Original Article

Histological and Immunohistochemical Study of the Effect of Topically Applied Phenytoin on Chemically Induced Buccal Mucosal Ulcer in Albino Rats

Nahla Wahba[1], Rabab Hassan[2] and Dina M. Abdelkhalik[2]

Department of Oral Biology, Faculty of Dentistry, [1]Fayoum University, [2]Ain Shams University, Egypt

ABSTRACT

Background: Mucosal ulcers are common lesions that arise from chemical damage to oral mucosa. Phenytoin is one of the promising wound healing agents that may have the ability to decrease the duration of the healing process of wounds.

Objectives: To evaluate the effect of topical application of phenytoin on chemically induced buccal mucosal ulcer of albino rat.

Material and Methods: 63 male albino rats were subjected to chemical ulcer, then rats were divided into 3 main groups. Group I (control): rats did not receive any treatment, right side buccal mucosa acted as a negative control (Group I -ve), while left side (ulcerated) served as a positive control (Group I +ve). Group II (plain gel) rats received topical application of plain gel twice daily on the ulcer from the day following ulcer induction till sacrification. Group III (phenytoin gel) rats received topical application of 1% phenytoin gel twice daily on the ulcer from the day following ulcer induction till sacrification. Each group was further divided into 3 subgroups A, B and C in which rats were sacrificed at 4, 7 and 12 days following ulcer induction respectively. Buccal mucosae were dissected and examined histologically and immunohistochemically.

Results: Histologically, group I+ve showed complete epithelial degeneration at day 4, re-epithelization at day 7, complete regeneration at day 12. Group II showed complete epithelial degeneration day 4, re-epithelization started at day 7, partial regeneration at day 12. Group III showed beginning of re-epithelization at day 4, continuous epithelial lining at day 7, complete regeneration at day 12. Immunohistochemically and statistically, group III showed the highest anti-PCNA expression regarding positive epithelial cells followed by group II then group I+ve. Group I-ve showed the lowest mean.

Conclusions: Topical application of phenytoin 1% accelerate healing of chemically induced ulcers through increased vascularization, decreased inflammatory cells and hastened re-epithelization.

Received: 03 January 2021, **Accepted:** 23 January 2021

Key Words: Buccal mucosa; oral ulcers; phenytoin; wound healing.

Corresponding Author: Nahla Wahba, MSc, Department of Oral Biology, Faculty of Dentistry, Fayoum University, Egypt, **Tel.**: +201 559969139, **E-mail:** nwh11@fayoum.edu.eg

ISSN: 1110-0559, Vol. 44, No.4

INTRODUCTION

The buccal mucosa comprises the intraoral mucosal coating of the inner side of the cheeks covering the buccal space attaching superiorly and inferiorly to the mucosa of the alveolar ridges[1].

Ulcers are the most frequent inflammatory conditions in the oral cavity. The most frequent site affected with ulceration is the cheek mucosa[2]. Antibiotic and corticosteroids are commonly used in management of massive chemically induced lesions[3]. However; treatment with antibiotic and corticosteroids may lead to development of oral candidiasis which is considered a common side effect[4].

Phenytoin is an antiepileptic drug available since 1937. Gingival overgrowth was a noticeable adverse effect of the drug, this suggested that phenytoin aggravates the normal tissue turn over and proposed the possibility of using phenytoin as a wound healing agent[5]. Experimental studies using phenytoin were first carried out in the field of dentistry by Shapiro in 1958 who observed reduced pain, less inflammation, and speeded healing in periodontal surgical wounds of patients previously treated with systemic phenytoin[6].

This study was designed to assess the effect of topical application of phenytoin on oral mucosal ulcer induced chemically in albino rats at different durations.

MATERIALS AND METHODS

Ethical Clearance

The proposal of the present study was reviewed and accepted by the Research Ethics committee of the Faculty of Dentistry, Ain Shams University, Egypt. Committee approval number: FDASU-RecIM031702.

Materials

Animals

Sixty three adult male albino rats weighing between 200-250 grams were used in the present study. The rats

 DOI: 10.21608/ejh.2021.54287.1402

were kept in cages (20cm X 40cm), 4 rats/cage, in the Animal House of "the Medical Research Center" in Ain Shams University under supervision of specialized veterinarian during the experimental period. Rats were provided with stable diet consisting of dried bread, fresh vegetables as well as tap water, with good aeration during the whole period of the experiment.

Chemicals

- Phenytoin powder from Nile Pharmaceuticals.
- Chitosan powder from Chitosan-Egypt.
- Polyvinylpyrrolidone (PVP) powder from Egyptian Group for Pharmaceutical Industries (EGPI).
- 99.7% acetic acid and ethanol from Faculty of Pharmacy, Ain Shams University.

Methods

Preparation of 1% phenytoin gel

0.5 gm of phenytoin was prepared in 10 ml of ethanol, then was added to a mixture of 15ml chitosan 6% solution and 25 ml PVP 4% polymer solution gradually while stirring until 50ml of viscous 1% phenytoin gel was formed[7].

Preparation of plain gel

25 ml of PVP polymer solution was added to 15 ml chitosan 6% solution while stirring to form a mixture[7].

All steps of preparation was done in Faculty of Pharmacy, Ain Shams University.

Ulcer induction

All animals in groups I+ve, II and III were anesthetized by ether. The animals were then restrained and their mouths were kept open for the topical application of 99.7% acetic acid (50ul) into the left side buccal mucosa using a micro syringe[8]. For group III; the rat's left cheek was retracted and 0.1ml of phenytoin gel was applied to the ulcer area via graduated plastic syringe[7]. Plain gel was applied similarly for rats of group II.

Animal grouping

After chemical ulcer induction, the 63 rats were divided randomly into three main groups (21 rats each) as follows:

● Group I (Control group):

Group I-ve (Negative control): The right side buccal mucosa was left without ulceration and received no treatment to act as negative control group.

Group I+ve (Positive control): The ulcerated left buccal mucosae of rats in the control group were left untreated to heal normally and serve as a positive control.

● Group II (Plain gel): rats in this group received plain mucoadhesive gel, which was applied twice daily on the ulcer of left side buccal mucosae only as a vehicle of the drug starting from the day following ulcer induction (day 1) till the day of scarification.

● Group III (Phenytoin gel): Rats in this group received 1% phenytoin mucoadhesive gel, which was applied twice daily on the ulcer of left side buccal mucosastarting from the day following ulcer induction (day 1) and till the day of scarification.

All groups were further subdivided into 3 subgroups 7 rats each according to sacrificing periods as follows:

- Subgroup (A): rats were sacrificed on day 4 after ulcer induction.
- Subgroup (B): rats were sacrificed on day 7 after ulcer induction.
- Subgroup (C): rats were sacrificed on day 12 after ulcer induction.

Sacrification of rats was done by overdose administration of ketamine. Buccal mucosa was collected from each rat and the rest of rat's body was eliminated by the incinerator of Ain Shams Hospital.

Specimen collection and preparation

All excised specimens were prepared to be examined histologically.

I- Haematoxylin and Eosin stain (H&E)

Samples were fixed in 10% formalin for 24hr. Specimens were dehydrated by conveying in a series of increasing ethanol concentrations (50%, 60%, 80%, 90%, 96%, and absolute ethanol), then transferred to xylene for clearance of alcohol. Then, specimens were embedded in the center of paraffin blocks of wax following paraffin wax infiltration. The specimens embedded in wax blocks were sectioned by rotary microtome into 5 micrometer thick serial sections and transferred in descending alcohol concentrates (96%, 70%, distilled water). Then, the sections were mounted on clean glass slides to be stained with H&E and examined under light microscope.

II- Immunohistochemical procedure

Following deparafinization and rehydration of paraffin embedded sections, the sections were stained using mouse anti-proliferating cell nuclear antigen (Anti-PCNA). For anti-PCNA staining, sections embedded in paraffin were dewaxed using xylene. Slides were then washed in ethanol to remove excess xylene. Following pepsin treatment of tissues for 20min at 37°C, the slides were washed using phosphate buffer saline (PBS). Following endogenous blocking using 3% hydrogen peroxide, protein blocking was done using protein blocking solution that consisted of 5% horse serum with 1% goat serum. The antigens retrieval was done using microwaving the sections in buffer of sodium citrate (10mM sodium citrate, pH 6.0) [9]. Samples were heated in a microwave for 10min. The sections were cooled at room temperature (25°C) for 30min, then the 10-min heating process was repeated using fresh buffer (25°C). After that addition of primary antibodies were done at 4°C for 18hr. Washing of slides with PBS was done followed by blocking again using

protein-blocking solution for 1hr, and incubation using horseradish peroxidase–conjugated anti-rat antibody at 1:200 dilutions for 1hr at room temperature. The slides were washed with PBS again for three times and then were incubated for 10min using 3, 3'diaminobenzidine. Hematoxylin was used for counterstaining after washing away excess 3, 3'diaminobenzidine[10]. Specimens were mounted in DPX and examined under light microscope. Positive reaction was detected as brown nuclear discoloration.

III- Histomorphometricanalysis

Three microscopic fields showing the highest anti-PCNA immunopositivity in both the epithelium and connective tissue were chosen and capture of photomicrographs was done at original magnification of X400.

Brightness of images was adjusted in order to only visualize anti-PCNA positive cells in epithelium and connective tissue. Conversion of images into binary black and white type was done. Then watershed was performed for separating two adjacent cells that had been formerly recognized as one. The icon of show ellipses was selected in order to see where and which cells recognized by the program. Then counting of positive cells was done automatically.

Capturing of all images was done using digital camera (EOS 650D, Cannon, Japan) that was mounted on a light microscope (BX60, Olympus, Japan). All images were transferred after that to the computer system to be analyzed using Image J, 1.41a, (NIH, USA) image analysis software.

IV- Statistical analysis

Recorded data was analyzed via the statistical package for social sciences, version 20.0 (SPSS Inc., Chicago, Illinois, USA). Expression of quantitative data was done as mean± standard deviation (SD). Comparing between more than two means was done using a one-way analysis of variance (ANOVA) test. Post Hoc test: Least Significant Difference (LSD) was used for multiple comparisons between different variables. Interval of confidence was set to 95% and the accepted margin of error was set to 5%. Accordingly, *P-value* <0.05 was considered significant.

RESULTS

Histological results (H&E) stain

Group I-ve (Negative control)

Right side buccal mucosa showed; keratinized stratified squamous epithelium with regular, broad and short rete pegs. The lamina propria showed well organized collagen fibers and blood vessels (B.Vs) of relatively average size (Figure 1a).

No difference was found regarding histological appearance of buccal mucosa in negative control subgroups during different experimental periods.

Ulcerated buccal mucosa after 4 days

Subgroup (I+ve)A (positive control)

Epithelium was completely lost over the ulcerated area with the presence of inflammatory cells infiltration and B.Vs in the underlying dense lamina propria. Keratinized stratified squamous epithelium was seen in the ulcer edges (Figures 1 b,c).

Subgroup IIA (Plain gel)

The ulcerated area showed complete loss of epithelium with detached keratin layer at ulcer edges. Few inflammatory cells were detected in the underlying loose lamina propria (Figure 1d).

Subgroup IIIA (Phenytoin gel): The ulcerated area showed beginning of re-epithelialization in the form of basal cell layer of epithelium. The ulcer edges showed well-developed rete pegs. The underlying lamina propria appeared loose with inflammatory cell infiltration and neovascularization (Figures 1 e,f).

Ulcerated buccal mucosa after 7 days

Subgroup (I+ve)B (positive control)

The ulcerated area showed beginning of re-epithelization in the form of basal and parabasal cell layers with marked discontinuity in epithelium in the center of the ulcer. Lamina propria appeared loose with irregularly arranged fibers and inflammatory cells infiltrate (Figure 2a).

Subgroup IIB (Plain gel)

Newly formed epithelial lining of basal and parabasal layers with marked discontinuity in some areas. Lamina propria appeared loose (Figure 2b).

Subgroup IIIB (Phenytoin gel)

Newly formed continuous epithelial lining was noticeable in the ulcerated area in the form of stratified squamous epithelium, keratin layer was detected in some areas (Figure 2c). The underlying lamina propria appeared loose in some areas and dense in others with few inflammatory cells and extravasated red blood cells (Figure 2d).

Ulcerated buccal mucosa after 12 days

Subgroup (I+ve)C (positive control)

The ulcerated area revealed newly formed epithelial lining in the form of basal and parabasal cell layers. The underlying lamina propria appeared dense in some areas and loose in others (Figure 3a).

Subgroup IIC (plain gel)

Re-epithelization in the form of basal and prickle cell layers was observed with marked discontinuity in epithelium in the center. The underlying lamina propria appeared dense in some areas and loose in others with B.Vs and inflammatory cells (Figure 3b).

Subgroup IIIC (phenytoin gel)

Complete epithelial regeneration with detectable rete pegs was seen in this subgroup. The newly formed epithelium showed detached keratin layer and marked keratohyaline granules in granular cell layer with hyperkeratosis in some areas. The lamina propria appeared loose with several newly formed B.Vs and few inflammatory cells infiltration (Figures 3 c,d).

Immunohistochemical results (Anti-PCNA antibody)

Group I-ve (negative control)

The epithelium showed positive reaction in basal cell layer. The connective tissue (c.t) cells showed detectable positive reaction (Figure 4a).

No difference was found regarding immunohistochemical stain distribution of buccal mucosa in negative control subgroups during different experimental periods.

Ulcerated buccal mucosa after 4 days

In subgroups (I+ve)A and IIIA, the epithelium of ulcer edges showed positive reaction in basal, prickle and granular cell layers (Figures 4 b,d). While in subgroup IIA the epithelium showed positive reaction in basal cell layer (Figure 4c). Some c.t cells in lamina propria in all these subgroups showed detectable positive reaction.

Ulcerated buccal mucosa after 7 days

In subgroups (I+ve)B and IIB, the epithelium of ulcer edges showed positive reaction in basal cell layer (Figures 5 a,b), while in subgroup IIIB the epithelium of ulcer edges showed positive reaction in basal and prickle cell layers in addition to positive reaction in basal cell layer of regenerated epithelium (Figure 5c). Lamina propria of all these subgroups showed positive reaction in some c.t cells.

Ulcerated buccal mucosa after 12 days

The regenerated epithelium in subgroups (I+ve)C and IIIC showed positive reaction in basal and prickle cell layers (Figures 6 a,c), while in subgroup IIC the regenerated epithelium showed positive reaction in the basal cell layer in addition to positive reaction in basal and prickle cell layers of epithelium of ulcer edges (Figure 6b). Positive reaction was detected in some c.t cells in all these subgroups.

Statistical results

Comparison of anti-PCNA positive epithelial cells

Subgroup A: There was no statistical significant difference between subgroups IIA and IIIA, subgroup IIIA showed the highest mean. There was no statistical significant difference between subgroups (I+ve)A and (I-ve)A, subgroup (I-ve)A showed the lowest mean (Table 1, Figure 7a).

Subgroup B: Subgroup IIIB showed the highest mean followed by subgroup (I+ve)B then subgroup IIB, subgroup (I-ve)B showed the lowest mean (Table 1, Figure 7b).

Subgroup C: Subgroup IIIC showed the highest statistically significant mean followed by subgroup IIC. There was no statistical significant difference between subgroups (I+ve)C and (I-ve)C, subgroup (I-ve)C showed the lowest mean (Table 1, Figure 7c).

Comparison of anti-PCNA positive c.t cells

Subgroup A: There was no statistical significant difference between subgroup (I+ve)A, subgroup IIA and subgroup IIIA. The highest mean was in subgroup (I+ve) A followed by subgroup IIIA then subgroup IIA. Subgroup (I-ve)A showed the lowest statistically significant mean (Table 2, Figure 8a).

Subgroup B: There was no statistical significant difference between subgroup (I+ve)B, subgroup IIB and subgroup IIIB. The highest mean was in subgroup IIIB followed by subgroup IIB then subgroup (I+ve)B. Subgroup (I-ve)B showed the lowest statistically significant mean (Table 2, Figure 8b).

Subgroup C: There was no statistical significant difference between subgroups IIC and IIIC. Subgroup IIIC showed the highest mean followed by subgroup IIC. There was no statistical significant difference between subgroup (I+ve)C and subgroup (I-ve) C. The lowest statistically significant mean was in subgroup (I-ve) C (Table 2, Figure 8c).

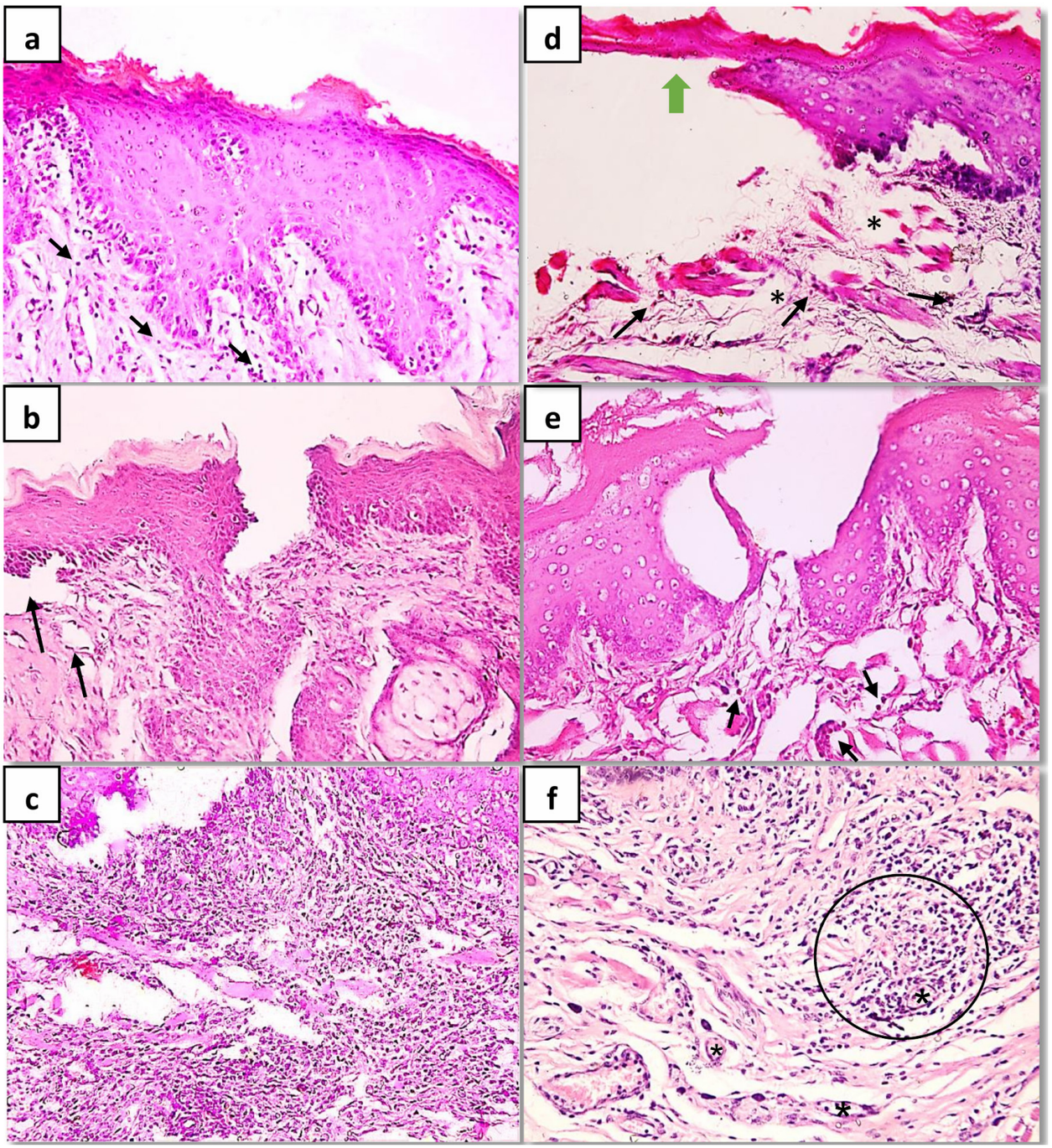

Fig. 1: Photomicrographs of subgroup A showing: a- Subgroup (I-ve)A: keratinized stratified squamous epithelium with few inflammatory cells (arrow) in lamina propria. b- Subgroup (I+ve)A showing complete loss of epithelium over the ulcerated area with some degenerated areas in lamina propria (arrow). c- Subgroup (I+ve)A showing inflammatory cells infiltration within lamina propria. d- Subgroup IIA showing detached keratin layer (green arrow) at ulcer edges with complete loss of epithelium over the ulcerated area and inflammatory cells (black arrow) in loose lamina propria with degenerated areas (asterisk).e- Subgroup IIIA showing ulcer edges with well-developed rete pegs. Ulcerated area showing beginning of re-epithelialization of basal layer with inflammatory cells (arrow) in loose lamina propria. f- Subgroup IIIA showing inflammatory cell infiltration (circle) and neovascularization (asterisk) in loose lamina propria (H&E orig. mag. X400).

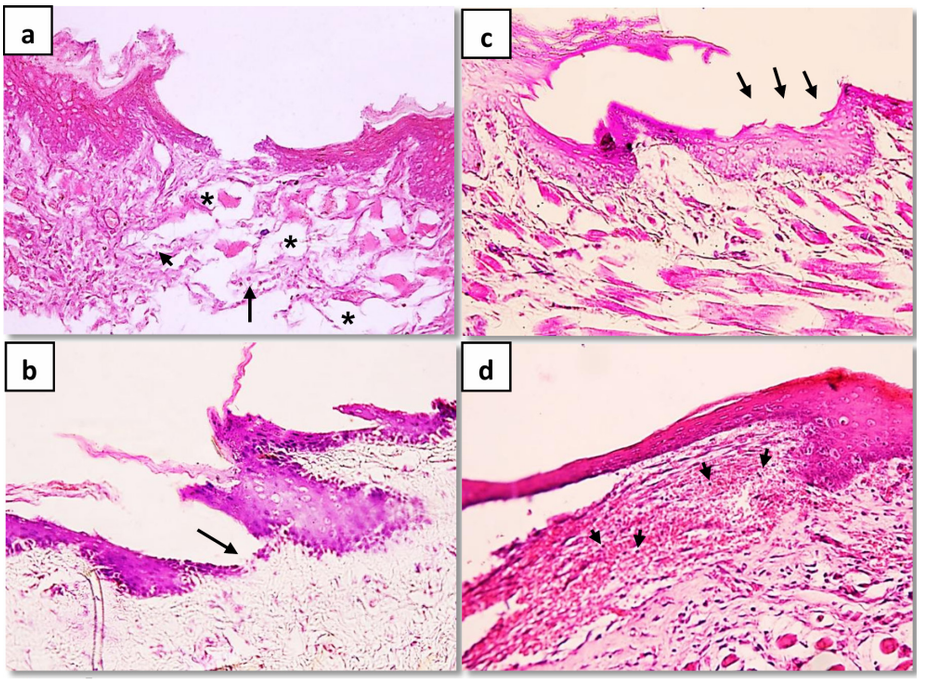

Fig. 2: Photomicrographs of subgroup B showing: a- Subgroup (I+ve)B showing beginning of re-epithelization of basal and parabasal layers. Loose lamina propria (asterisk) infiltrated with inflammatory cells (arrows). b- Subgroup IIB showing epithelial lining in the form of basal and parabasal layers with marked discontinuity in epithelium in some areas (arrow) and loose lamina propria. c- Subgroup IIIB showing newly formed continuous epithelial lining (arrows) formed of: basal, prickle and granular cell layers. d- Subgroup IIIB showing extravasated red blood cells and inflammatory cells infiltration (arrows) (H&E orig. mag. X400).

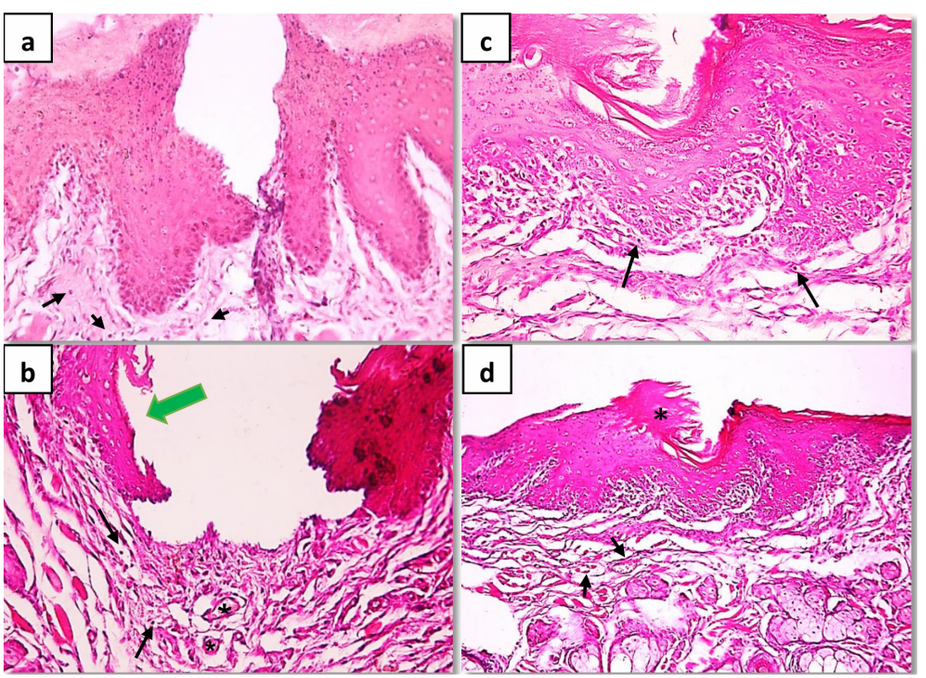

Fig. 3: Photomicrographs of subgroup C showing: a- Subgroup (I+ve)C with newly formed basal and parabasal layers covering the ulcer area. Few scattered inflammatory cells (arrows) in the lamina propria. b- Subgroup IIC showing re-epithelization in the form of basal and prickle cell layers (green arrow). Lamina propria appeared loose in some areas with B.Vs (asterisk) and inflammatory cells (black arrows). c- Subgroup IIIC showing complete epithelium regeneration of keratinized stratified squamous epithelium. The newly formed epithelium showing detached keratin layer and marked keratohyaline granules in the granular cell layer. Lamina propria with few inflammatory cells (arrows). d- Subgroup IIIC showing increase in the keratin layer (hyperkeratosis) in some areas (asterisk) in newly formed epithelium. Detectable rete pegs. Lamina propria appeared loose with several newly formed B.Vs (arrows) (H&E orig. mag. (a,b,c X400) (dX200)).

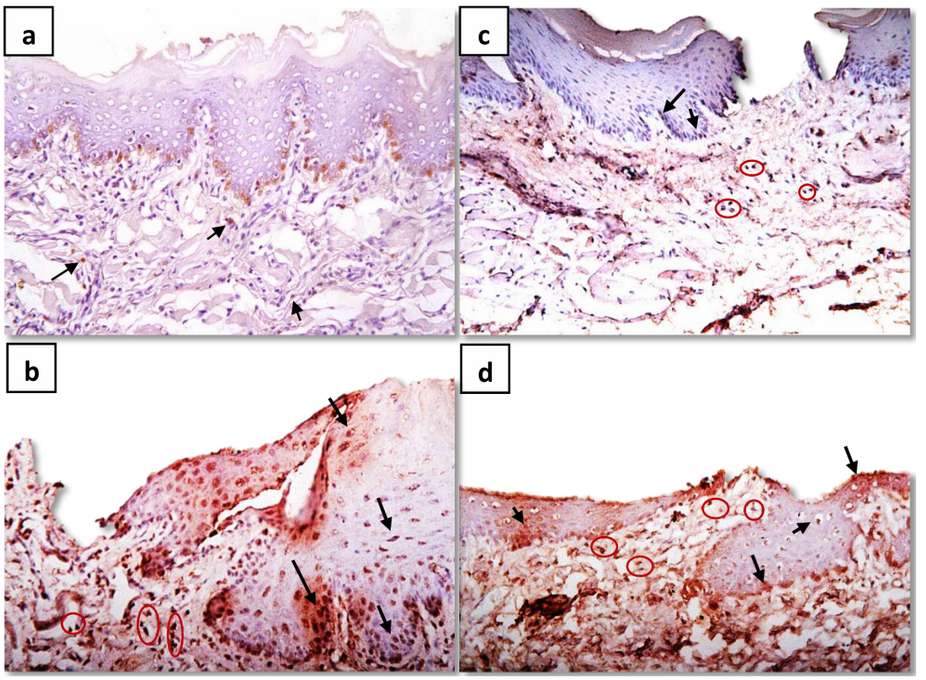

Fig. 4: A photomicrograph of subgroup A showing a- Subgroup I-ve with positive anti-PCNA reaction in the basal cell layer and few positive cells in the underlying lamina propria (arrows). b- Subgroup (I+ve)A showing positive reaction in basal, prickle and granular cell layers of epithelium of ulcer edges (arrows) with positive reaction in some c.t cells (circles). c- Subgroup IIA showing few positive reaction in basal cell layer of epithelium of ulcer edges (arrows). Some c.t cells with positive reaction (circles). d- Subgroup IIIA showing positive reaction in basal, prickle and granular cell layers of epithelium of ulcer edges (arrows) with detectable positive reaction in c.t cells (circles) (Anti-PCNA orig. mag. X400).

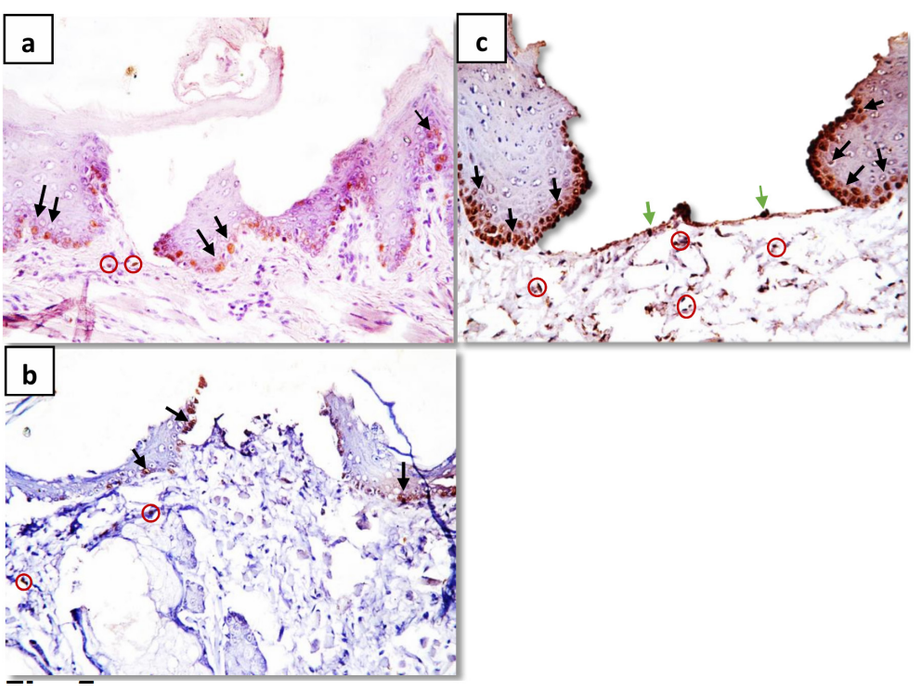

Fig. 5: Photomicrographs of subgroup B showing: a- subgroup (I+ve)B showing some positive reaction in basal cell layer of epithelium lateral to the ulcer (arrows) with few positive c.t cells (circles). b- Subgroup IIB showing positive reaction in the basal cell layer of epithelium lateral to the ulcer (arrows). Few c.t cells with positive reaction (circles). c- Subgroup IIIB showing positive reaction in basal and prickle cell layers of epithelium lateral to the ulcer (black arrows) and in the basal layer of regenerated epithelium (green arrows). Some c.t cells with positive reaction (circles) (Anti-PCNA orig. mag. X400).

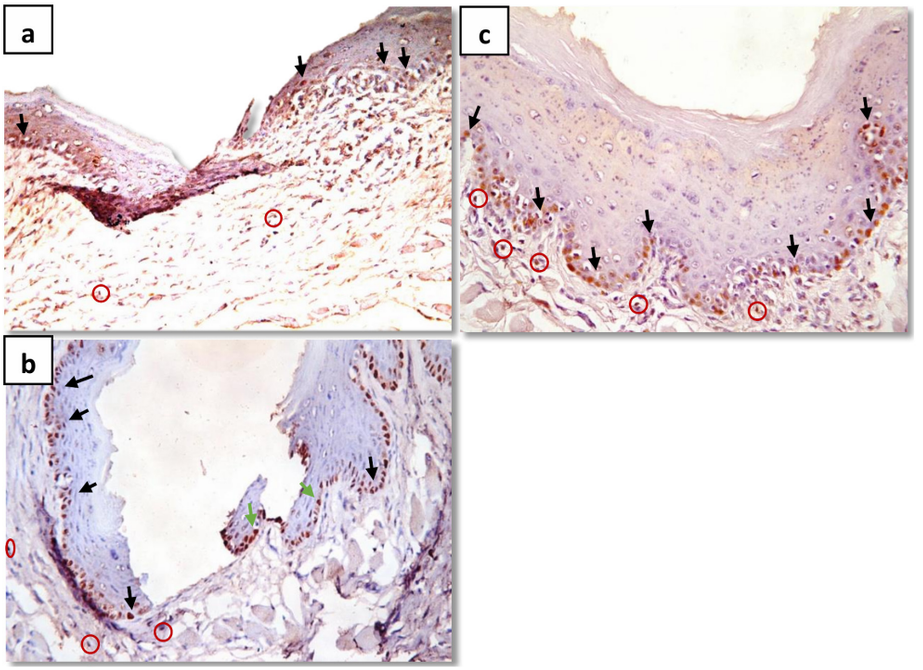

Fig. 6: Photomicrographs of subgroup C showing: a- Subgroup (I+ve)C with positive reaction in basal and prickle cell layers of regenerated epithelium (arrows) with some positive c.t cells (circles). b- Subgroup IIC showing positive reaction in the basal and prickle cell layers of epithelium of ulcer edges (black arrows) and basal layer of regenerated epithelium (green arrows). Some c.t cells showed positive reaction (circles). c- Subgroup IIIC showing positive reaction in basal and prickle cell layers of the regenerated epithelium (arrows) with positive reaction in some c.t cells (circles) (Anti-PCNA orig. mag. X400).

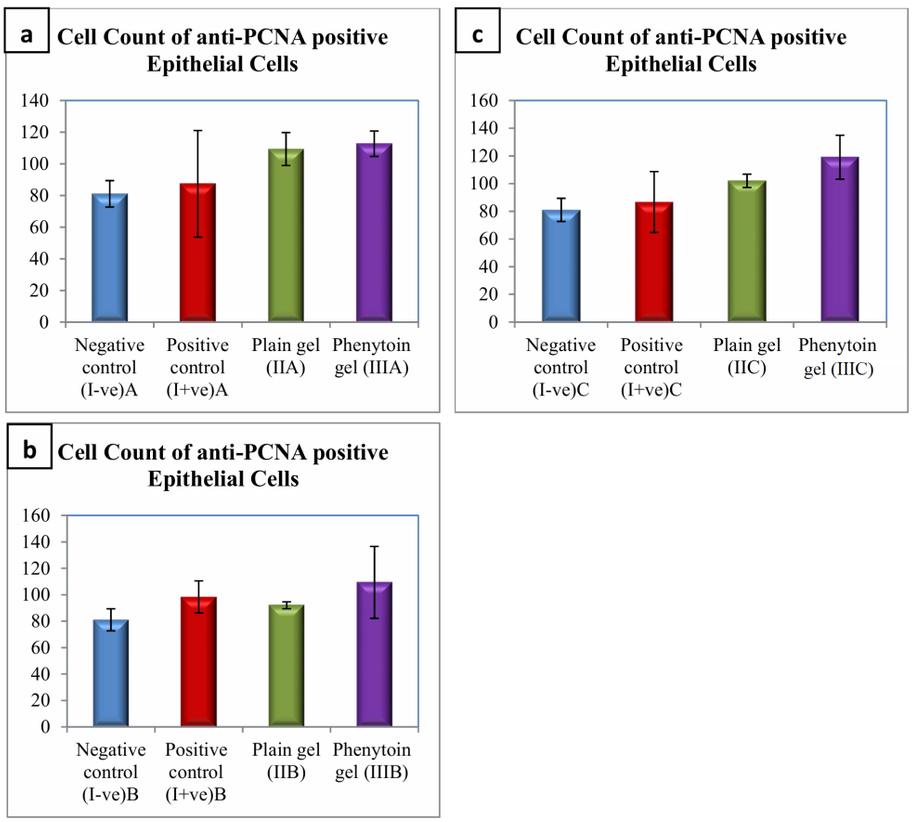

Fig. 7: Bar charts showing comparison of anti-PCNA positive epithelial cells in: a- Subgroup A. b- Subgroup B. c- Subgroup C.

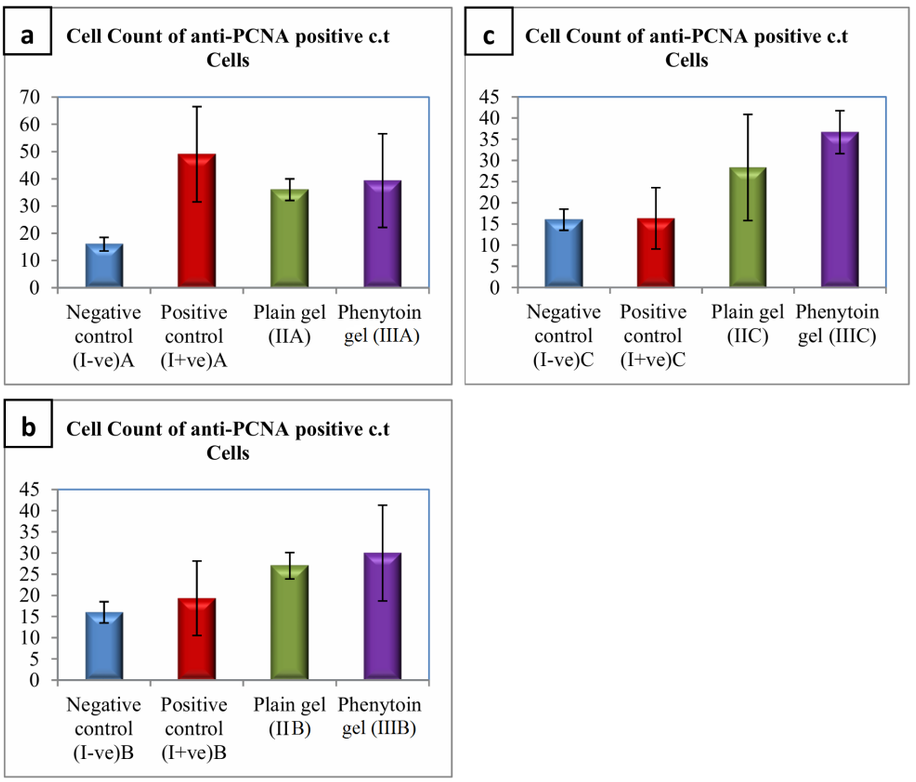

Fig. 8: Bar charts showing comparison of anti-PCNA positive c.t cells in: a-Subgroup A. b- Subgroup B. c- Subgroup C.

Table 1: showing the mean ± SD values, results of ANOVA and post hoc tests for the comparison of anti-PCNA positive epithelial cells in different subgroups

Cell Count of anti-PCNA positive epithelial cells		Negative control (-ve)	Positive control (+ve)	Plain gel	Phenytoin gel	ANOVA	*p-value*
Subgroup A	Mean	81.00^{b}	87.33^{b}	109.33^{a}	112.67^{a}	3.891	0.034*
	±SD	8.36	33.78	10.44	8.05		
Subgroup B	Mean	81.00^{c}	98.33^{ab}	92.00^{bc}	109.33^{a}	2.326	0.039*
	±SD	8.36	12.13	2.60	27.18		
Subgroup C	Mean	81.00^{c}	86.67^{c}	102.00^{b}	119.00^{a}	9.312	<0.001**
	±SD	8.36	21.98	4.82	15.87		

Different superscript letters in the same row indicate significant difference between each subgroup.
**p-value* <0.05 is significant. ***p-value* <0.001 is highly significant.

Table 2: showing the mean ± SD values, results of ANOVA and post hoc tests for the comparison of anti-PCNA positive c.t cells in different subgroups

Cell Count of anti-PCNA positive c.t cells		Negative control (-ve)	Positive control (+ve)	Plain gel	Phenytoin gel	ANOVA	*p-value*
Subgroup A	Mean	16.00^{b}	49.00^{a}	36.00^{a}	39.33^{a}	1.991	0.158
	±SD	2.5	17.51	3.97	17.20		
Subgroup B	Mean	16.00^{b}	19.33^{a}	27.00^{a}	30.00^{a}	1.611	0.221
	±SD	2.5	8.08	3.12	11.30		
Subgroup C	Mean	16.00^{b}	16.33^{b}	28.33^{a}	36.67^{a}	7.318	0.003*
	±SD	2.5	7.24	12.53	5.07		

Different superscript letters in the same row indicate significant difference between each subgroup.
**p-value* <0.05 is significant

DISCUSSION

Ulcerations of the oral mucosa are common conditions that may severely affect life quality. Oral ulcers are caused by various conditions including trauma, infections, systemic disease, adverse drug reaction or allergic reaction[11].

Phenytoin application is one of the approaches that has been used to decrease the duration of wound healing process in various tissues and has revealed promising results in different types of wounds[12].

Buccal mucosa was chosen in the present study because it is the most common site affected by oral ulceration[3].

Topical application of phenytoin was chosen in the current study rather than systemic administration due to entrance of the drug directly to the targeted site without getting systemic adverse effects[13]. Gel formulation has been prepared and used in this study, as it was reported to be the most appropriate for clinical trials[14].

PVP used in the present study as component of drug base is one of the most known chemically inert polymer in physiological reaction[15]. While chitosan which was used as an ingredient in the same drug base may have a possible synergistic effect. So, drug base group was included in this study for the elimination of the interfering effect of chitosan polymer used to prepare phenytoin gel[16].

Anti-PCNA antibody has been used in the present study to detect proliferating cells as it is considered a valuable tool for assessing cell proliferation throughout wound healing[17].

In this study, group I-ve showed ortho-keratinized stratified squamous epithelium with regular, broad and short rete pegs. The lamina propria showed well organized collagen fibers and B.Vs of relatively average size. All of these findings are in accordance with Ahlfors & Larsson[18].

In the present study, subgroup (I+ve)A showed ortho-keratinized stratified squamous epithelium at the ulcer edges, while the ulcerated area had marked discontinuity of all epithelial layers with the presence of inflammatory cells infiltration and blood vessels in the underlying dense lamina propria, these results are in accordance with Ishiyama *et al*.,[8] who reported complete epithelium at ulcer edges in control group after 5 days of buccal mucosal ulcer induction in albino rats by acetic acid injection, with the presence of inflammatory cells, B.Vs and dense fibers in lamina propria of the ulcer base.

In the current study, subgroup IIA showed complete loss of epithelium at the ulcerated area with the presence of few inflammatory cells in the underlying loose lamina propria, these results are similar to Hagh *et al*.,[16] who reported decrease of inflammatory cells count on the 3rd day in chitosan group in hard palate wound of albino rat, they accredited this result to antimicrobial and bacteriostatic activity of chitosan. Obvious discontinuity of epithelium at ulcerated area reported in this study in the same subgroup is in accordance with Mahattanadul *et al*.,[19] who detected largest oral ulceration size in chitosan vehicle group in buccal mucosa of hamster on the 4th day without epithelization at the ulcer surface.

In the present study, subgroup IIIA showed heavy inflammatory cells infiltration and neovascularization in the underlying lamina propria, these results are in accordance with Sayar *et al*.,[20] who observed formation of new capillaries in a burn wound of rat's skin at a higher level in the phenytoin cream group compared to other groups starting from 3rd day of treatment. The partial re-epithelization which was observed in the basal layer of the ulcerated area in the same subgroup is in accordance with Assareh *et al*.,[7] who reported enhancement of re-epithelization in hard palate wound of albino rat increasing from day 3 to day 14.

Moreover, the inflammatory cells infiltration and neovascularization in subgroup IIIA in this study could be related to the ability of phenytoin to induce fibroblasts to release cytokines that lead to accumulation of lymphocytes and formation of B.Vs. Furthermore, cytokines released by lymphocytes may lead to angiogenesis according to Pitiakoudis *et al*.,[21].

In the current study, subgroup (I+ve)B presented beginning of re-epithelization in the form of basal and parabasal cell layers with marked discontinuity in epithelium in the center of the ulcer, lamina propria had inflammatory cells infiltrate. These findings are in accordance with Cavalcante *et al*.,[22] who observed through microscopic evaluation re-epithelization and decrease in untreated ulcer area without complete healing of buccal mucosa of mice from the 5th day till the 8th day with moderate inflammatory cells in c.t.

In the present study, subgroup IIB showed newly formed epithelial lining of basal and parabasal layers with marked discontinuity in some areas, this result is in parallel with Hagh *et al*.,[16] who reported lower rate of epithelialization in chitosan base group on the 7th day when compared to phenytoin gel treated group. The same subgroup showed loose lamina propria, this result is in accordance with Stone *et al*.,[23] who reported looser c.t stroma in papillary dermis at skin graft donor sites covered by chitosan dressing in comparison to control group biopsies.

In the present study, subgroup IIIB showed newly formed continuous epithelial lining, this result is in accordance with Hagh *et al*.,[16] who noticed higher rate of epithelialization on 7th day in phenytoin gel group compared to control group. The same subgroup in this study showed few inflammatory cells in lamina propria. This finding is in parallel with Aminifard *et al*.,[24] who reported decreased inflammatory cells in corneal ulcer of rabbits treated with phenytoin, and this was related to the antibacterial effect of topical phenytoin. In addition, extravasated red blood cells from congested B.Vs in lamina propria were seen in the same subgroup. This result is similar to Gurgel *et al*.,[25] who observed areas of erythrocytes extravasation in some areas of congested B.Vs of phenytoin induced gingival enlargement (PIGE) of epileptic patient.

Relative increase in B.Vs density observed in subgroups IIIA & IIIB in the current study is also in agreement with Sayar *et al*.,[20]; Turan *et al*.,[26] who reported increased amounts of B.Vs from 3rd to 7th day following phenytoin treatment. This could be explained due to increased expression of vascular endothelial growth factor (VEGF) which is consistent with proliferative phase in process of wound healing.

In the present study, the ulcerated area of subgroup (I+ve)C showed epithelial lining in the form of basal and parabasal cell layers with underlying loose lamina propria. These results are in accordance with Karavana *et al*.[27] and Miao *et al*.,[28] respectively. Moreover, Idrus *et al*.,[29] reported complete healing of acetic acid induced ulceration of the buccal mucosa from 11 to 14 days and that was due to variation in healing capability and wound depth.

In the current study, subgroup IIC showed re-epithelization up to prickle cell layer with discontinuity in epithelium in the center. The underlying lamina propria appeared dense in some areas and loose in others with B.Vs and inflammatory cells. Re-epithelization is in similarity to Ribeiro *et al*.[30] and El Sadik *et al*.,[31] who observed progressive increase in epithelial layer thickness from day 7 till complete epithelialization on day 21 in skin wound of rat receiving chitosan hydrogel, while inflammatory cells is in accordance with El Sadik *et al*.,[31]; Alsarra[32] who attributed this to chitosan releasing active dimer that chemotracts inflammatory cells, these inflammatory cells then release lysozymes that promote the release of active dimer from chitosan once again through catalysis of chitosan degradation, subsequently chitosan turns into a constant source of active dimer during the time the wound has inflammatory cells until complete healing.

In the present study, the newly formed epithelium in subgroup IIIC showed keratohyalin granules in granular cell layer with hyperkeratosis in some areas, this result is similar to Sayar, *et al*.,[20] who observed keratohyalin granules in stratum granulosum of a burn wound in rat treated with topical phenytoin. The visibility and demarcation of keratohyalin granules in stratum granulosum are due to the abundance of constituents of these granules "profilaggrin/filaggrin", indicating tendency toward hyper-keratinization[33]. Complete epithelial regeneration in the same subgroup with detectable rete pegs, is in similarity to Gurgel *et al*.,[25] who observed numerous well developed rete pegs in biopsies of PIGE. In addition to complete epithelial regeneration, lamina propria in the same subgroup appeared loose with apparently several newly formed B.Vs and few inflammatory cells. These findings are in accordance with Taheri *et al*.,[34] who reported healing of excisional wound in skin of rat with completely formed orthokeratinized stratified squamous epithelium as well as few lymphocytic infiltration in c.t on day 14. Blood vessels in the underlying lamina propria in the same subgroup in the present study is similar to Assareh *et al*.,[7] where they observed increased density of B.Vs in hard palate wound of rat on days 3, 7 and 14 in topical phenytoin group.

In this study, complete epithelium regeneration in subgroup IIIC is in accordance with Das & Olsen,[35] who attributed this to phenytoin ability to increase keratinocyte growth factor (KGF) secretion and receptor expression in epithelial cells by more than 150%. The earlier complete ulcer healing attained in the present study in subgroup IIIC rather than other subgroups is in agreement with Qunaibi *et al*.,[36] who concluded that phenytoin needs much less days than placebo to achieve the same histological wound healing effect in skin wound of rat.

In the present study, the immunohistochemical and statistical analysis results of group I-ve (negative control) revealed insignificant positive reaction in the basal cell layer only of epithelium. Lack of suprabasal positive reaction could be related to absence of special proliferative cellular state in normal oral mucosa as reported by Keshav & Narayanappa[37].

Less anti-PCNA expression in epithelial cells in group I+ve in all days compared to group III is in accordance with Jainu *et al*.,[38] who reported reduction of anti-PCNA detected following administration of acetic acid and accredited this to acetic acid ability to inhibit angiogenic factors at ulcer margin and base.

In the current study, reduced anti-PCNA expression in group II relative to phenytoin group is in accordance with Othman *et al*.,[39] who reported that group treated with chitosan encapsulated drug showed significantly reduced anti-PCNA expression relative to other group treated with drug free from chitosan.

In this study, the highest anti-PCNA expression regarding positive epithelial cells was observed in group III. This finding indicated the presence of great number of proliferative epithelial cells. This result is in accordance with Turan *et al*.,[26] who reported increase in epithelialization in incisional wound of rat skin topically treated with phenytoin. Furthermore, this finding is also in accordance with Simsek *et al*.,[40] who observed increased levels of anti-PCNA in rat nasal wound treated with phenytoin.

There was no statistically significant difference in this study concerning anti-PCNA positive c.t cells which indicated insignificant proliferation of c.t cells within all subgroups. This finding is in parallel with Kantarci *et al*.,[41] who reported reduction of apoptosis in fibroblasts rather than proliferation due to decreased inflammation attained by phenytoin action. Furthermore, Kim *et al*.,[42] reported that phenytoin did not significantly increase proliferation of c.t cells, and suggested that PIGE was only due to increased matrix deposition.

CONCLUSIONS

Histological, immnunohistochemical and statistical results suggested that the topical application of 1% phenytoin help in accelerating healing of chemically induced ulcer through hastened re-epithelization, increased vascularization and decreased inflammation. The healing

rate was increased in time dependent manner in comparison to other groups.

CONFLICT OF INTERESTS

There are no conflicts of interest.

REFERENCES

1. Chhetri DK, Rawnsley JD and Calcaterra TC: Carcinoma of the buccal mucosa. Otolaryngol. Head Neck Surg. (2000) 123(5):566-71.
2. Dewedar O, Farid M and Adawy H: Potential Effect of Fibroblast Growth Factor (FGF2) Versus Epidermal Growth Factor (EGF) on Healing of Induced Oral Ulcer in Albino Rats (A Histological and Immunohistochemical Study). Al-Azhar Dent. J. Girls. (2018) 5(1):23-7.
3. Gilvetti C, Porter SR and Fedele S: Traumatic chemical oral ulceration: a case report and review of the literature. BDJ. (2010) 208(7):297-300.
4. Marable DR, Bowers LM, Stout TL, Stewart CM, Berg KM, Sankar V, DeRossi SS, Thoppay JR and Brennan MT: Oral candidiasis following steroid therapy for oral lichen planus. Oral Dis. (2016) 22(2):140-147.
5. Hasamnis AA, Mohanty BK and Patil S: Evaluation of wound healing effect of topical phenytoin on excisional wound in albino rats. J Young Pharm. (2010) 2(1):59-62.
6. Firmino F, Almeida AM, Alves GD, Grandeiro DD and Penna LH: Scientific production on the applicability of phenytoin in wound healing. Rev Esc Enferm USP. (2014) 48(1):162-169.
7. Assareh F, Yousefimanesh H, Saleh F, Zadeh M and Taherzadeh P: Histological comparison of efficacy of topical phenytoin and topical honey in wound healing of rat's hard palate: A pilot study. Int. J. Comp Leading Res. Sci. (2015) 1(1): 163–174.
8. Ishiyama H, Kawai K, Azuma A and Nagano C: Therapeutic effect of rebamipide in a modified acetic acid-induced buccal mucosal ulcer model. Inflammopharmacology. (2002) 10(4-6):391-399.
9. Kawai K, Serizawa A, Hamana T and Tsutsumi Y: Heat-induced antigen retrieval of proliferating cell nuclear antigen and p53 protein in formalin-fixed, paraffin-embedded sections. Pathol. Int. (1994) 44(10-11):759-764.
10. Kitana N, Won SJ and Callard IP: Reproductive deficits in male freshwater turtle Chrysemys picta from Cape Cod, Massachusetts. Biol. Reprod. (2007) 76(3):346-52.
11. Chen P, Yao H, Su W, He Y, Cheng K, Wang Y, Peng W and Li P: Sleep deprivation worsened oral ulcers and delayed healing process in an experimental rat model. Life Sci. (2019) 1(232):116594.
12. Fakhim A, Babaei H, Zarringhalam M and Ashrafi, J: Wound healing effect of topical phenytoin on rat palatal mucosa. Int J Curr Res. (2015) 3(6):463-476.
13. Hajong R, Naku N, Hajong D, Anand M, Singh K & Majumdar, N: Effect of topical phenytoin on wound healing. IOSR-JDMS. (2016) 15(9): 161-164.
14. Abdou AG, Abdelwahed Gaber M, Elnaidany NF and Elnagar A: Evaluation of the effect and mechanism of action of local phenytoin in treatment of vitiligo. J Immunoassay Immunochem. (2017) 38(5):523-537.
15. Teodorescu, M. & Bercea, M: Poly (vinylpyrrolidone) –a versatile polymer for biomedical and beyond medical applications. Polym Plast Technol Eng. (2015) 54(9): 923-943.
16. Hagh LG, Karimi B, Moghimipour E, Abdi Z, Abdolalian F and Rohani A: Histological Evaluation of Wound Healing Effect of Topical Phenytoin on Rat Hard Palate Mucosa. J. Res. Med. Dent. Sci. (2018)6(2):466-472.
17. Onuma H, Mastui C and Morohashi M: Quantitative analysis of the proliferation of epidermal cells using a human skin organ culture system and the effect of DbcAMP using markers of proliferation (BrdU, Ki-67, PCNA). Arch. Dermatol. (2001) 293(3):133-138.
18. Ahlfors EE and Larsson Å: Chemically induced inflammation in rat oral mucosa. Eur J Oral Sci. (1988) 96(5): 428-34.
19. Mahattanadul S, Mustafa MW, Kuadkaew S, Pattharachayakul S, Ungphaiboon S and Sawanyawisuth K: Oral ulcer healing and anti-candida efficacy of an alcohol-free chitosan-curcumin mouthwash. Eur. Rev. Med. Pharmacol. Sci. (2018) 22:7020-7023.
20. Sayar H, Gergerlioglu N, Seringec N, Ozturk P, Bulbuloglu E and Karabay G: Comparison of efficacy of topical phenytoin with hypericin in second-degree burn wound healing: an experimental study in rats. Med. Sci. Monit. Basic Res. (2014) 20:36.
21. Pitiakoudis M, Giatromanolaki A, Iliopoulos I, Tsaroucha AK, Simopoulos C and Piperidou C: Phenytoin-induced lymphocytic chemotaxis, angiogenesis and accelerated healing of decubitus ulcer in a patient with stroke. J Int Med Res. (2004) 32(2):201-205.
22. Cavalcante GM, Paula RJ, Souza LP, Sousa FB, Mota MR and Alves AP. Experimental model of traumatic ulcer in the cheek mucosa of rats. Acta Cir Bras. (2011) 26(3):227-34.
23. Stone CA, Wright H, Devaraj VS, Clarke T and Powell, R: Healing at skin graft donor sites dressed with chitosan. Br. J. Plast. Surg. (2000) 53(7): 601-606.

24. Aminifard MN, Yazdi H and Pahlavanzadeh S: Evaluation of Topical Phenytoin Eye Drop 1% Therapeutic Effects on Corneal Alkali Burns in Rabbit Model. Iran J Ophthalmol. (2012) 24(3): 33-38.

25. Gurgel BC, Morais CR, Rocha-Neto PC, Dantas EM, Pinto LP and Costa AD: Phenytoin-induced gingival overgrowth management with periodontal treatment. Braz. Dent. J. (2015) 26(1):39-43.

26. Turan M, Ünver Saraydýn S, Eray Bulut H, Elagöz S, Çetinkaya Ö, Karadayi K, Canbay E and Sen M: Do vascular endothelial growth factor and basic fibroblast growth factor promote phenytoin's wound healing effect in rat? An immunohistochemical and histopathologic study. Dermatol. Surg. (2004) 30(10):1303-1309.

27. Karavana SY, Sezer B, Güneri P, Veral A, Boyacioğlu H, Ertan G and Epstein JB. Efficacy of topical benzydamine hydrochloride gel on oral mucosal ulcers: an in *vivo* animal study. Int. J. Oral Maxillofac. (2011) 40(9): 973-978.

28. Miao M, Peng M, Xing Z and Liu D: Effect of Shuangjinlian mixture on oral ulcer model in rat. Saudi J. Biol. Sci. (2019) 26(4):790-794.

29. Idrus E, Hartanti PD, Suniarti DF, Prasetyo SR, Wimardhani YS and Subarnbhesaj A: An experimental model of chemically-induced ulceration of the buccal mucosa of Mus musculus. Makara J. Sci. (2019) 23(3):10.

30. Ribeiro MP, Espiga A, Silva D, Baptista P, Henriques J, Ferreira C, Silva JC, Borges JP, Pires E, Chaves P, and Correia IJ: Development of a new chitosan hydrogel for wound dressing. Wound Repair Regen. (2009) 17(6): 817-24.

31. El Sadik AO, El Ghamrawy TA and Abd El-Galil TI: The effect of mesenchymal stem cells and chitosan gel on full thickness skin wound healing in albino rats: histological, immunohistochemical and fluorescent study. PLOS one. (2015) 10(9):e0137544.

32. Alsarra IA: Chitosan topical gel formulation in the management of burn wounds. Int. J. Biol. Macromol. (2009) 45(1):16-21.

33. McLean WH: Filaggrin failure–from ichthyosis vulgaris to atopic eczema and beyond. Br. J. Dermatol. (2016) 175: 4-7.

34. Taheri JB, Bagheri F, Mojahedi M, Shamloo N, Nakhostin MR, Azimi S and Namazi Z: Comparison of the effect of low-level laser and phenytoin therapy on skin wound healing in rats. J Lasers Med Sci. (2015) 6(3):124.

35. Das SJ and Olsen I: Up-regulation of keratinocyte growth factor and receptor: a possible mechanism of action of phenytoin in wound healing. Biochem. Biophys. Res. Commun. (2001) 282(4): 875-881.

36. Qunaibi EA, Disi AM and Taha MO: Phenytoin enhances collagenization in excision wounds and tensile strength in incision wounds. Pharmazie. (2009) 64(9): 584-586.

37. KeShav R and Narayanappa U. Expression of proliferating cell nuclear antigen (PCNA) in oral submucous fibrosis: an immunohistochemical study. J Clin Diagn Res. (2015) 9(5): ZC20-ZC23.

38. Jainu M, Vijaimohan K and Kannan K: Cissus quadrangularis L. extract attenuates chronic ulcer by possible involvement of polyamines and proliferating cell nuclear antigen. Pharmacogn. Mag. (2010) 6(23):225.

39. Othman AI, El-Sherbiny IM, ElMissiry MA, Ali DA and AbdElhakim E: Polyphenon-E encapsulated into chitosan nanoparticles inhibited proliferation and growth of Ehrlich solid tumor in mice. Egypt. J. basic appl. sci. (2018) 5(1):110-120.

40. Şimşek G, Ciftci O, Karadag N, Karatas E and Kizilay A: Effects of topical phenytoin on nasal wound healing after mechanical trauma: An experimental study. Laryngoscope. (2014) 124(12): E449-E454.

41. Kantarci A, Augustín P, Firatli E, Sheff MC, Hasturk H, Graves DT and Trackman PC: Apoptosis in gingival overgrowth tissues. J. Dent. Res. (2007) 86(9): 888-892.

42. Kim SS, Michelsons S, Creber K, Rieder MJ and Hamilton DW: Nifedipine and phenytoin induce matrix synthesis, but not proliferation, in intact human gingival connective tissue ex *vivo*. Cell Commun. Signal. (2015) 9(4): 361-375.

الملخص العربى

دراسة هستولوجية وهيستوكيميائية مناعية لتأثير الفينيتوين المُستخدم موضعياً على قرحة الغشاء المخاطي الشدقي المستحثة كيميائيا في الفئران البيضاء

نهلة وهبه[١]، رباب حسن[٢]، دينا محمد عبدالخالق[٢]

قسم بيولوجيا الفم كلية طب الأسنان، [١]جامعة الفيوم، [٢]جامعة عين شمس

المقدمة: تقرحات الغشاء المخاطي الشدقي هي واحدة من الحوادث الشائعة الناتجة عن التعرض للكيماويات. يعتبر الفينيتوين أحد العوامل الواعدة في التئام الجروح والتي قد يكون لها القدرة على تقليل مدة عملية التئام الجروح.
هدف البحث: تقييم تأثير الإستخدام الموضعي للفينيتوين على القرحة المخاطية الشدقي المستحثة كيميائياً في الفئران البيضاء على فترات زمنية مختلفة.
مواد و طرق البحث: تم احداث قرحة كيميائية فى ثلاثة وستين فأراً أبيضاً ذكر. بعد ذلك ، تم تقسيم الفئران بشكل عشوائي إلى ثلاث مجموعات رئيسية كالتالى: المجموعة الضابطة: (المجموعة الضابطة السلبية): ترك الجانب الأيمن من الغشاء المخاطي الشدقي دون احداث قرحة ليكون بمثابة مجموعة ضابطة سلبية. (المجموعة الضابطة الإيجابية): تم إحداث قرحة في الغشاء المخاطي الشدقي الأيسر وتركها دون علاج لكي تلتئم بشكل طبيعي. المجموعة الثانية (معالجة بمادة هلامية بسيطة) تم استخدام مادة هلامية بسيطة خالية من الفينيتوين بشكل موضعي على القرحة مرتين يوميا بداية من اليوم التالي ليوم إحداث القرحة (يوم ١) وحتى يوم قتل الفئران. المجموعة الثالثة (معالجة بهلام الفينيتوين) تم استخدام الفينيتوين في صورة مادة هلامية بشكل موضعي على القرحة مرتين يوميا بداية من اليوم التالي ليوم إحداث القرحة (يوم ١) وحتى يوم قتل الفئران. تم تقسيم كل مجموعة بالتساوي إلى ٣ مجموعات فرعية (أ) و(ب) و(ج) حيث تم قتل الفئران بعد ٤ و ٧ و ١٢ يومًا من إحداث القرحة على التوالي. تم تشريح الغشاء المخاطي الخدي وفحصه نسيجيا ومناعيا.
النتائج: من الناحية الهستولوجية ، أظهرت المجموعة الضابطة الإيجابية تقرحاً كاملاً في النسيج الطلائي في اليوم ٤، وبدأت إعادة التكوُّن في اليوم ٧ ، ولوحظ التجدد الكامل في اليوم ١٢. كما أظهرت المجموعة الثانية تقرحاً كاملاً في النسيج الطلائي في اليوم ٤ ، وبدأت إعادة تكوين النسيج الطلائي في اليوم ٧ ، و لوحظ التجدد الجزئي للنسيج الطلائي المفقود في اليوم ١٢. أظهرت المجموعة الثالثة بداية إعادة تكوين النسيج الطلائي في اليوم ٤ ، وتم الكشف عن وجود نسيج طلائي كامل في اليوم ٧ ، ولوحظ تجديد كامل للنسيج الطلائي مع زيادة في طبقة الكيراتين في اليوم ١٢. من الناحية المناعية والإحصائية ، لوحظ أعلى تفاعل ايجابي فيما يتعلق بالخلايا الطلائية في المجموعة الثالثة.
الاستنتاج: الاستخدام الموضعي لـ ١٪ من عقار الفينيتوين يساعد في تعجيل إلتئام القرحة المُحدثة كيميائيًا من خلال إعادة تكوين النسيج الطلائي بشكل أسرع وزيادة الأوعية الدموية وتقليل الإلتهاب