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Effectiveness of lenalidomide as a topical ointment in mouse models of imiquimod-induced psoriasis

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***Abstract*---**Background: Psoriasis is an inflammatory skin disorder whose cause is unknown. Psoriasis appears to be an immune-mediated disease, according to growing evidence. The immunomodulatory effects of lenalidomide inhibit the production of pro-inflammatory cytokines that have been associated with several hematologic cancers. Lenalidomide enhances the host's immune system by regulating T cell proliferation, which causes alterations in inflammation that are associated with the etiology of psoriasis. The purpose of the study: In this trial, the aim was to study the effectiveness of lenalidomide as an ointment in the treatment of psoriasis-induced in mice and to examine the histological differences between the tested groups. Materials and methods: This study was conducted from November 2021 to June 2022. 70 healthy male albino mice, which were randomly divided into seven groups of 10 mice each. Psoriasis was induced by imiquimod in groups (1, 2, 3, 4, 5 and 6). Group 1 received only 5% imiquimod cream, Group 2 received Clobetasol ointment, Group 3 received only the base containing lenalidomide and Groups (4, 5, and 6) received lenalidomide ointment (1%, 2% and 3%, respectively). In Group 7, healthy mice were used as a comparison control. Statistical analysis of the data was performed using SAS (Statistical Analysis System-version 9.1). One-way ANOVA and least significant differences (LSD) post hoc tests were performed

to assess significant differences between means. Results: The psoriatic area improved after treatment with lenalidomide. Baker's scoring system explains the histopathological differences between the tested groups. Baker's scoring system (which indicates a combination of nine histological characteristics) reveals significant differences between the groups. Due to its immunomodulatory and anti-inflammatory effects, our results suggest that lenalidomide can treat imiquimod-induced psoriasis in mice. Conclusions: According to the study, different concentrations of lenalidomide can worsen or improve psoriasis in mice. It is effective at a certain dose and has efficacy comparable to that of a standard drug.

Keywords---lenalidomide, immunomodulatory, anti-inflammatory, baker's scoring system, imiquimod-induced psoriasis in mice.

Introduction

Psoriasis is one of the most common, persistent and non-contagious diseases that is characterized by highly proliferative keratinocytes and widespread leukocyte infiltration[1]. In North America and Europe, Psoriasis is the most common immune-mediated skin disease in adults[2]. It is characterized by an overactive cellular immune system. It is similar to Crohn's disease, rheumatoid arthritis, multiple sclerosis, and type 1 diabetes in that "A clinical syndrome produced by the activation of T lymphocytes and b lymphocytes, or even both, in the absence of a chronic infection or other identifiable cause," [3]. Inflammatory cytokines such as tumor necrosis factor (TNF) are believed to have a major pathogenic role in psoriasis, and other forms of inflammatory leukocytes may also play a role. Infiltrating leucocytes, resident skin cells, and a variety of pro-inflammatory cytokines, chemokines, and chemical mediators released in the skin under the control of the cellular immune system are all associated with psoriasis[4].

Lenalidomide is a thalidomide analogue that was synthesized by altering the chemical structure of thalidomide to enhance potency and minimize side effects such as side effects of the nervous system (sedation and neuropathy)[5]. Its activity has been shown to be effective as a treatment for a variety of solid and hematological tumors[5]. The Food and Drug Administration (FDA) has approved it for the treatment of patients with myelodysplastic syndromes and multiple myeloma[6]. Lenalidomide has been shown to be an immunomodulator, affecting both the cellular and humoral components of the immune system. Lenalidomide has been shown to modulate cytokine production, T cell co-stimulatory activity, and NK cell cytotoxicity[6]. Lenalidomide has been shown to inhibit the production of pro-inflammatory cytokines TNF-α, IL-1, IL-6, IL-12 and increase the production of anti-inflammatory cytokines IL-10 from human peripheral blood mononuclear cells7. Downregulation of TNF-α secretion is particularly striking and is up to 50,000 times greater compared to thalidomide[8]. TNF-α is a highly pleiotropic cytokine produced primarily by monocytes and macrophages and plays an important role in protective immune responses against bacterial and viral infections. Elevated TNF-α production is involved in the pathogenesis of psoriasis[9].

The purpose of the study

In this trial, the aim was to study the effectiveness of lenalidomide as an ointment in the treatment of psoriasis-induced in mice and to examine the histological differences between the tested groups.

Materials and Methods

Chemicals and reagents

The chemicals and reagents used in this experiment were as follows: linalidomide powder (Hangzhou hyper chemicals-China), 5% IMQ propionate cream (Meda-Sweden), Clobetasol 17-propionate ointment (GSK), Castor oil (KTC/Germany), petroleum jelly (Battles, Hayward, Bower Ltd/UK), Diethyl Ether (Fluka-Switzerland), Phosphate buffer solution (Pure chemistry–Germany), Distilled water (Iraq) , Paraffin wax (Medite-USA), ethanol99.9%) (Hayman®/UK), Xylene (Scharlau-Spain), Hematoxylin stain, Eosin stain, DPX mounting medium (SyrBio-Switzerland), Scott‘s tap water (bioGnost®/Croatia), hydrochloric acid (HCl) 1% in 70% ethanol (Riedel-de Han®/Germany). All of the chemicals were of analytical reagent grade.

Preparation of lenalidomide ointment

Linalidomide powder was purchased from Hangzhou Hyper Chemicals-China Limited as white powder and then prepared at the College of Pharmacy, University of Baghdad, as an ointment. The lenalidomide powder is dissolved in castor oil[12], which acts as a levigating agent[13], and then mixed with petroleum jelly by geometric mixing[14] to make three different concentrations of lenalidomide ointment (1%, 2% and 3%).

Mice and Treatments

70 healthy albino mice, 6-8 weeks old, were purchased from the Iraqi center for cancer research and medical genetics. The Experimental Animal Ethics Committee of the Baghdad Medical College of Medicine approved all procedures, which met national guidelines for the care and use of experimental animals (1697, 6/12/2021). The animals were housed in pathogen-free environments. Water and food are provided ad libitum. The mice are randomly divided into seven groups. Group 1 received Aldara cream (imiquimod 5%) once a day for 6 days on the shaved backs of mice to produce a skin-inflammatory animal model similar to psoriasis (induction group)[16]. After imiquimod induction of psoriasis, Group 2 received topically clobetasol ointment once a day for 8 days on the shaved backs of mice. Group 3 received only the base containing lenalidomide (castor oil and petroleum jelly) (free of drugs). Groups 4, 5, and 6 received lenalidomide ointment (1%, 2% and 3%, respectively) to apply topically to the shaved backs of mice once daily for 6 days. In Group 7, only mice without any application (apparently healthy) were used as a comparison control group, as shown in tab.1.

Table 1
Mice and Treatments

Group	Application	No. mice
G1	Induction	10
G2	standard treated with clobetasol ointment	10
G3	Vehicle treatment (drug-free), placebo treatment	10
G4	Treated with lenalidomide ointment 1%	10
G5	Treated with lenalidomide ointment 2%	10
G6	Treated with 3% lenalidomide ointment	10
G7	Control with no application	10

One animal per cage was kept in plastic cages measuring 20x25x35 cm. Before beginning the study procedure, the animals were housed for two weeks in a controlled environment with a temperature of 22 ± 1^0C and a light schedule of 12–12 hour light/dark cycles, as well as an air vacuum to acclimate to the environment of the animal house. The animals had free access to food, commercial pellets and water[15].

Histological Examination
Preparation of the Samples

After 6 days of application of IMQ on the skin for the induction group, the animals were sacrificed humanely by cervical dislocation and the skin treatment region was excised and stored in 10% Phosphate Buffered Saline (PBS) for histopathological examination. On day 14, groups of mice that started treatment on day 7 of induction (day 1 after induction) with lenalidomide, clobetasol and placebo for 8 days were also sacrificed humanely by cervical dislocation, and the skin treatment region was excised and stored in 10% Phosphate Buffered Saline (PBS) for histopathological examination[17].

Preparation of formalin-fixed paraffin-embedded tissues[18] (FFPE)

- Tissue Fixation
- Dehydration
- Impregnation
- Embedding tissues in paraffin blocks
- Tissue sectioning and slide preparation
- Hematoxylin and Eosin (H&E) staining of paraffin sections

Assessment of histological analysis of skin tissue

- The light microscope (Genx ®/ USA) was used to capture the zones of a slide that were randomly selected at magnifications of X 10 and X 20.
- Barker's scoring system is used for histopathological examination of wound samples depending on the hematoxylin and eosin staining system to confirm healing as in (Table.2) [17].

Table 2
Baker's scoring system

Item		Score
keratin	Munro abscess	2.0
	Hyperkeratosis	0.5
	Parakeratosis	1.0
epidermis	Thining above the papillae	0.5
	Lengthening and Clubbing of rete Ridges	1.5
	Acanthosis	0.5
	Lack of granular layer	1.0
dermis	Lymphocytic infiltrate(mild)	0.5
	Moderate	1.0
	Marked	2.0
	Papillary congestion	0.5

Statistical analysis

Statistical analysis of the data was performed using SAS (Statistical Analysis System-version 9.1)[11]. One-way ANOVA and least significant differences (LSD) post hoc tests were performed to assess significant differences between means. $P < 0.05$ is considered statistically significant[11].

Results

After 6 days of IMQ application on the skin on the shaved back of the mice, the charactrisics of psoriasis of round, red papules or plaques with a gray or silvery white dry scale appear on the applied area as shown in (Fig. 1.A). On day 14 (day 8 after induction), the results improved completely with respect to psoriasis after the application of clobetasol (Fig. 1.C) and also completely improved after the application of 1% lenalidomide (Fig. 1.D), but mice treated with clobetasol appeared to lose weight and had secondary skin infections. Mice that were treated with lenalidomide 2% (Fig.1.E) and lenalidomide 3% (Fig.1.F) showed no good improvement, as well as mice treated with placebo showed approximately similar effects to (lenalidomide 2%, 3%) (Fig.1.G). (Fig.1.B) shows a mouse without any applications and appears to be healthy.

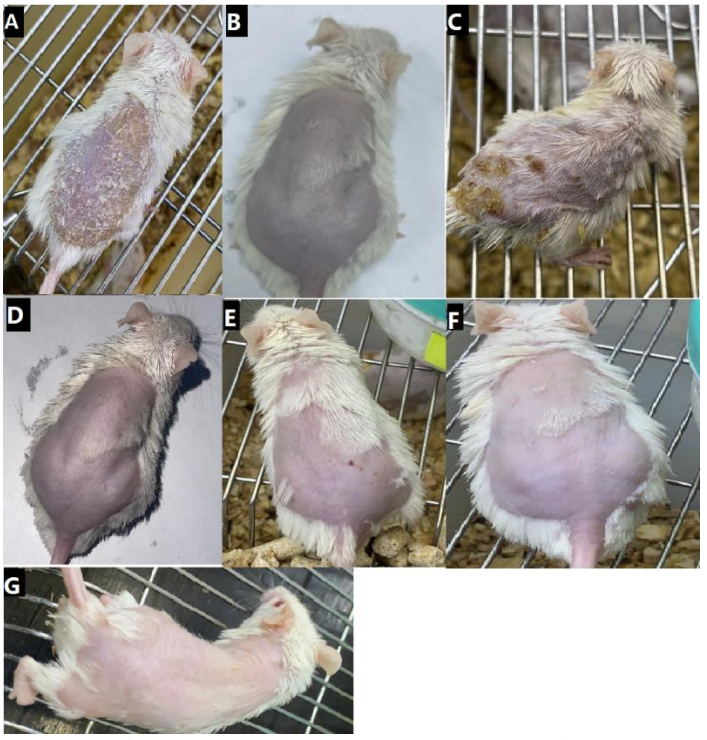


Figure 1. Various treatments on mice

The results of the skin stained with IMQ showed increased epidermal thickness (hyperkeratosis) and subcutaneous tissue compared to the control group. In addition, abnormal differentiation of keratinocytes with marked parakeratosis (nuclei in the stratum corneum) was observed. Thinning above the papillae, lengthening and clubbing of the rete ridges, acanthosis, and the lack of a granular layer are also observed (Fig. 2.A). In the dermis layer, there is marked lymphocytic infiltrate and papillary congestion (Fig. 2.A). The average score of the induction group was statistically 9.20±0.15a**.** Clobetasol treatment completely inhibited the increase in thickness. Stratum corneum dropouts were observed in IMQ-treated skin. Although thin above Papillae, the lengthening and clubbing of rete ridges and acanthosis disappear, the granular layer returns. There is no lymphocytic infiltrate or papillary congestion 0.00±0.00d (fig.2.B). Histological examination shows approximately similar results when inducted mice are treated with lenalidomide 1% 0.10±0.10d (fig.2.D). The difference between the clobetasol group and the lenalidomide 1% group was not significantly less than LSD (0.54) as the statistical results show, so the groups show approximately similar results.

The mice group that was treated with 2% lenalidomide showed hyperkeratosis, parakeratosis, lengthening and clubbing of the rete ridges, and acanthosis. There is also lymphocytic infiltrate and papillary congestion 4.25±0.35b (fig.2.E, F). Hyperkeratosis, lengthening and clubbing of rete ridges, and acanthosis were observed in the 3% lenalidomide group. There is also a lymphocytic infiltrate 2.90±0.31c (fig.2.G, H). The group treated with placebo, vehicle of lenalidomide (drug-free) showed lengthening and clubbing of rete ridges and acanthosis. Lymphocytic infiltrate and papillary congestion are found 3.20±0.08b (fig.2.C).

There is a significant difference between the groups (lenalidomide 2%, 3% and placebo groups) and the groups (clobetasol and lenalidomide 1%) groups. As shown in Figure 2.J, no abnormal phenotype was observed in the control groups 0.00±0.00d. These results may provide evidence to suggest that treatment with lenalidomide 1% was effective and approximately similar to standard. Treatment with lenalidomide 2% or lenalidomide 3% did not cause improvement and the characteristics of psoriasis were still present.

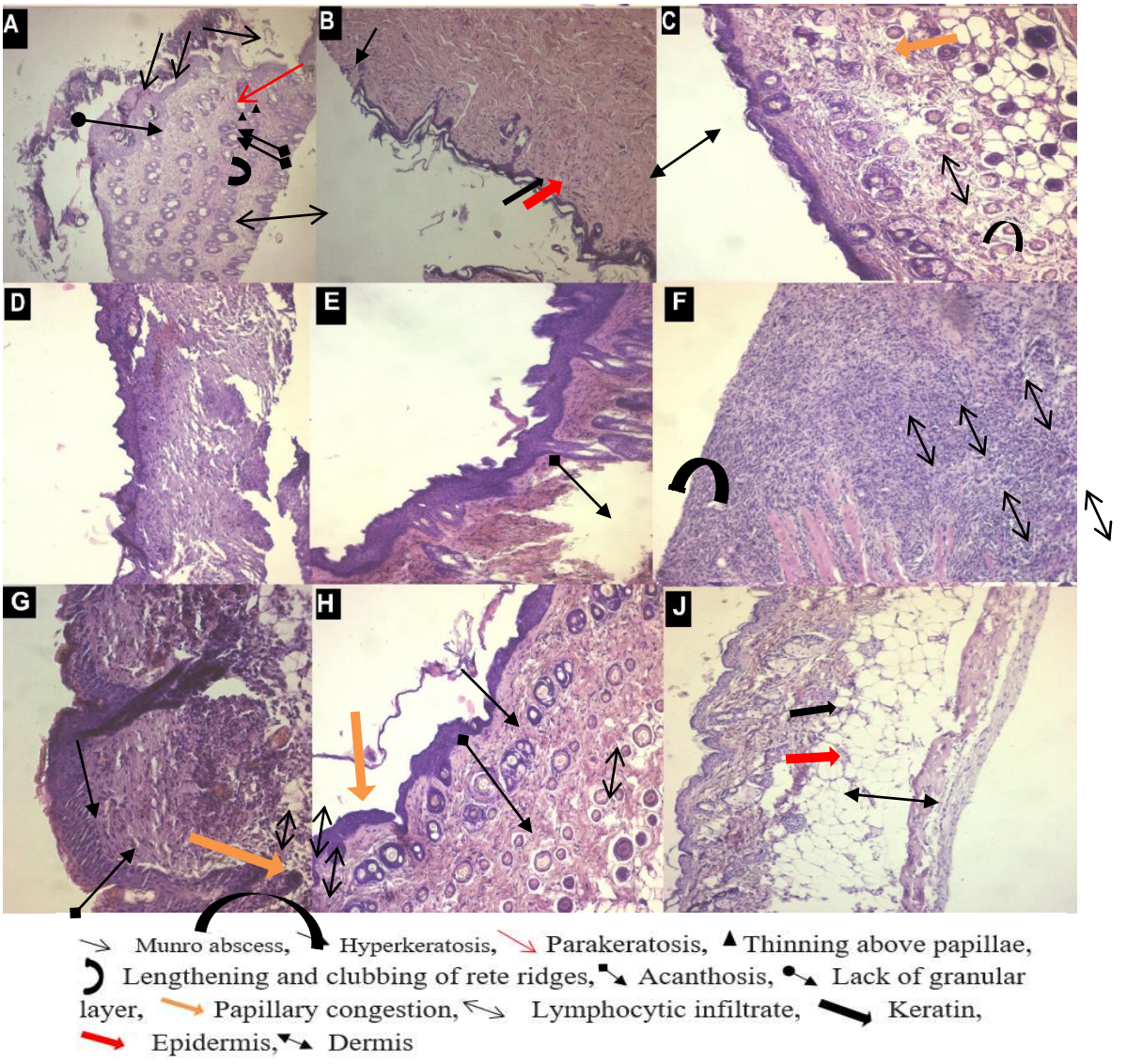


Figure 2. Histological examination of excised skin

Table 3
Statistical analysis of Baker's scoring

Groups	Scores
Group 1 Induction	9.20±0.15a
Group 2 Standard	0.00±0.00d
Group 3 Placebo	3.20±0.08b
Group 4 Lenalidomide 1%	0.10±0.10d
Group 5 Lenalidomide 2%	4.25±0.35b
Group 6 Lenalidomide 3%	2.90±0.31c
Group 7 Normal	0.00±0.00d
LSD	0.54

Means with a different letter are significantly different (P<0.05)

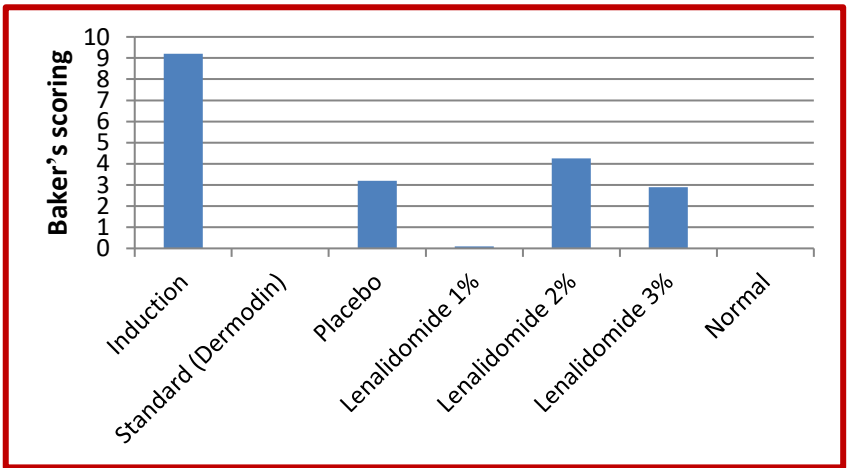


Figure 3. Statistical analysis of Baker's scoring

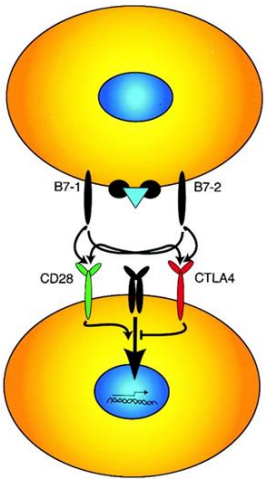
Discussion

Although the cause of psoriasis is uncertain, some characteristics suggest that it is caused by an immunological response[1]. Following skin absorption, Imiquimod can activate monocytes, macrophages, and dendritic cells by binding to Toll-like receptors (TLRs) in plasmacytoid dendritic cells (pDCs) and activating them, leading to massive release of downstream factors such as IFN, IL-17A, IL-22, and TNF in psoriasis lesions and peripheral blood[19]. The use of a 5% imiquimod cream generated apparent pathological changes similar to psoriasis in the present investigation. Furthermore, Baker's scores in the imiquimod group were significantly higher than in the control group, indicating progression similar to psoriasis in the imiquimod group. These results revealed that imiquimod therapy can cause and aggravate psoriasis in mice. (Fig.3)

Imiquimod can activate the canonical NF-κB signaling pathway (Nuclear factor kappa-light-chain-enhancer of activated B cells), which has been linked to the pathogenesis of psoriasis induced by imiquimod[20]. NF-κB signaling pathway is responsible for the transcriptional induction of pro-inflammatory cytokines,

chemokines, and additional inflammatory mediators in different types of innate immune cells. These inflammatory mediators can both directly participate in inflammation induction and act indirectly by promoting the differentiation of inflammatory T cells[21]. NF-κB signaling activates Macrophages rapidly and promotes the secretion of a wide variety of pro-inflammatory cytokines, such as IL-1, IL-6, IL-12, TNF-α and chemokines[21]. NF-κB also plays a role in the regulation of T cell differentiation and effector function. Upon activation, CD4+ T cells differentiate into different subsets of effector T cells, including Th1, Th2, Th17 and T follicular (Tfh) cells, which secrete different cytokines and mediate different aspects of immune responses, such as IL-17 secretion by Th17 cells, an inflammatory cytokine that recruits monocytes and neutrophils to the site of inflammation in response to invasion by pathogens or self-antigens[21]. Although NF-κB involved in the generation of Treg cells, Treg cells acquire Th17 inflammatory effector functions under lymphopenic conditions[21].

T cells are important immune response effectors, and their activation is tightly regulated to avoid auto-reactivity. T cell activation involves the presentation of the peptide fragments displayed by antigen-presenting cells (APCs) to the T cell receptor (TCR), and this interaction gives specificity to the response[22]. However, this interaction alone is not sufficient if a T cell has to generate an effective response against antigen[22]. A secondary interaction of B7/CTLA4 on the surface of T cells has a negative signal that regulates T cell activation by inhibiting T cell activation by restricting the availability of B7 for interaction with CD-28[23]. CTLA4 has a higher affinity for B7 (20 times) than CD-28. Blocked of this interaction provides the co-stimulatory signal that increases the T cell response and aids in T cell proliferation, differentiation, and survival, followed by a cascade of cytokine and cellular responses[23]. In certain situations, T cells or B cells that encounter their antigens in the absence of costimulation can be anergized or deleted by programmed cell death[24]. Immunomodulatory drugs (IMiDs), including lenalidomide, act on T cells via the B7/CD28 co-stimulatory pathway by blocking the interaction of B7/CTLA4 Ig[25]. IMiDs do not up-regulate CD28 and B7 expression in T cells and APCs, respectively, but they can directly induce tyrosine phosphorylation of CD28 in T cells, leading to activation of downstream targets such as PI3K, GRB-2-OS and NF-κb. This might explain their ability to partially overcome CTLA4 Ig blockade[26,34,35]. T cell costimulation with lenalidomide increases the Th1 cytokine response, leading to increased IFN- and IL-2 secretion, which stimulates clonal T cell proliferation and NK cell activity[27].



This model proposes that T-cell activation requires two independent signals. The first is transduced through the T cell receptor (TCR) after engagement by antigen; and the second costimulatory signal is delivered by ligation of a distinct receptor present on the surface of the T cell. This model predicts that the engagement of TCR in the absence of costimulation will not activate T cell[28]. For decades, topical corticosteroids, particularly high-potency corticosteroids, have been the standard for the treatment of psoriasis. Their success can be linked to a variety of mechanisms of action, including anti-inflammatory, immunosuppressive, and antiproliferative effects. Clobetasol was shown to have a considerable impact. Clobetasol has been shown to have an effect on the IL-23/IL-17A axis in the genesis of psoriasis-like inflammation in mice, reducing the levels of IFN, IL-17A, IL-22, and TNF[29]. This explains why there is a significant difference between the Clobetasol group and the induction group. (Fig.3), (tab.3)

The results of this study do not show significant differences between the Clobetasol group and the 1% lenalidomide group. The immunomodulatory properties of lenalidomide reduce the generation of pro-inflammatory cytokines, which have been linked to the pathogenesis of psoriasis by altering cytokine production and regulating T cell co-stimulation[31,32]. Lenalidomide at the lowest concentration of 1% has the lowest or absence of a co-stimulatory signal, leading to T cell death by programing cell death[24,33,37]. This explains why lenalidomide at 1% concentration has similar results to Clobetasol (Fig.3), (tab.3). As lenalidomide concentrations increased, as in 2% and 3%, co-stimulatory signals increased, leading to T-cell activation. This promotes proliferation and differentiation. As a result, the production of pro-inflammatory cytokines such as TNF and IL-17 increased[27,34]. (Fig.3), (tab.3)

Conclusions

These findings show that lenalidomide ointment at a certain concentration can improve Imiquimod-induced psoriasis in mice. It is effective and has an efficacy similar to that of a standard drug, with a less thinning effect on the skin by reducing the generation of pro-inflammatory cytokines, which have been linked to

the pathogenesis of psoriasis by altering cytokine production and regulating T cell co-stimulation.

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