ethyl acetate applied topically alone as a control footpad.

Preparation of skin samples for Langerhans cells enumeration

Epidermal sheets from the footpads of the mice were prepared as follows: the skin was excised from the footpads at the end of the experiment and then separated into two parts. The upper part was fixed in 10% buffered neutral for-

malin for hematoxylin and eosin staining. The later part was incubated at 37°C in PBS-EDTA buffered for 2 hours according to Scaletta and MacCallum (1972). Whole epithelial sheets were carefully separated from the dermis with the aid of a fine forceps. The epidermal sheets obtained were fixed and incubated by using a method similar to that of Wolff and Winkelmann (1967a,b) to demonstrate the ATPase activities of Langerhans cells.

ATPase staining of epidermal Langerhans cells

The epidermal sheets were washed in 3 changes of cold 0.2 M trisimal buffer (with 6.84% sucrose) at pH 7.3 for a total of 20 minutes; fixed in cold (4°C) cacodylate buffered formaldehyde for 20 minutes; re-washed in 3 changes of cold trisimal buffer for 30 minutes; incubated at 37°C for 30 minutes in a substrate containing 10 mg ATP (Sigma-Aldrich, St Louis, USA), 5 ml 5% MgSO₄, 3 ml 2% PbNO₃ in 42 ml trisimal buffer (with 8.55% sucrose) at pH 7.3; washed in buffer and treated with dilute ammonium sulphide, the developing solution, for 5 minutes. The epidermal sheets were washed again for 3 changes in distilled water. The washed epidermal sheets were mounted in buffered glycerine (9:1), with the dermal side up, for light microscopy.

Histopathological staining

The upper part of the footpads was cut, trimmed and processed in a Histomatic™ Tissue Processor model 166MP (Fisher Scientific, Co, Pittsburgh, Pennsylvania, USA) and then stained

with hematoxylin and eosin via the standard technique.

Langerhans cells enumeration

The epidermal specimens were examined with the aid of a calibrated optical grid fitted into the eye piece at a magnification of 400x. One outlined field corresponded to an area of 0.0156 mm². The dendritic positive cells were randomly chosen and counted in 20 consecutive fields and expressed as the number of cells per mm² of surface area.

Statistical analysis

For statistic analysis of the differences between the right hind footpad and left hind footpad, the unpaired two tailed Student's *t*-test was used and a p-value of <0.05 was regarded as significant.

RESULTS

The Langerhans cells density (LC/mm²) of all the experimental groups is shown in Table 1. There was no significant difference in Langerhans cells density in GVO, on the right footpad compared to the left footpad. The Langerhans cells density was increased at the highest level at day 3 on the right footpad (1,624), and on the left footpad (1,674). Langerhans cells in this group showed normal morphology characterized by numerous, fine, ramified, and darkening by ATPase, staining both on the left footpad and the right footpad (Fig 1A).

Langerhans cells were not only significantly

Table 1
Number of Langerhans cells after topical application of GVO, T-2 toxin, GVO/T-2 toxin and T-2 toxin/GVO on mice footpad.

Experimental groups	Number of Langerhans cells in each interval							
	Day 1		Day 3		Day 5		Day 7	
	Rt	Lt	Rt	Lt	Rt	Lt	Rt	Lt
GVO	1,351	1,374	1,624	1,674	1,500	1,533	1,097	1,143
T-2	629 ^a	962	861 ^a	1,220	1,010 ^a	1,254	1,090 ^a	1,392
GVO/T-2	1,203 ^a	1,498	1,033 ^a	1,142	1,047 ^a	1,508	1,121 ^a	1,388
T-2/GVO	1,123 ^a	1,436	992 ^a	1,156	1,088 ^a	1,205	1,077 ^a	1,325

^aSignificant difference from the corresponding left footpad (p<0.05).

decreased in density after the T-2 toxin application, but also changed in their morphology. They showed a decrease in size with shortened and rounded dendritic processes (Fig1B). There was a significant decrease in the Langerhans cells density on the right footpad (629-1,090) compared to the left footpad (962-1,392) on days 1, 3, 5 and 7 ($p<0.05$). Langerhans cells density was markedly decreased on day 1 (629), and gradually recovered on days 3 through 7.

In GVO/T-2 toxin, there was a significant decrease in Langerhans cells density on the right footpad (1,033-1,203) compared to the left footpad (1,142-1,508) on days 1,3,5 and 7 ($p<0.05$) after initial application. Langerhans cells density was markedly decreased on day 3 (1,033) and gradually recovered on day 5. Langerhans cells showed a decrease in size, with shortened,

rounded dendritic processes (Fig1C).

In T-2 /GVO, Langerhans cells on the right footpad were significantly decrease in density compared to GVO alone. They showed a decrease in size and shorten, rounded dendritic processes (Fig1D). There was a significant decrease in Langerhans cells density on the right footpad (992-1,123) compared to the left footpad (1,156-1,436). Langerhans cells on the right footpad were markedly decreased on day 3 and gradually increased on days 5 and 7. It revealed that the Langerhans cells density on the right footpad on days 1,3 and 5 were $GVO>GVO/T-2>T-2/GVO>T-2$ toxin, which gradually recovered at day 7.

The histopathological findings of the footpads of the control mice showed normal epidermal appearance (Fig 2A). The GVO treated footpad did not show an affect on the normal archi-

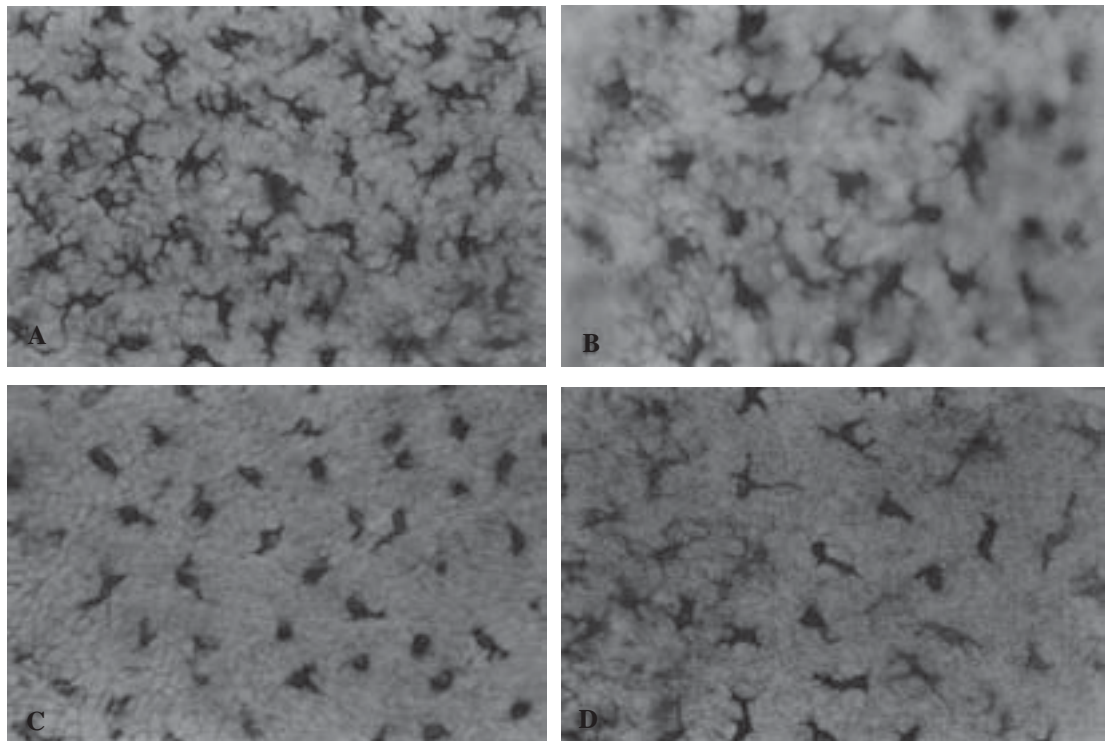


Fig 1—Photomicrographs of mouse skin with ATPase stained : (A) Langerhans cell in a normal epidermal sheet showing numerous fine dendrites and ramified. (B) Epithelial sheet treated with T-2 toxin on day 7 showing marked decreased in Langerhans cells density and cell size with rounded dendrites. (C) Epidermal sheet of mouse treated with GVO/T-2 toxin on day 7 showing numerous Langerhans cells with moderately fine dendrites. (D) Epidermal sheet of mouse treated with T-2 /GVO on day 7, also showing marked decreased in Langerhans cell density (x400).

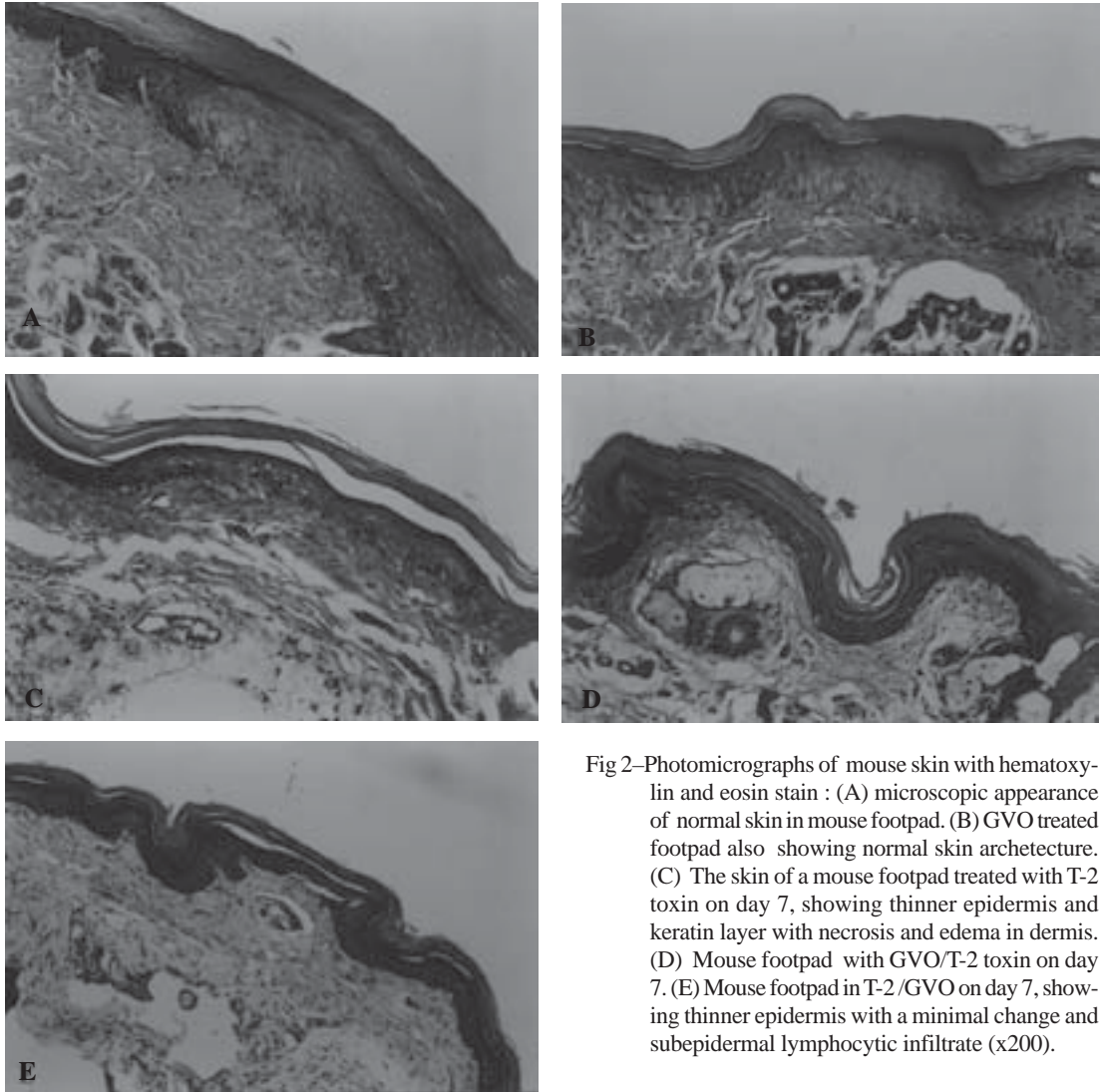


Fig 2—Photomicrographs of mouse skin with hematoxylin and eosin stain : (A) microscopic appearance of normal skin in mouse footpad. (B) GVO treated footpad also showing normal skin architecture. (C) The skin of a mouse footpad treated with T-2 toxin on day 7, showing thinner epidermis and keratin layer with necrosis and edema in dermis. (D) Mouse footpad with GVO/T-2 toxin on day 7. (E) Mouse footpad in T-2/GVO on day 7, showing thinner epidermis with a minimal change and subepidermal lymphocytic infiltrate (x200).

texture of the mice skin and did not show any irritant property on the skin; there were no inflammatory infiltrating cells present (Fig 2B). The T-2 toxin treated footpad showed subsequent redness and swelling with flat hyperemic plaques on day 3 after topical application. The plaques become covered with a dry friable exudate. Scaly superficial flakes were frequently present on the application sites on days 5 and 7. The histopathological appearance showed disorganised skin architecture and desquamation of the keratinized epithelium on days 1 through 7. The treated footpad revealed early cellular necrosis with a pyknotic nucleus including karrhyorrhexis of the

epidermis. Hyperplasia of the epidermis was evident on day 3. The affected tissue became edematous and contained an inflammatory cell infiltrate. Focal areas of coagulative necrosis and hemorrhage were evident in the more severe reaction (Fig 2C). In the GVO/T-2 and T-2/GVO treated groups there was less severity than in the T-2 toxin treated group (Fig 2D and 2E).

DISCUSSION

Garlic volatile oil has many constituents which may have different activities (Vernin *et al*, 1986; Lawson *et al*, 1991). In this study, GVO,

T-2 toxin, GVO/T-2 toxin, T-2 toxin/GVO were used as topical application on the right footpad of Swiss albino mice. The results showed that GVO could protect the intensive dermatological changes and epidermal Langerhans cell density and morphological changes induced by the T-2 toxin. Langerhans cells play an important role in the cutaneous immunological response while common garlic is found to act as an immune system modulator and biological response modifier. It was found that the phagocytic activity of macrophages were enhanced when garlic was administered to rats (Belman, 1983). Saponin, found in common garlic with the aged garlic extract, enhanced cytotoxicity and proliferation of human peripheral blood lymphocytes (Morioka *et al*, 1993). They suggested that the active substances from garlic extract may have an efficient immunopotentiator and could be used for immunotherapy. GVO have many allyl sulfides (Vernin *et al*, 1986) of which the major compound is diallyl sulfide (DAS). Allicin, a component of garlic can inhibit some sulphhydryl enzymes *ie* succinic dehydrogenase, xanthine oxidase, hexokinase but not adenosine triphosphatase (Wills, 1956). The garlic extract from common garlic (*Allium sativum*) stimulated the activity of liver adenosine triphosphatase in intact mitochondria but had no effect on this enzyme after disruption of mitochondria (Bogin and Abrams, 1976). It is possible that these components facilitate the transport of ATP into the mitochondria where ATPase is located.

The T-2 toxin treated group had a reduction in the epidermal Langerhans cell density and was associated with morphological changes in skin as evidence by epidermal cell damage. The maximum reduction in Langerhans cells density resulting from topical application of T-2 toxin to the skin was 20-35% when compared to the opposite non-treated left footpad. Carcinogens, such as DMBA (Muller *et al*, 1985), can reduce the Langerhans cells density by 50% within 7 days following initial treatments. The tumor promoter, *ie* croton oil, 12-O-tetradecanoylphorbol-13-acetate (TPA) and teleocidine can also decrease the epidermal Langerhans cells (Halliday *et al*, 1987). UVB radiation has been shown to reduce Langerhans cells density at least 50% on ATPase

staining (Bergstresser *et al*, 1980). Topically applied immunosuppressants *ie* cyclosporin, prednisolone, have been shown to deplete Langerhans cells by 30-50% (Halliday *et al*, 1986).

Thus the T-2 toxin produced a moderate reduction in epidermal Langerhans cells density, comparable to immunosuppressants but less than carcinogens or UV light. In the T-2 toxin treatment, the decreased Langerhans cells gradually recovered until day 7. It was found that Langerhans cells can recover to normal numbers in about 60 days after treatment with a high dose of UV radiation (Bergstresser *et al*, 1980), DMBA or croton oil application (Muller *et al*, 1985). It has been suggested that skin carcinogens and high dose UV radiation induce migration of Langerhans cells from the epidermis and cause a relatively slow repopulation. T-2 toxin is a potent inhibitor of protein synthesis (Rosenstein and Lafarge-Frayssinet, 1983), suppresses antibody synthesis against RBC and enhances delayed type hypersensitivity (Masuko *et al*, 1977), reduces the level of both MHC class II (Ia) on Langerhans cells and inhibits protein synthesis in epidermal cells culture induced by cyclohexamide (Blaylock *et al*, 1993).

We propose that one mechanism of action for T-2 toxin in reducing the contact dermatitis response is via inhibition of protein synthesis and effective antigen presentation by epidermal Langerhans cells. This may be associated with decreased class II antigen expression. The results show impaired skin immunologic functioning in T-2 toxin exposed skin. Langerhans cells play an important role in the immune system of skin (Weiss, 1988). In T-2 toxin, GVO/T-2 and T-2/GVO treated, the Langerhans cells density gradually recovered on days 5 through 7 similar to the DMBA treated group in the previous report (Muller *et al*, 1985). Langerhans cells reappear in the epidermis during recovery and may be repopulation either from bone marrow precursors or from mitosis of residual Langerhans cells. Morphological alteration in the skin due to T-2 toxin, GVO/T-2 and T-2/GVO correlated well with the reduction of the Langerhans density. The histopathological observation revealed a non-specific acute inflammatory reaction characterized by hemorrhage, edema, and an inflammatory cell infiltrate with variable degree of necrosis of the epidermis.

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