The Effect Of Topical Dapsone In Comparison With Tacrolimus On Dnbc Induced Atopic Dermatitis In Mice

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ABSTRACT
To assess the effect of topical dapsone in the treatment of atopic dermatitis in comparison with tacrolimus. A case-control study was done on 80 male Albino mice (20-30g) recruited from local markets, were treated with 1-Chloro-2,4-dinitrobenzene (DNCB)-induced atopic dermatitis and compared with age and the sex-matched control group comprised of 20 healthy male Albino mice. The topical applications of Tacrolimus 0.1% ointment and Dapsone 5% gel were performed on atopic dermatitis area for seven days and fourteen days once daily starting from the fourth day of induction and Complete blood count, IL-4 and IL-13 were measured in skin tissue homogenate in addition to histopathological evaluation of atopic dermatitis skin lesion and assessment of observational severity score and compared with those of controls. The results revealed that the levels of IL-4 and IL-13, total WBC, observational severity score, and the score of all histopathological markers were increased significantly in the group of mice treated with 1-Chloro-2,4-dinitrobenzene (DNCB) for A.D. induction in comparison with controls. These markers were reduced significantly after the topical application of tacrolimus. They showed a comparable reduction but after a longer duration of topical treatment with dapsone after two weeks of treatment. Topical application of dapsone on induced A.D. mice showed a significant reduction in the levels of IL-4 and IL-13, total WBC, observational severity score, and histopathological markers score and these effects were compared to those of topical tacrolimus.

INTRODUCTION
Atopic dermatitis (A.D.) is a common, familial, chronic inflammatory skin disease characterized by intense pruritus, erythematous lesions, scaly skin lesions, and xerosis. The pathophysiology of atopic dermatitis based on skin barrier dysfunction combined with complex interactions of genetic, immunologic, psychologic, and environmental factors (Lyons et al., 2015). Atopic dermatitis is count as a primarily T cell-driven disorder in which infiltrating T cells, especially T helper 2 cells, are essential. Cytokines related to T helper 2, IL-4, and IL-13 are the significant parameters in this disease. Topical corticosteroids, calcineurin inhibitors (TCIs); tacrolimus and pimecrolimus are the mainstay of the treatment for moderate to severe atopic dermatitis, both in children and adults (Fuxench, 2017).

Tacrolimus ointment is an immunosuppressive medication which considered as newer formulation used for the treatment of acute flares and maintenance therapy of atopic dermatitis, by binding to the cytosolic immunophilin receptor (macrophilin-12) to form a complex that inhibits the activity of ...
of the enzyme calcineurin it will block T-cells activation (Gutfreund et al., 2013). Dapsone (4,4₀-
 diamino-diphenyl sulfone) is an aniline derivative belonging to the group of synthetic sulfones with
 microbial activity. The use of dapsone to treat non-pathogen- caused diseases revealed alternate
 anti-inflammatory mechanisms that initially were elucidated by inflammatory animal models. Thus,
 dapsone has dual functions of both: antimicrobial/antiprotozoal effects and anti-inflammatory
 features (Wozel and Blasum, 2014).

This study was aimed to investigate the effect of topi-
cal dapsone in treatment of induced atopic derma-
titis in mice through measure the levels of IL-4, IL-
13 in skin tissue homogenate, CBC, histopathologi-
cal markers, and assessment of observational sever-
ity score, and compare them with those topically
 treated with tacrolimus and non-treated induced
 atopic dermatitis group.

MATERIALS AND METHODS

Study design
A case-control study was done on 80 male Albino
 mice (20-30g) recruited from local markets, were
 treated with 1-Chloro-2,4-dinitrobenzene (DCNB)-
 induced atopic dermatitis (Hamad et al., 2017) and
 compared with age and the sex-matched control
group comprised of 20 healthy male Albino mice.
The study was started in June and finished in Decem-
ber 2019. The experimental animals were per-
formed in compliance with the principles guide of
 ethical in laboratory animals approved by the Uni-
versity of Al-Naharain. The practical part of the
 study was conducted at Department of pharma-
cology, College of Medicine, Al-Nahrain University,
Baghdad- Iraq. Eighty-four animals have continued
the study successfully. Sixteen animals died dur-
during the study because of environmental condi-
tions and induction. Albino mice were sub-categor-
ized into three groups; Group I represent 20 induced
 atopic dermatitis mice without treatment, Group II
represent 20 induced atopic dermatitis mice treated
with tacrolimus 0.1% ointment topically once daily
at 9:00 AM for 7 and 14 days. Group III represent
20 induced atopic dermatitis mice treated with Dap-
sone 5% gel topically once daily at 9:00 AM for 7 and
14 days.

Treatment protocols
The topical applications of treatments (Tacrolimus
0.1% ointment and Dapsone 5% gel) were applied
on atopic dermatitis area of animal for 7 days and
14 days once daily at 9 AM morning starting from
the fourth day of induction.

The following listed parameters are used to com-
pare in results between experimental groups on day
7 and 14 of the treatment
1. Complete blood count.
2. IL-4 and IL-13 were measured in skin tissue
homogenate for mice with an atopic dermatitis skin
lesion and compared with those of controls.
3. Histopathological evaluation of atopic dermatitis
skin lesion and compared with those of controls.
4. Assessment of observational severity score.
Table 1: Demographic characteristics of the mice for all groups in comparison with controls.

| Variables       | Control | Group I  | Group II | Group III | Group IV | P-value |
|-----------------|---------|----------|----------|-----------|----------|---------|
| Age (days)      | 20      | 20       | 20       | 20        | 20       |         |
| Weight (g)      | 37±1.83 | 35±1.49  | 35.7±1.89| 36.1±2.28 | 35.5±1.96| 0.204   |

Table 2: Levels of skin tissue homogenate of IL-4 and IL-13, complete blood count in A.D. induced mice in comparison with controls

| Variables       | After one week Control | Non-treated Induced AD | p-value | After two weeks control | Non-treated Induced AD | p-value |
|-----------------|------------------------|------------------------|---------|-------------------------|------------------------|---------|
| IL-4            | 24.19±3.04             | 47.67±2.35             | <0.001  | 26.38±3.07              | 43.87±2.9              | <0.001  |
| IL-13           | 51.13±6.23             | 71.07±4.2              | <0.001  | 54.52±8.11              | 69.82±10.65            | <0.001  |
| WBC (x103/μl)   | 5.33±0.93              | 7.09±1.57              | <0.001  | 5.03±0.81               | 7.01±2.03              | <0.001  |
| Neutrophils (x103/μl) | 0.83±0.063             | 1.03±0.12              | 0.061   | 0.77±0.073              | 0.97±0.11              | 0.073   |
| Basophils (x103/μl) | 0.023±0.0070.041±0.011 | <0.001                | 0.023±0.007 | 0.039±0.01              | <0.001                |         |
| Monocytes (x103/μl) | 0.39±0.042             | 0.58±0.071             | 0.04    | 0.41±0.038              | 0.54±0.065             | 0.075   |
| Eosinophils (x103/μl) | 0.007±0.001            | 0.024±0.011            | <0.001  | 0.008±0.002             | 0.022±0.06            | 0.008   |
| Lymphocytes (x103/μl) | 4.09±0.79              | 5.28±1.1               | 0.043   | 4.13±0.85               | 5.07±1.14             | 0.047   |
| RBC (x 106/μl)  | 8.83±0.94              | 8.62±1.87              | 0.462   | 8.69±1.03               | 8.92±1.09             | 0.537   |
| Hemoglobin (g/dl) | 13.71±5.31             | 13.37±6.1              | 0.613   | 14.01±5.81              | 13.51±5.8             | 0.249   |
| Platelets (x103/μl) | 793.4±107.6            | 984±134                | 0.009   | 802.7±111.3             | 979±97                | 0.011   |

Table 3: Comparison between controls and non-treated A.D. induced group regarding observational severity score, and histopathological score.

| Variables               | After one week Control | Non-treated Induced A.D. | p-value | After two weeks control | Non-treated Induced A.D. | p-value |
|-------------------------|------------------------|--------------------------|---------|-------------------------|--------------------------|---------|
| Observational severity score | 0.3±0.48               | 2.8±0.42                 | <0.001  | 0.2±0.42                | 2.7±0.48                 | <0.001  |
| Epidermal thickness     | 0.0±0.0                | 3.0±0.0                  | <0.001  | 0.0±0.0                  | 2.5±0.0                  | <0.001  |
| Hyperkeratosis          | 0.0±0.0                | 3.0±0.0                  | <0.001  | 0.0±0.0                  | 2.5±0.0                  | <0.001  |
| Parakeratosis           | 0.0±0.0                | 3.0±0.0                  | <0.001  | 0.0±0.0                  | 3.0±0.0                  | <0.001  |
| Erosion                 | 0.0±0.0                | 2.25±0.74                | <0.001  | 0.0±0.0                  | 2.0±0.0                  | <0.001  |
| Inflammation            | 0.0±0.0                | 3.0±0.0                  | <0.001  | 0.0±0.0                  | 3.0±0.0                  | <0.001  |
| Edema                   | 0.0±0.0                | 2.75±0.54                | <0.001  | 0.0±0.0                  | 2.5±1.1                  | <0.001  |
Table 4: Levels of skin tissue homogenate of IL-4 and IL-13, complete blood count in A.D. induced mice in comparison with tacrolimus treated group.

| Variables | After one week | After two weeks | p-value | After one week | After two weeks | p-value |
|-----------|----------------|----------------|---------|----------------|----------------|---------|
| IL-4      | 47.67±2.35     | 36.48±4.74     | 0.01    | 43.87±2.9      | 32.54±7.53     | <0.001  |
| IL-13     | 71.07±4.2      | 66.18±9.11     | 0.028   | 69.82±10.65    | 62.58±7.63     | 0.01    |
| WBC (x10³/µl) | 7.09±1.57   | 6.4±1.89       | 0.079   | 7.01±2.03      | 6.1±1.92       | 0.018   |
| Neutrophils | 1.03±0.12    | 0.96±0.09      | 0.103   | 0.97±0.11      | 0.88±0.087     | 0.037   |
| Basophils (x10³/µl) | 0.041±0.011 | 0.037±0.019    | 0.21    | 0.039±0.01     | 0.0.33±0.009   | 0.028   |
| Monocytes (x10³/µl) | 0.58±0.071   | 0.53±0.08      | 0.318   | 0.54±0.065     | 0.5±0.091      | 0.271   |
| Eosinophils (x10³/µl) | 0.024±0.011  | 0.019±0.006    | 0.091   | 0.022±0.06     | 0.014±0.002    | 0.012   |
| Lymphocytes (x10³/µl) | 5.28±1.1     | 5.1±1.04       | 0.538   | 5.07±1.14      | 4.92±0.71      | 0.439   |
| RBC (x 10⁶/µl) | 8.62±1.87    | 8.57±4.11      | 0.395   | 8.92±1.09      | 8.43±1.02      | 0.251   |
| Hemoglobin (g/dl) | 13.37±6.1    | 12.84±4.97     | 0.407   | 13.51±5.8      | 12.78±5.31     | 0.383   |
| Platelets (x10³/µl) | 984±134      | 965±86         | 0.042   | 979±97         | 959±92        | 0.031   |

Table 5: A comparison in observational severity score, and histopathological score between tacrolimus treated group and non-treated A.D. induced group.

| Variables          | After one week | After two weeks | p-value | After one week | After two weeks | p-value |
|--------------------|----------------|----------------|---------|----------------|----------------|---------|
| observational severity score | 2.8±0.42       | 1.6±0.52       | 0.042   | 2.7±0.48       | 1.4±0.52       | 0.036   |
| Epidermal thickness | 3.0±0.0        | 1.25±0.56      | 0.031   | 2.5±0.0        | 1.0±0.0        | <0.001  |
| Hyperkeratosis      | 3.0±0.0        | 1.5±0.88       | 0.045   | 2.5±0.0        | 1.25±0.48      | 0.001   |
| Parakeratosis       | 3.0±0.0        | 1.75±0.44      | 0.01    | 3.0±0.0        | 1.5±0.44       | 0.001   |
| Erosion            | 2.25±0.74      | 1.5±0.66       | 0.045   | 2.0±0.0        | 1.0±0.0        | 0.021   |
| Inflammation       | 3.0±0.0        | 1.0±0.0        | <0.001  | 3.0±0.0        | 1.0±0.0        | <0.001  |
| Oedema             | 2.75±0.54      | 1.5±0.64       | 0.046   | 2.5±1.1        | 1.2±0.34       | 0.001   |
### Table 6: Levels of skin tissue homogenate of IL-4 and IL-13, complete blood count in A.D. induced mice in comparison with dapsone treated group.

| Variables            | After one week | Dapsone treated group | p-value | After two weeks | Dapsone treated group | p-value |
|----------------------|----------------|------------------------|---------|----------------|------------------------|---------|
| IL-4                 | 47.67±2.35     | 45.73±5.9              | 0.761   | 39.52±7.26     | 0.041                  |
| IL-13                | 71.07±4.2      | 68.41±9.25             | 0.442   | 64.13±13.22    | 0.028                  |
| WBC (x103/μl)        | 7.09±1.57      | 6.12±2.76              | 0.029   | 6.06±1.93      | 0.012                  |
| Neutrophils (x103/μl)| 1.03±0.12      | 0.83±0.13              | 0.047   | 0.79±0.092     | 0.038                  |
| Basophils (x103/μl)  | 0.041±0.011    | 0.038±0.019            | 0.523   | 0.036±0.021    | 0.61                   |
| Monocytes (x103/μl)  | 0.58±0.071     | 0.55±0.082             | 0.419   | 0.46±0.069     | 0.044                  |
| Eosinophils (x103/μl)| 0.024±0.011    | 0.02±0.017             | 0.397   | 0.015±0.009    | 0.047                  |
| Lymphocytes (x103/μl)| 5.28±1.1       | 5.11±1.08              | 0.352   | 4.87±1.11      | 0.264                  |
| RBC (x 106/μl)       | 8.62±1.87      | 8.32±0.84              | 0.41    | 8.2±0.98       | 0.037                  |
| Hemoglobin (g/dl)    | 13.37±6.1      | 12.53±4.26             | 0.178   | 12.36±4.58     | 0.109                  |
| Platelets (x103/μl)  | 984±134        | 1012±79                | 0.316   | 1076±86        | 0.203                  |

### Table 7: A comparison in observational severity score and histopathological score between dapsone treated group and non-treated A.D.induced group

| Variables            | After one week | Dapsone treated group | p-value | After two weeks | Dapsone treated group | p-value |
|----------------------|----------------|------------------------|---------|----------------|------------------------|---------|
| observational severity score | 2.8±0.42 | 2±0.67 | 0.071 | 2.7±0.48 | 1.7±0.48 | 0.047 |
| Epidermal thickness  | 3.0±0.0 | 2.32±0.86 | 0.062 | 2.5±0.0 | 1.25±0.3 | 0.011 |
| Hyperkeratosis       | 8.0±0.0 | 1.75±0.86 | 0.056 | 2.5±0.0 | 1.25±0.68 | 0.019 |
| Parakeratosis        | 3.0±0.0 | 1.75±0.44 | 0.01 | 3.0±0.0 | 1.5±0.44 | 0.001 |
| Erosion              | 2.25±0.74 | 1.5±0.66 | 0.54 | 2.0±0.0 | 1.0±0.0 | 0.021 |
| Inflammation         | 3.0±0.0 | 1.5±0.35 | 0.024 | 3.0±0.0 | 1.11±0.43 | 0.002 |
| Oedema               | 2.75±0.54 | 2.1±0.74 | 0.086 | 2.5±1.1 | 1.25±0.52 | 0.007 |
Table 8: Comparison between the levels of studied variables in the first and second week of induced A.D. group and with tacrolimus group

| Variables                        | Non-treated Induced A.D. First week | Non-treated Induced A.D. Second week | Tacrolimus treated group First week | Tacrolimus treated group Second week | p-value |
|----------------------------------|-------------------------------------|------------------------------------|-------------------------------------|--------------------------------------|---------|
| IL-4                             | 47.67±2.35                          | 43.87±2.9                          | 36.48±4.74                          | 32.54±7.53                           | 0.07    |
| IL-13                            | 71.07±4.2                           | 69.82±10.65                        | 66.18±9.11                          | 62.58±7.63                           | 0.11    |
| WBC (x103/μl)                    | 7.09±1.57                           | 7.01±2.03                          | 6.4±1.89                            | 6.1±1.92                             | 0.23    |
| Neutrophils (x103/μl)            | 1.03±0.12                           | 0.97±0.11                          | 0.96±0.09                           | 0.88±0.08                            | 0.35    |
| Basophils (x103/μl)              | 0.041±                               | 0.039±                              | 0.037±                              | 0.33±                                | 0.27    |
| Monocytes (x103/μl)              | 0.58±                                | 0.54±                              | 0.53±0.08                           | 0.51±0.09                            | 0.36    |
| Eosinophils (x103/μl)            | 0.024±0.011                         | 0.022±0.06                         | 0.019±                              | 0.014±                               | 0.56    |
| Lymphocytes (x103/μl)            | 5.28±1.1                            | 5.07±1.14                          | 5.1±1.04                            | 4.92±0.71                            | 0.33    |
| RBC (x 106/μl)                   | 8.62±1.87                           | 8.92±1.09                          | 8.57±4.11                           | 8.43±1.02                            | 0.51    |
| Haemoglobin (g/dl)               | 13.37±6.1                           | 13.51±5.8                          | 12.84±4.97                          | 12.78±5.31                           | 0.63    |
| Platelets (x103/μl)              | 984±134                             | 979±97                             | 965±86                              | 959±92                               | 0.35    |
| RBC (x 106/ μl)                  | 8.62±1.87                           | 8.92±1.09                          | 8.57±4.11                           | 8.43±1.02                            | 0.51    |
| Haemoglobin (g/dl)               | 13.37±6.1                           | 13.51±5.8                          | 12.84±4.97                          | 12.78±5.31                           | 0.63    |
| Platelets (x103/μl)              | 984±134                             | 979±97                             | 965±86                              | 959±92                               | 0.35    |
| Observational severity score     | 2.8±0.42                            | 2.7±0.48                           | 1.6±0.52                            | 1.4±0.52                             | 0.41    |
| Epidermal thickness              | 3.0±0.0                             | 2.5±0.0                            | 1.25±0.56                           | 1.0±0.0                              | 0.21    |
| Hyperkeratosis                   | 3.0±0.0                             | 2.5±0.0                            | 1.5±0.88                            | 1.25±0.48                            | 0.14    |
| Parakeratosis                    | 3.0±0.0                             | 3.0±0.0                            | 1.75±0.44                           | 1.5±0.44                             | 0.27    |
| Erosion                          | 2.25±0.74                           | 2.0±0.0                            | 1.5±0.66                            | 1.0±0.0                              | 0.12    |
| Inflammation                     | 3.0±0.0                             | 3.0±0.0                            | 1.0±0.0                             | 1.0±0.0                              | 0.79    |
| Oedema                           | 2.75±0.54                           | 2.5±1.1                            | 1.5±0.64                            | 1.2±0.34                             | 0.23    |

Animal sacrificing, dissection, histological analysis, skin tissue homogenate preparation, and assessment of observational severity score

At the 7th day of the treatment, we took half number of mice from each study groups and anaesthetized through a piece of cotton soaked with ether put with the mouse inside a closed jar for few minutes to be anaesthetized by inhalation, blood sample collected (1ml) in EDTA tube then sacrificed by cervical dislocation and the sharp blade cut atopic dermatitis skin area; this skin wound was dissection into two equal pieces one for the histological analysis and the second for the preparation of skin homogenate. The remaining mice from each group were subjected to the same procedure on the 14th day of the treatment.

Histological analysis

Dorsal skin samples were collected from each animal in study groups and fixed in 10% formaldehyde, paraffin-embedded and cut into 6 μm sections. Deparaffinized sections were stained with hematoxylin and eosin (H and E) to determine the inflammatory degree and histological changes associated with atopic dermatitis.

Assessment of histopathological changes in skin sections

Histopathologic follow-up procedures were used for the skin samples taken from each group on the 7th and 14th days of treatment. Histopathological changes of skin of each specimen were evaluated and scored by semiquantitative scoring systems for the evaluation of mouse model histopathology include Epidermal hypertrophy, hyperkeratosis, parakeratosis, erosion, inflammation, oedema, ulcer (each 0–4) were examined by a pathologist (Watan-
Table 9: Comparison between the levels of studied variables in the first and second week of treatment with dapsone.

| Variables               | Dapsone treated group |            |            | p-value |
|-------------------------|-----------------------|------------|------------|---------|
|                         | First week            | Second week|            |         |
| IL-4                    | 45.73±5.9             | 39.52±7.26 | 0.07       |
| IL-13                   | 68.41±9.25            | 64.13±13.22| 0.15       |
| WBC (x103/µl)           | 6.72±2.76             | 6.36±1.93  | 0.36       |
| Neutrophils (x103/µl)   | 0.83±0.13             | 0.79±0.092 | 0.26       |
| Basophils (x103/µl)     | 0.038±0.019           | 0.036±0.021| 0.79       |
| Monocytes (x103/µl)     | 0.55±0.082            | 0.46±0.069 | 0.052      |
| Eosinophils (x103/µl)   | 0.02±0.017            | 0.015±0.009| 0.54       |
| Lymphocytes (x103/µl)   | 5.11±1.08             | 4.87±1.11  | 0.39       |
| RBC (x 106/µl)          | 8.32±0.84             | 8.2±0.98   | 0.47       |
| Hemoglobin (g/dl)       | 12.53±4.26            | 12.36±4.58 | 0.18       |
| Platelets (x103/µl)     | 1012±79               | 1076±86    | 0.071      |
| observational severity score | 2±0.67            | 1.7±0.48   | 0.37       |
| Epidermal thickness     | 2.32±0.86             | 1.25±0.3   | 0.053      |
| Hyperkeratosis          | 1.75±0.86             | 1.25±0.68  | 0.14       |
| Parakeratosis           | 1.75±0.44             | 1.5±0.47   | 0.41       |
| Erosion                | 1.5±0.66              | 1.0±0.0    | 0.19       |
| Inflammation            | 1.5±0.35              | 1.11±0.43  | 0.16       |
| Edema                   | 2.1±0.74              | 1.25±0.52  | 0.06       |

Table 10: Comparison between the studied variables in the first week among all groups by ANOVA test.

| Variables               | Non-treated Induced A.D. | Tacrolimus treated group | Dapsone treated group | p-value |
|-------------------------|--------------------------|--------------------------|-----------------------|---------|
| IL-4                    | 47.67±2.35               | 36.48±4.74               | 45.73±5.9             | 0.031   |
| IL-13                   | 71.07±4.2                | 66.18±9.11               | 68.41±9.25            | 0.069   |
| WBC (x103/µl)           | 7.09±1.57                | 6.4±1.89                 | 6.72±2.76             | 0.027   |
| Neutrophils (x103/µl)   | 1.03±0.12                | 0.96±0.09                | 0.83±0.13             | 0.43    |
| Basophils (x103/µl)     | 0.041±0.011              | 0.037±0.019              | 0.038±0.019           | 0.21    |
| Monocytes (x103/µl)     | 0.58±0.071               | 0.53±0.08                | 0.55±0.082            | 0.079   |
| Eosinophils (x103/µl)   | 0.024±0.011              | 0.019±0.006              | 0.02±0.017            | 0.191   |
| Lymphocytes (x103/µl)   | 5.28±1.1                 | 5.1±1.04                 | 5.11±1.08             | 0.314   |
| RBC (x 106/µl)          | 8.62±1.87                | 8.57±4.11                | 8.32±0.84             | 0.048   |
| Hemoglobin (g/dl)       | 13.37±6.1                | 12.84±4.97               | 12.53±4.26            | 0.107   |
| Platelets (x103/µl)     | 994±134                  | 965±86                   | 1012±79               | 0.081   |
| observational severity score | 2.8±0.42            | 1.6±0.52                 | 2±0.67                | 0.01    |
| Epidermal thickness     | 3.0±0.0                  | 1.25±0.56                | 2.32±0.86             | 0.021   |
| Hyperkeratosis          | 3.0±0.0                  | 1.5±0.88                 | 1.75±0.86             | 0.033   |
| Parakeratosis           | 3.0±0.0                  | 1.75±0.44                | 1.75±0.44             | 0.06    |
| Erosion                | 2.25±0.74                | 1.5±0.66                 | 1.5±0.66              | 0.027   |
| Inflammation            | 3.0±0.0                  | 1.0±0.0                  | 1.5±0.35              | 0.008   |
| Edema                   | 2.75±0.54                | 1.5±0.64                 | 2.1±0.74              | 0.071   |
Table 11: Comparison between the studied variables in the second week among all groups by ANOVA test

| Variables                  | Non-treated A.D. | Induced A.D. | Tacrolimus treated group | Dapsone treated group | p-value |
|----------------------------|------------------|--------------|--------------------------|----------------------|---------|
| IL-4                       | 43.87±2.9        | 32.54±7.53   | 39.52±7.26               |                      | 0.009   |
| IL-13                      | 69.82±10.65      | 62.58±7.63   | 64.13±13.22              |                      | 0.043   |
| WBC (x103/µl)              | 7.01±2.03        | 6.1±1.92     | 6.36±1.93                |                      | 0.021   |
| Neutrophils (x103/µl)      | 0.97±0.11        | 0.88±0.087   | 0.79±0.092               |                      | 0.13    |
| Basophils (x103/µl)        | 0.039±0.01       | 0.33±0.009   | 0.036±0.021              |                      | 0.039   |
| Monocytes (x103/µl)        | 0.54±0.065       | 0.51±0.091   | 0.46±0.069               |                      | 0.081   |
| Eosinophils (x103/µl)      | 0.022±0.06       | 0.014±0.002  | 0.015±0.009              |                      | 0.015   |
| Lymphocytes (x103/µl)      | 5.07±1.14        | 4.92±0.71    | 4.87±1.11                |                      | 0.091   |
| RBC (x 106/ µl)            | 8.92±1.09        | 8.43±1.02    | 8.2±0.98                 |                      | 0.037   |
| Hemoglobin (g/dl)          | 13.51±5.8        | 12.78±5.31   | 12.36±4.58               |                      | 0.071   |
| Platelets (x103/µl)        | 979±97           | 959±92       | 1076±86                  |                      | 0.03    |
| Observational severity     | 2.7±0.48         | 1.4±0.52     | 1.7±0.48                 |                      | 0.001   |
| Epidermal thickness        | 2.5±0.0          | 1.0±0.0      | 1.25±0.3                 |                      | 0.005   |
| Hyperkeratosis             | 2.5±0.0          | 1.25±0.48    | 1.25±0.68                |                      | 0.011   |
| Parakeratosis              | 3.0±0.0          | 1.5±0.44     | 1.5±0.47                 |                      | 0.039   |
| Erosion                    | 2.0±0.0          | 1.0±0.0      | 1.0±0.0                  |                      | 0.041   |
| Inflammation               | 3.0±0.0          | 1.0±0.0      | 1.11±0.43                |                      | 0.024   |
| Oedema                     | 2.5±1.1          | 1.2±0.34     | 1.25±0.52                |                      | 0.018   |

Table 12: Correlations between the studied markers with the age and weight of mice.

|                      | Weight (g) | Age (days) | IL-4 | IL-13 |
|----------------------|------------|------------|------|-------|
| Weight (g) r         | 1          | -0.121     | 0.315*| 0.035 |
|                      | r          | 0.404      | 0.026| 0.811 |
| Age (days) r         | 1          | -0.292*    | 0.040| 0.100 |
| IL-4 r               | 1          | 0.681**    | 0.000|      |

* Correlation is significant at the 0.05 level (2-tailed).
** Correlation is significant at the 0.01 level (2-tailed).

abe et al., 2009).

Skin tissue homogenate preparation

The second piece of skin obtained were washed with normal saline, and rinsed with chilled phosphate buffer saline (1X PBS), put with filter paper and weighed. Each 100 mg of skin wound tissue was homogenized with 1 ml of (1X PBS) with the aid of tissue homogenizer for 1 minute at 4 °C and must be stored overnight at 20 °C. Two freeze-thaw cycles must be performed to break the cell membranes; the homogenates were centrifuged for ten minutes at 2000 RPM at 2-8 °C. The supernatant was obtained and stored at −20°C to the assay of IL-4 and IL-13 levels in the tissue.

Biochemical assays

Quantitative measurement of IL-4 and IL-13 (principle of the assay)

ELISA Kit for the estimation of IL-4 and IL-13 was obtained from Mybiosource/USA. The kit was based on sandwich enzyme-linked immune-sorbent assay technology. Anti- IL-4 and Anti- IL-13 antibodies were pre-coated onto 96-well plates. And the biotin conjugated anti-IL-4 and anti-IL-13 antibodies were used as detection antibodies. The standards, test samples and biotin conjugated detection antibodies were added to the wells subsequently.
and washed with wash buffer. HRP-Streptavidin was added, and unbound conjugates were washed away with wash buffer.

TMB substrates were used to visualize HRP enzymatic reaction. TMB was catalyzed by HRP to produce a blue colour product that changed into yellow after adding the acidic stop solution. The density of yellow is proportional to the IL-4 and IL-13 amount of sample captured in plate. Read the O.D. absorbance at 450nm in a microplate reader. Then the concentration of IL-4 and IL-13 can be calculated by comparing the optical density of the samples to that of the standard curve in the corresponding microtiter plate (Voller et al., 1978). The concentration of IL-4 and IL-13 in each sample was expressed in pg/ml for comparison of results with those of controls concentration.

Assessment of observational severity score

The severity of AD on the dorsal area was evaluated for each group on the 7th and 14th days of treatment. The evolution of erythema, dryness, erosion and edema will scored as 0 (none), 1 (mild), 2 (moderate), and 3 (severe) (Suto et al., 1999).

Statistical analysis

The data of the study were stored in Microsoft Excel spreadsheet and analyzed on the computer using the SPSS software 20 and Microsoft excel program (2010). Numeric variables were expressed as mean ± S.D., and all statistical comparisons were made using independent t-test, and ANOVA test with P ≤0.05 was considered statistically significant. Categorical variables were expressed as numbers and analyzed by cross-tabulation to assess the frequency and percentage of each variable among studied groups. The correlation was done between all parameters using Pearson correlation test (Norman, 2010), eta (η) test between numerical and categorical variables (given that values ranged from 0-0.5 considered as weak correlation while values above 0.5 considered as strong correlation) and Chi-square to test the relationships between categorical variables.

RESULTS AND DISCUSSION

General characteristics of the study groups

Some demographic characteristics of the studied groups were summarized in Table 1 that showed non-significant differences in age and weight among all studied groups.

Table 2 revealed that the levels of IL-4 and IL-13 were increased significantly (p<0.05) in the group of mice with an induced A.D. in comparison with controls after the first and second week of sampling. It was demonstrated that the values of complete blood count showed a significant increase in the total WBC count, basophils, eosinophils, lymphocytes and platelets after one and two weeks of atopic dermatitis induction when compared with control. The only exception was the monocytes that showed a significant high count in the first week of induction in comparison with controls at the same time while a non-significant difference was obtained in the second week of application.

Table 3 and Figure 1 obviated the effect of A.D. induction on the histopathological score after one and two weeks. It revealed that observational severity score, Epidermal thickness, Hyperkeratosis, Parakeratosis, Erosion, Inflammation and Edema score showed significantly higher scores than controls after one and two weeks of A.D. induction.

Results postulated in the Table 4 elucidate clearly that the levels of IL-4 and IL-13 in mice received tacrolimus topically were significantly lower than the corresponding levels in A.D. induced non-treated group after one and two weeks of treatment. Furthermore, total WBC, neutrophils, basophils and eosinophil count showed a significant reduction after two weeks of topical application of tacrolimus. In contrast, platelets count decreased significantly after one week of treatment with topical tacrolimus which reduced further after the second week of treatment.

Table 5 and Figure 2 showed that observational severity score, epidermal thickness, hyperkeratosis, parakeratosis, inflammation and oedema score were significantly reduced in tacrolimus treated group in comparison with those non-treated A.D. induced group after one week of treatment. Additionally, observational severity score, epidermal thickness, hyperkeratosis, parakeratosis, erosion, inflammation and oedema score showed significant reduction after two weeks of treatment with tacrolimus.

Data postulated in the Table 6 illustrated that the levels of all studied variables after the application of dapsone topically were non-significantly differ from those of corresponding non-treated induced A.D. group except the significant decrease in the count of WBC and neutrophils after one week of treatment. On the other hand, levels of IL-4 and IL-13 in addition to the count of WBCs, neutrophils, Monocytes, Eosinophils and RBC were significantly decreased in comparison with a non-treated A.D. induced group after two weeks of treatment.

Results illustrated in the Table 7 and Figure 3 showed that the Parakeratosis and Inflammation...
scores of topically dapsone treated group after one week of treatment were significantly lower than that of non-treated A.D. induced group whereas all studied histopathological scores including epidermal thickness, hyperkeratosis, parakeratosis, erosion, inflammation and oedema score in addition to the observational severity score were significantly lower than those of mice group with induced A.D. that did not receive treatment.

The influence of sampling time on the studied variables:

All data presented in Tables 8 and 9 revealed that there was a non-significant difference between the levels of all studied variables that including IL-4, IL-13, CBC, observational severity score and A.D. histopathological scores obtained after one or two weeks of treatment, i.e. levels obtained after two weeks of induction or treatment were non-significantly differ from those obtained after the first week.

Comparison between all studied groups by ANOVA test

Following the data postulated in the Table 10, the levels of IL-4 were significantly differed among all studied groups, whereas IL-13 showed a non-significant change among these groups in the first week of sampling. CBC values showed that WBC and RBC counts were significantly changed among the studied group during the first week of A.D. induction and treatment. Additionally, Observational severity score and histopathological scores including, Epidermal thickness, Hyperkeratosis, Erosion, and Inflammation score were significantly changed in the first week.

Furthermore, after two weeks of treatment with different topical application, more significant differences were obtained including significant differences in the levels of both studied cytokines (IL-4 and IL-13) in addition to the CBC values that showed significant differences in WBCs, Basophils, Eosinophils, RBCs and platelets counts with significant changes in observational severity and all histopathological scores (Table 11).

Correlations

Table 12 revealed that the levels of IL-4 and IL-13 were correlated positively and significantly with each other. Moreover, levels of IL-4 showed a significant positive correlation with the weight of mice and correlated significantly and negatively with the age of mice subjected to the present study.

Results of the present study revealed that the atopic dermatitis induction by DNCB cause significant elevations in the levels of IL-4 and IL-13 in skin homogenate in comparison with controls and also cause a significant increase in total WBC count, basophils, eosinophils, lymphocytes and platelets after one week. Many previous kinds of research used DNCB as an inducer for atopic dermatitis that causes an elevation in the levels of IL-4 and IL-13 (Kitamura et al., 2018) which is consistent with the results obtained in this study. The ability of DNCB to induce atopic dermatitis originate mainly from its role in increase type-2 helper T cell activity, IL-4 and IL-13 that result in the inflammatory response that elevate the levels of IgE and affect the histological scores of the affected skin which appear clearly via the histopathological investigations, which explained the high significant increase in observational severity scores (Kim et al., 2018). It was noticed that the levels of studied markers were reduced slightly after the application of DNCB by two weeks in comparison with the same markers after one week from the application which may be contributed to the healing process that occurs after more than ten days which is agreed with the results illustrated in recent work published in 2020 (Lee et al., 2020).

In a consistent with a majority of the articles that dealt with the use of tacrolimus in A.D. treatment (Crissinger and Nguyen, 2014), our recent work obviates the effect of tacrolimus on atopic dermatitis tissue markers, histological and observational severity scores and also on the CBC of the affected mice. Levels of IL-4 and IL-13 were decreased significantly in topically tacrolimus treated group in comparison with those suffered from atopic dermatitis induced by DNCB which occur after one week of treatment and persist to the second week with the slight non-significant difference between these two sampling time. The assumed mechanism of action of tacrolimus was discussed in several types of research, and they postulated that the primary mechanism of action of tacrolimus is its immunosuppression activity (Sengoku et al., 2000) which then subjected to more profound studies to elucidate this mechanism precisely.

Tacrolimus known to can bind specific receptors on T cells leading to an increase in intracellular calcium which, in turn, lead to series of reactions inhibiting the transcription of several genes for several cytokines such as IL-4 (Russell, 2002). Furthermore, more recent study postulated that tacrolimus bind to the F.K. binding protein-12 (FKBP12) to inhibit calcineurin, a protein phosphatase responsible for the dephosphorylation of the nuclear factor of activated T-cells (NF-AT) (Crissinger and Nguyen, 2014). Without dephosphorylation, NF-AT cannot be translocated into the nucleus, and thus the pro-
duction of inflammatory interleukins is inhibited. Adjunctive mechanisms of action have been proposed for tacrolimus including binding at cell surface steroid receptors, inhibition of mast cell mediator release, and downregulation of chemoattractant IL-8 receptors, intracellular adhesion molecule-1, E-selectin, and Langerhans cells IgE receptors (Ohtsuki et al., 2018).

The proposed mechanisms for tacrolimus activity explain its anti-inflammatory and anti-chemotactic role that may provide an acceptable explanation about the other variables and histological scores that reduced after treatment with it for one and two weeks such as the reduction in the counts of total WBCs, neutrophils, eosinophils and basophils in agreement with the previous review (Kandikattu and Mishra, 2018). Additionally, the results illustrated in the current study showed a significant reduction in the count of platelets inconsistency with previous results (Arai et al., 2016). The second treatment group that examined throughout the current study was topical dapsone which is an aniline derivative belonging to the group of synthetic sulfones with microbial activity. Shortly after that, the use of dapsone to treat non-pathogen-caused diseases revealed alternate anti-inflammatory mechanisms that initially were elucidated by inflammatory animal models. Thus, dapsone has dual functions of both: antimicrobial/antiprotzoal effects and anti-inflammatory features similarly to non-steroidal anti-inflammatory drugs (Kridin, 2018). that is the cause of our study on topical dapsone to confirm its role in treating atopic dermatitis regarding a case report and brief assay which describe its use in A.D. (Lee et al., 2005).

Results illustrated in the present work obviate the possible role of topical dapsone in the treatment of atopic dermatitis as it showed a significant effect on the levels of IL-4, IL-13, CBC, histopathological scores, and observational severity scores that appear more clearly after two weeks of treatment with dapsone topically. No obvious explanation found about the effect of IL-4 and IL-13 levels. Still, the assumed effect of dapsone on Th2 cells may be the cause of the significant decrease in these cytokines levels in agreement with previous work (Wozel and Blasum, 2014).

The postulated decrease in the counts of total WBCs, Monocytes, eosinophils, neutrophils, and RBCs may be considered as a possible mechanism of an anti-inflammatory which also considered as a side effect for dapsone which can be used in future as a promising topical application for the treatment of A.D. which is inconsistent with previous works of literature that consider this activity as an adverse effect (Halim and Ogbeide, 2002). In eosinophil-mediated inflammation dapsone may act by several mechanisms, its anti-inflammatory actions involve several effects on neutrophils such as: reduced chemotactic attraction, inhibition of neutrophil myeloperoxidase, reduced secretion of reactive oxygen species, and reduced generation of 5-lipoxygenase and other lysosomal enzymes. Additionally, dapsone has a significant effect on eosinophil function. It has been shown to inhibit eosinophil peroxidase, which is a cytotoxic granule protein of eosinophils that induces mast cell degranulation (Bokotas et al., 2014). The inhibitory effect of dapsone on eosinophil peroxidase as a previous explanation of the therapeutic efficacy of dapsone in eosinophil-mediated diseases showed that the dapsone inhibitory effect reduces the effect of eosinophil peroxidase on mast cells which lead to decreasing the release of the inflammatory mediators; this inhibitory effect might be due to the inhibition of lipoxygenase and ROS production (Smith and Cox, 2008).

These effects of dapsone may be the explanation of the reduction in the histopathological and observational severity scores recorded in the current study which require a further focusing on elucidating the obscure about the use of topical dapsone in atopic dermatitis treatment.

Results demonstrated in this study revealed that there were non-significant differences between the levels of all studied variables in the first week and those in the second, which may indicate that our work needs longer time to obviate the differences with the application of treatment.

Results obtained in the current work showed that IL-4, CBC and RBC were the only variables that significantly varied among the studied group in addition to observational severity and histopathological scores of epidermal thickness, Hyperkeratosis, Erosion and Inflammation which is consistent with the above-discussed results. Moreover, the majority of studied variables showed significant differences among all studied groups after two weeks of treatment that owned to the longer duration of topical application that causes a different rate of response in mice subjected to the current research. Results demonstrated in the present study showed clearly that the elevation of IL-4 was accompanied by an increase in IL-13 which is inconsistent with the previously mentioned source of them from Th2 cells that activated in atopic dermatitis (Bao and Reinhardt, 2015).
CONCLUSIONS

Levels of the majority of studied variables were increased in response to induction of atopic dermatitis with topical DNCB which then reduced generally after topical application of tacrolimus. Topical application of dapsone showed an anti-inflammatory effect that may be used in future as a promising treatment for A.D.

Conflict of Interest

The authors declare that they have no conflict of interest for this study.

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