

## **The Possible Healing Effect of Aloe Vera versus Silver Nanoparticles on Acid Induced Lip Ulcer in Albino Rats (Histological and Immuno-histochemical Study)**

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### **Abstract**

**Background:** Aloe Vera (AV), a tropical plant has been used for wound healing. Silver nanoparticles (AgNPs) are also considered one of the most promising nanomaterials in wound healing.

**Objectives:** To compare between the possible healing effect of application of AV and AgNPs on chemically-induced labial mucosal ulcer in Albino rats at different durations.

**Material and methods:** After chemical induction of ulcer on labial mucosa, forty-two adult male Albino rats were divided equally into three main groups: Group I (untreated ulcerated): rats didn't receive any treatment. Group II (AV gel): rats received topical application of AV gel (0.25ml/kg) once daily. Group III (AgNPs gel): rats received topical application of AgNPs gel (2mg/kg) once daily. Each group was divided into two subgroups named A and B according to time of sacrifice at three and nine days respectively. Labial mucosae were dissected and examined histologically and immunohistochemically for cell proliferation.

**Results:** Histologically, subgroup IA, IB and IIA showed epithelial degeneration, while subgroup IIB and IIIA showed continuous epithelial lining. Complete epithelial regeneration was seen in subgroup IIIB. Immunohistochemically and statistically, subgroup IIIA showed higher mean area fraction (AF%) of anti-PCNA immunopositivity in epithelium and lamina propria than subgroup IA. The highest mean AF % was shown in subgroup IIIB when compared to subgroup IB and IIB.

**Conclusions:** Both AV and AgNPs groups showed healing effects after ulcer induction on labial mucosa through enhanced re-epithelialization and their anti-inflammatory effect. However, AgNPs group showed better healing effect than AV group.

Key words: Ulcer; Labial mucosa; Aloe Vera; Silver nanoparticles.

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## INTRODUCTION

The oral mucosa shows thousands of diseases and lesions and the most common lesions are oral ulcers which are characterized by defects in the epithelium, underlying connective tissue, or both [1]. Traumatic ulcers are the most common ulcers occurring in the lip, they are caused by mechanical damage, thermal, electrical or chemical burns [2]. Corticosteroids and antibiotics are commonly used in management of massive chemically induced lesions [3], however, their use may cause oral candidiasis [4].

Great attention has been directed toward therapeutic natural products as they result in less side effects. The gel extracted from the leaf of AV contains a wide range of natural components that were reported to improve wound healing [5]. Aloe Vera gel has anthraquinone components such as aloin A and isobarbaloin which are potent antimicrobials and analgesic agents. Auxins and gibberellins also are found in AV gel, considered as anti-inflammatory hormones and have wound healing effect [6].

Nowadays, the use of new medicinal preparations at nanoscale levels is widespread. Silver nanoparticles (AgNPs) of 100 nm dimension or less have unique physical, chemical and biological properties. Their effectiveness in the treatment of burns, skin wounds and pemphigus were demonstrated [7].

So, this study was done to evaluate and compare between the healing potential of AV and AgNPs on acid-induced lip ulcer in rat model.

## MATERIAL 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-RECIM 041804

### •Animals

Forty-two male adult Albino rats (weighing 200-250 gms) were used in this study. Rats were housed in the animal research center of Ain Shams University, Cairo, Egypt in wire mesh cages under controlled temperature and dark-light cycle. All animals were fed with fresh vegetables diet and tap water ad libitum throughout the experimental period.

### Materials:

#### •Acetic acid

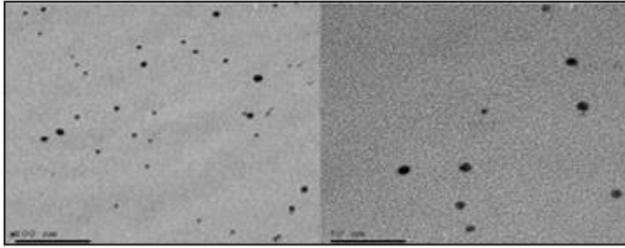
It was prepared with a concentration of 50% diluted in water and obtained from Al-Gomhoria Company, Cairo, Egypt [8].

#### •Aloe Vera gel

It was prepared in faculty of Pharmacy Ain Shams University. The AV leaves were cleaned and disinfected by means of distilled water and a mild chlorine solution (0.5% sodium hypochlorite solution). Then the inner gel was obtained by making an incision in the leaves and was collected by sterile spatula. 70% inner gel of AV, 10% purified water were mixed to obtain a homogenous gel and 1% preservative solution of methylparaben was added to prevent oxidation and give AV a longer shelf life. 19% hydroxyethylcellulose which is a highly inert non-ionic and water-soluble agent was added for gel thickening [9],[10].

#### •Silver nanoparticles

It was prepared by Nano Gate Company, Cairo, Egypt by chemical reduction method guided by Mulfinger et al. (2007) [11]. 0.4gm of Carboxymethyl cellulose from (Loba CHIME, india) was sprinkled gently and gradually over the solution of AgNPs 100ppm under mild temperature with vigorous stirring to get homogenous gel [12]. The obtained AgNPs were spherical in shape, optical properties:  $\lambda_{max}$ ~410 nm, with 500ppm concentration and average size less than 20nm (fig. 1).



**Fig. (1):** transmission electron microscope image of AgNPs (orig. mag. x200000).

## Methods:

### •Ulcer induction

All animals were anesthetized by intraperitoneal injection with a mixture of xylaject (10 mg/ kg) and ketamen (50 mg /kg). The animals were restrained and their mouths were kept open for the topical application using clamp forceps then a round filter paper 3 mm in diameter was soaked in 15ml of 50% acetic acid and was used to cause aseptic tissue necrosis. The acid-soaked paper was pressed onto labial mucosa for 30 seconds [13]. The pain was controlled by administration of Diclofenac sodium at a dose of five mg/kg once daily [14].

### •Animal grouping

After ulcer induction, the animals were randomly divided into three main groups: Group I (ulcerated untreated group): Consisted of 14 rats in which the ulcers did not receive treatment.

Group II (AV group): Consisted of 14 rats that received daily topical application of AV gel (0.25ml/kg) [15] on the ulcer from the day following ulcer induction till the day of sacrifice.

Group III (AgNPs group): Consisted of 14 rats that received daily topical application of AgNPs gel (2mg/kg) [15] on the ulcer from the day following ulcer induction till the day of sacrifice.

Each group was subdivided equally into two subgroups (seven rats each) according to time of sacrifice:

Subgroup A: Rats were sacrificed after three days from ulcer induction.

Subgroup B: Rats were sacrificed after nine days from ulcer induction.

Sacrification of rats was done by overdose of ketamine. Labial mucosa was collected from each rat and the rest of rat's body was

eliminated by the incinerator of Ain Shams Hospital.

All excised specimens were prepared to be examined histologically with routine H&E [16] and immunohistochemically for anti-proliferating cell nuclear antigen (anti-PCNA) mouse monoclonal purchased from Dako Company.

In immunohistochemical procedure, after treating the tissue with pepsin for 20min at 37°C, the slides were washed with phosphate buffer saline (PBS). Endogenous blocking was done with 3% hydrogen peroxide followed by protein blocking using 5% horse serum with 1% goat serum (protein blocking solution). The antigens were retrieved by microwaving the sections in sodium citrate buffer (10mM sodium citrate, pH 6.0). The samples were heated in a microwave (800W, 2450MHz) at 800W for 3min to initiate boiling of the solution and then for 7min at 400W to continue boiling. The sections were cooled for 30min at room temperature 25°C. After that, the 10min heating procedure was repeated with fresh buffer 25°C. The primary antibodies were then added for 18hrs at 4°C. The slides were then washed with PBS, blocked again with protein-blocking solution for 1hr, and incubated with horseradish peroxidase-conjugated anti-rat antibody at 1:200 dilutions for 1hr at room temperature. The slides were washed again in PBS thrice and then incubated with 3,3'diaminobenzidine for 10 min. After the excess 3, 3'diaminobenzidine was washed off, counterstaining was done with hematoxylin [17]. Specimens were mounted in DPX and examined under light microscope. Positive reaction was detected as nuclear brown coloration.

### Immunohistochemical Assessment:

Three microscopic fields showed anti-PCNA positive immunoreaction and photomicrographs were captured by digital camera (EOS 650D, Canon, Japan) which was mounted on a light microscope (BX60, Olympus, Japan). The area fraction % of immunopositivity area to the total area of the microscopic field was measured automatically using Image J, 1.41a, (NIH, USA) analysis software. The mean area fraction for each

sample was calculated and it was performed in the Precision Measurement Unit, Oral Pathology Department, Faculty of Dentistry, Ain Shams University.

#### 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  $\pm$  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 insignificant,  $*p$ -value  $< 0.05$  was considered significant and  $**p$ -value  $< 0.001$  was considered highly significant.

## RESULTS

### I- H&E stain results:

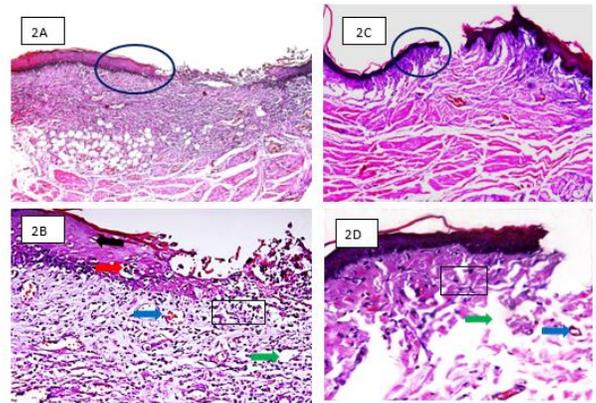
#### • Untreated ulcerated group I:

##### 1-Subgroup IA (three days after ulcer induction):

The epithelium and lamina propria (LP) were completely lost over ulcerated area (fig. 2A). Higher magnification of the area lateral to the ulcer showed keratinized stratified squamous epithelium with poorly defined basement membrane (BM), disorganized basal cell layer, area of epithelial degeneration and several clear cells in the prickle and granular cell layers. The LP at the sides of the ulcerated area showed many inflammatory cells, dilated blood vessels (BVs) engorged with RBCs and some areas of degeneration (figs. 2B).

##### 2-Subgroup IB (nine days after ulcer induction):

Discontinuity of epithelium and LP of the ulcerated area was detected with apparently thick epithelium with elongated rete pegs at one side (fig. 2C). Higher magnification at the other side showed apparently thin keratinized stratified squamous epithelium with flat rete pegs. The LP showed some inflammatory cells infiltration, normal BVs and large areas of degeneration (fig. 2D).



**Fig. (2A):** A photomicrograph of rat's labial mucosa of subgroup IA showing loss of the epithelium and LP (H & E orig. mag. x100).

**Fig. (2B):** Higher magnification of the inset in fig.(2A) showing poorly defined BM, disorganized basal cell layer, area of epithelial degeneration (red arrow) and several clear cells (black arrow). LP showing many inflammatory cells (black rectangle), dilated BVs engorged with RBCs (blue arrow) and areas of degeneration (green arrow) (H & E orig. mag. x400).

**Fig. (2C):** A photomicrograph of rat's labial mucosa of subgroup IB showing discontinuity of the epithelium and LP of the ulcerated area. Note the apparently thick epithelium with elongated rete pegs at one side of the ulcer (H & E orig. mag.x100).

**Fig. (2D):** Higher magnification of the inset in fig. (2C) showing apparently thin epithelium with flattened rete pegs. LP showing some inflammatory cells infiltration (black rectangle), normal BVs (blue arrow) and large areas of degeneration (green arrow) (H & E orig. mag. x400).

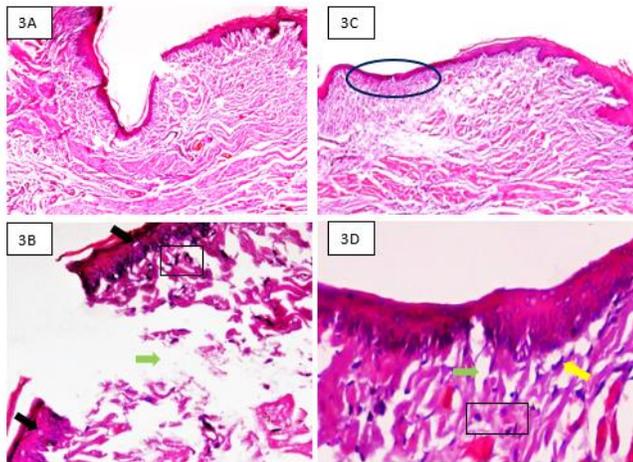
#### • Aloe vera group II:

##### 1- Subgroup IIA (three days after ulcer induction):

This subgroup revealed discontinuity of the epithelium and LP of the ulcerated area (fig. 3A). Higher magnification showed keratinized stratified squamous epithelium with poorly defined BM and some vacuolated cells at both sides of the ulcer. The LP showed sparse inflammatory cells as well as areas of degeneration (fig. 3B).

## 2-Subgroup IIB (nine days after ulcer induction):

This subgroup showed continuity of the epithelium at the healed area (fig. 3C). Higher magnification showed keratinized stratified squamous epithelium with poorly developed epithelial rete pegs. The LP showed apparently few inflammatory cells, fibroblasts and areas of degeneration (fig. 3D).



**Fig. (3A):** A photomicrograph of rat's labial mucosa of subgroup IIA showing discontinuity of the epithelium and the underlying LP of the ulcerated area (H & E orig. mag. x100).

**Fig. (3B):** Higher magnification of the previous photomicrograph showing epithelium with poorly defined BM and some vacuolated cells at both sides of the ulcer (black arrows). LP showing sparse inflammatory cells (black rectangle) and areas of degeneration (green arrow) (H & E orig. mag. x400).

**Fig. (3C):** A photomicrograph of rat's labial mucosa of subgroup IIB showing continuity of epithelium at the healed area (blue circle) (H & E orig. mag. x100).

**Fig. (3D):** Higher magnification of the inset in fig.(3C) showing epithelium with indistinct rete pegs. LP showing few inflammatory cells (black rectangle), fibroblasts (yellow arrow) and areas of degeneration (green arrow) (H & E orig. mag. x400).

## • Silver Nanoparticles group III:

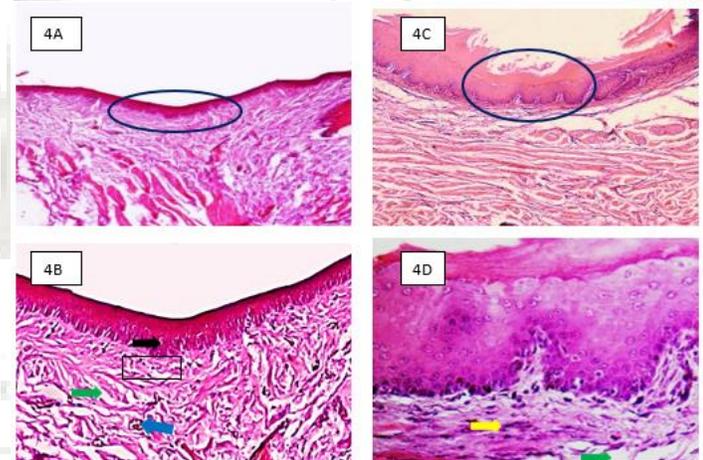
### 1-Subgroup IIIA (three days after ulcer induction)

This subgroup showed continuity of the epithelium at the healed area (fig. 4A). Higher

magnification showed thin keratinized stratified squamous epithelium with few clear cells. Epithelial rete pegs were poorly developed. The presence of few inflammatory cells, BVs engorged with RBCs and minimal areas of degeneration were observed in the underlying LP (fig. 4B).

### 2-Subgroup IIIB (nine days after ulceration):

This subgroup revealed continuous keratinized stratified squamous epithelium at the healed area (fig. 4C). By higher magnification, the epithelium was apparently thick with broad rete pegs. LP showed many fibroblasts with areas of degeneration were noted (fig. 4D).



**Fig. (4A):** A photomicrograph of rat's labial mucosa of subgroup IIIA showing continuity in epithelium at the healed area (blue circle) (H & E orig. mag. x100).

**Fig. (4B):** Higher magnification of the inset in fig.(4A) showing thin epithelium with few clear cells (black arrow). Epithelial rete pegs are poorly developed. Few inflammatory cell infiltration (black rectangle), BVs engorged with RBCs (blue arrow) and minimal areas of degeneration are seen in LP (green arrow) (H & E orig. mag. x400).

**Fig. (4C):** A photomicrograph of rat's labial mucosa of subgroup IIIB showing continuous keratinized stratified squamous epithelium at the healed area (blue circle) (H & E orig. mag. x100).

**Fig. (4D):** Higher magnification of the inset in fig.(4C) showing apparently thick epithelium with broad rete pegs. LP showing many fibroblasts (yellow arrow) and areas of degeneration (green arrow) (H & E orig. mag. x400).

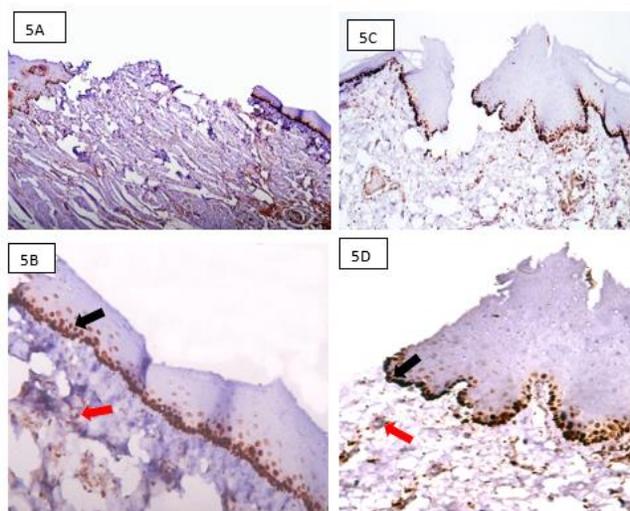
## II -Immunohistochemical results:

### • Untreated ulcerated group I: 1-Subgroup IA (three days after ulcer induction)

This subgroup revealed discontinuity of epithelium at the ulcer site with immunopositivity in epithelium and LP (fig.5A). Higher magnification showed few anti-PCNA immunostaining positive nuclei in basal and prickle cell layers of the side of ulcer with the presence of few immuno-positive nuclei in the underlying LP (fig. 5B).

### 2-Subgroup IB (nine days after ulcer induction):

It revealed discontinuity of epithelium at the ulcer site with immunopositivity in epithelium and LP (fig.5C). Higher magnification showed anti-PCNA immunostaining positive nuclei in basal and prickle cell layers of the side of ulcer with the presence of some immuno-positive nuclei in the underlying LP (fig. 5D).



**Fig. (5A):** A photomicrograph of rat's labial mucosa of subgroup IA showing discontinuity of epithelium at the ulcer site with immunopositivity in epithelium and LP (anti-PCNA orig. mag. x200).

**Fig. (5B):** Higher magnification of fig.(5A) showing the side of ulcer with few anti-PCNA immunostaining positive nuclei in basal and prickle cell layers (black arrow). LP showing few positive nuclei (red arrow) (anti-PCNA orig. mag. x400).

**Fig. (5C):** A photomicrograph of rat's labial mucosa of subgroup IB showing discontinuity of epithelium at the ulcer site with immunopositivity in epithelium and LP (anti-PCNA orig. mag. x200).

**Fig. (5D):** Higher magnification of fig.(5C) showing the side of ulcer with anti-PCNA immunostaining positive nuclei in basal and prickle cell layers (black arrow). LP showing some positive nuclei (red arrow) (anti-PCNA orig. mag. x400).

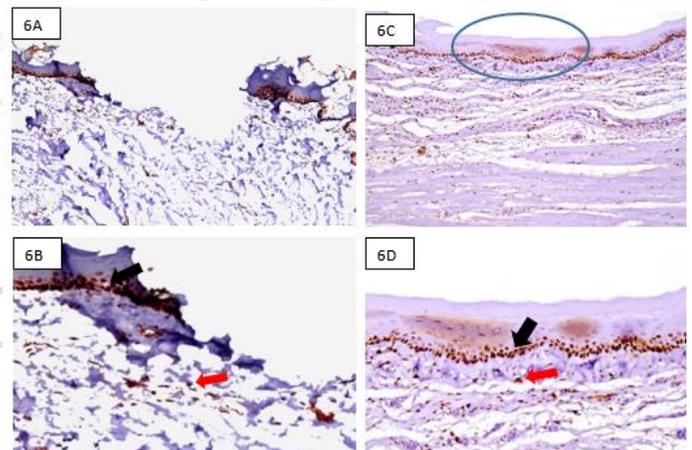
### Aloe Vera group II:

### 1-Subgroup IIA (three days after ulcer induction):

This subgroup revealed discontinuity of epithelium at the ulcer site with immunopositivity in epithelium and LP (fig.6A). Higher magnification showed anti-PCNA immunoreaction in nuclei of basal and prickle cell layers lateral to the ulcer, with the presence of few positive nuclei in the underlying LP (fig. 6B).

### 2-Subgroup IIB (nine days after ulceration):

This subgroup revealed continuity of epithelium at the healed ulcer with immunopositivity in epithelium and LP (fig.6C). Higher magnification showed immunoexpression of anti-PCNA in this subgroup revealed moderate positive stained nuclei in basal and prickle cell layers of the healed area with the presence of some positive nuclei in the underlying LP (fig. 6D).



**Fig. (6A):** A photomicrograph of rat's labial mucosa of subgroup IIA showing discontinuity of epithelium at ulcer site with immunopositivity in epithelium and LP (anti-PCNA orig. mag. x200).

**Fig. (6B):** Higher magnification of fig.(6A) showing the side of the ulcer with positive immunostaining nuclei in basal and prickle cell layers of the labial epithelium lateral to the ulcer (black arrow) with the presence of few positive immunostaining nuclei in the underlying LP (red arrow) (anti-PCNA orig. mag. X400).

**Fig. (6C):** A photomicrograph of rat's labial mucosa of subgroup IIB showing continuity of epithelium at the healed ulcer with immunopositivity in epithelium and LP (anti-PCNA orig. mag. x200).

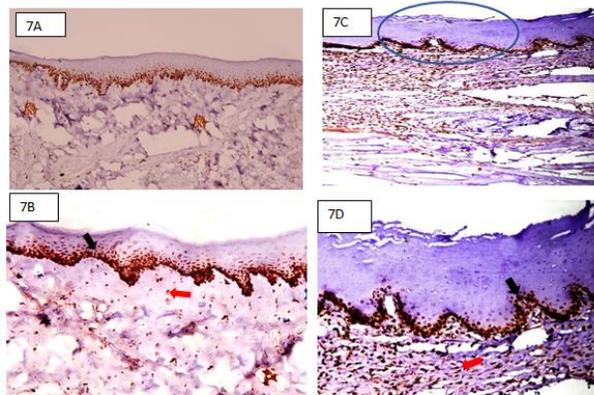
**Fig.(6D):** Higher magnification of the inset of fig.(6C) showing moderate immunostained nuclei in basal, and prickle cell layers of the reepithelized region (black arrow). Note the presence of some immuno-positive nuclei in the underlying LP (red arrow) (anti-PCNA orig. mag. X400).

• **Silver Nanoparticles group III: 1-subgroup IIIA (three days after ulcer induction):**

This subgroup revealed continuity of epithelium at the healed ulcer with immunopositivity in epithelium and LP (fig.7A). Higher magnification showed marked anti-PCNA immunopositivity was noted in nuclei in basal and prickle cell layers of the reepithelized region of the ulcerated area with the presence of some positive nuclei in the underlying LP (fig. 7B).

**2-Subgroup IIIB (nine days after ulcer induction):**

This subgroup revealed continuity of epithelium at the healed ulcer with immunopositivity in epithelium and LP (fig.7C). Higher magnification showed positive anti-PCNA immunostaining in this subgroup was revealed in many nuclei of basal and prickle cell layer of the healed area in epithelium and in many nuclei in the underlying



LP (fig. 7D).

**Fig. (7A):** A photomicrograph of rat's labial mucosa of subgroup IIIA showing continuity of epithelium at the healed ulcer with immunopositivity in epithelium and LP (anti-PCNA orig. mag.x200).

**Fig. (7B):** Higher magnification showing marked positive stained nuclei in the regenerated basal and prickle cell layer of the healed area in epithelium (black arrow). Some positive immunostained nuclei in the underlying LP are observed (red arrow) (PCNA orig. mag. X400).

**Fig. (7C):** A photomicrograph of rat's labial mucosa of subgroup IIIA showing continuity of epithelium at the healed ulcer with immunopositivity in epithelium and LP (anti-PCNA orig. mag.x200).

**Fig. (7D):** Higher magnification of the inset in fig. (7C) showing many positive stained nuclei in basal and prickle cell layer of complete reepithelization area (black arrow). Many positive nuclei are observed in the underlying LP (red arrow) (anti-PCNA orig. mag. x 400).

**III - Statistical results:**

Using Post HOC, and LSD test. At three days, significant difference was found between untreated ulcerated and AgNPs subgroups where area fraction (AF%) of anti-PCNA immunoreactivity was higher in AgNPs than untreated ulcerated subgroups. No significant difference was found between untreated ulcerated and AV subgroups nor between AV and AgNPs subgroups (Table 1, fig. 8).

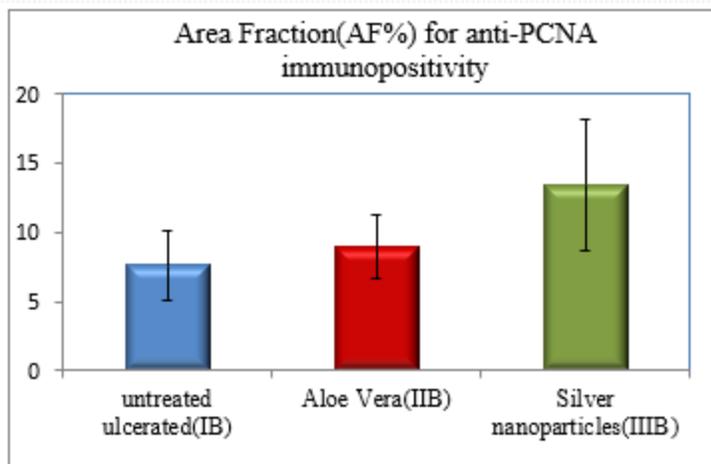
At nine days, significant difference between untreated ulcerated and AgNPs and significant difference between AV and AgNPs subgroups were found where AF % of anti-PCNA immunoreactivity was higher in AgNPs subgroups than untreated ulcerated and in AgNPs than AV subgroups. No significant difference was detected between untreated ulcerated and AV subgroups (Table 2, fig. 9).

Using Paired Sample t-test, highly statistically significant increase in mean AF% of anti-PCNA immunopositivity at nine days compared with three days in group III was detected. Group II showed significant increase in AF% of anti-PCNA immunopositivity at nine days compared with the same group at three days. Group I didn't show significant difference between three and nine days (table 3 and fig.10).

**Table (1):** Showing area fraction% of anti-PCNA immunopositivity of control, AV and AgNPs subgroups at three days post ulceration

Area Fraction (%)	Mean SD	Range	ANOVA	p-value
Untreated ulcerated (IA)	6.42B±1.50	4.07-8.51	4.579	0.046*
Aloe vera (IIA)	7.00AB±2.67	4.13-11.12		
Silver nanoparticles (IIIA)	8.70A±2.32	4.98-11.35		

Different letters indicate significant difference at (p<0.05) among means \* Significant.

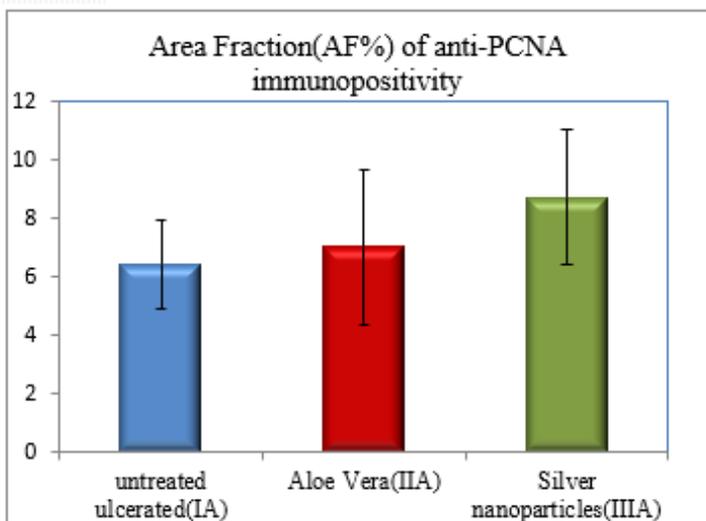


**Fig. (9):** Bar chart showing area fraction% of anti-PCNA immunopositivity of untreated ulcerated, AV and AgNPs subgroups at nine days post ulceration.

**Table (3):** Comparison between three days and nine days of the experimental groups according to area fraction% of anti-PCNA immunopositivity.

Area Fraction (AF%)	3 Days	9 Days	Paired Sample t-test		
	Mean±SD	Mean±SD	Mean Diff.	t-test	p-value
Untreated ulcerated (I)	6.42±1.50	7.56±2.50	1.14	1.042	0.328
Aloe Vera (II)	7.00±2.67	8.98±2.33	1.98	2.171	0.044*
Silver nanoparticles (III)	8.70±2.32	13.41±4.74	4.71	4.279	<0.001**

\* Significant. \*\* Highly significant.

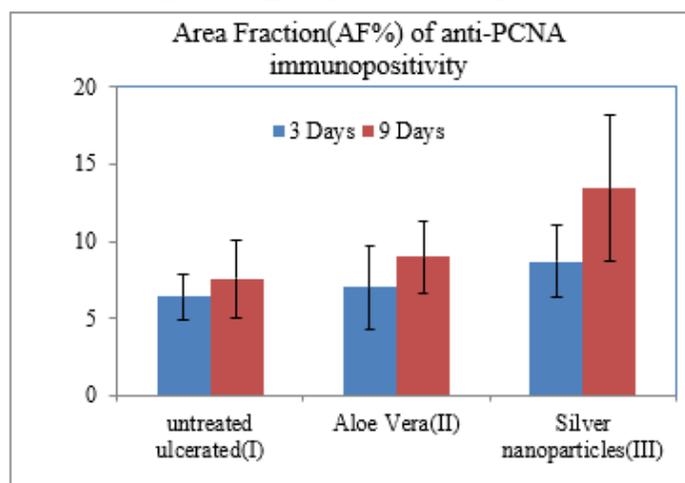


**Fig. (8):** Bar chart showing area fraction% of anti-PCNA immunopositivity of untreated ulcerated, AV and AgNPs subgroups at three days post ulceration.

**Table (2):** Showing area fraction% of anti-PCNA immunopositivity of control, AV and AgNPs subgroups at nine days post ulceration.

Area Fraction (AF%)	Mean SD	Range	ANOVA	p-value
Untreated ulcerated (IB)	7.56B±2.50	4.05-10.41	7.370	0.003*
Aloe vera (IIB)	8.98B±2.33	6.06-12.11		
Silver nanoparticles (IIIB)	13.41A±4.74	10.2-25.06		

Different letters indicate significant difference at (p<0.05) among means.\* Significant.



**Fig. (10):** Bar chart showing comparison between three days and nine days according to area fraction% of anti-PCNA immunopositivity in each group.

## Discussion

In the current study, acetic acid was used for ulcer induction as it produces ulcer in the oral mucosa and is suitable for evaluating the efficacy of new drugs in promoting healing of oral ulcer [18].

In the present study, the topical application of AV was used as it was reported to be the safety form of drug delivery [19]. Silver nanoparticles were also used topically to overcome its systemic adverse effects as Liao et al. (2019) [20] documented that AgNPs could precipitate in liver and kidney following intravenous, intraperitoneal and intratracheal routes. Gel formulation of AV and AgNPs were used for topical application since they have the ability to release drug molecules from the systems, are more easily applied to mucosal surfaces than creams and ointments, so it is the most appropriate form for clinical trials [21].

In the current study, histological examination of labial mucosa of untreated ulcerated subgroup IA revealed marked discontinuity of the epithelium at three days after ulcer induction and heavy inflammatory cells infiltration in LP with areas of degeneration and many dilated BVs. These findings were in parallel with Chen et al. (2019) [22] who found loss of surface epithelium continuity and LP was infiltrated with massive inflammatory cells in chemically induced ulcer by phenol acid in rat buccal mucosa at third day after ulcer induction. Gefen et al. (2020) [23] attributed these results to release of inflammatory chemokines by the damaged cells which dilated capillaries and attracted neutrophils and monocytes to the ulcerated area. Neutrophils and monocytes produced high concentration of reactive oxygen species (ROS) in mucosal cells resulting in oxidative stress, then damaged epithelial cells and also produced matrix metalloproteinases (MMPs), leading to destruction of extracellular matrix (ECM).

In the present study, histological examination of the labial mucosa of untreated ulcerated subgroup IB showed discontinuity of the epithelium, inflammatory cells infiltration with areas of degeneration in LP and normal BVs at nine days post-ulceration. It was earlier

explained by (Menke et al., 2007) [24] that the insistent presence of inflammatory cells in ulcers is due to tissue trauma by pressure, bacterial overgrowth or ischemic-reperfusion injury. The ulcer appeared to be decreased in size and the inflammatory cells apparently decreased in subgroup IB compared to subgroup IA. These findings were in agreement with a previous study which reported discontinuity of epithelium with less amount of inflammatory cells infiltration in untreated ulcerated group at day nine compared to the same group at day three in mice tongue ulcer induced chemically by phenol [25].

In our study, the lesion apparently decreased in size with mild inflammatory cells infiltration in LP in AV treated subgroup IIA at three days after ulcer induction compared to untreated ulcerated subgroup. Similar results were reported by El-Batal and Ahmed (2018) [15] who found less inflammatory cells infiltration in AV treated group than the untreated group in labial mucosal ulcer in mice induced by acetic acid after radiation at three days. The anti-inflammatory effect of AV was reported to be associated with a decrease in the production of nitric oxide which decreases the release of inflammatory mediators such as tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) and increase in anti-inflammatory mediators like inter-leukin-10 (IL-10). Also, AV can inhibit the production of Prostaglandin-E (PG-E), IL-2 and IL-8 responsible for inflammatory reactions. In addition, AV has antioxidant property due to the presence of vitamin A, E, C and Selenium which strongly clear free radicals especially  $\alpha$ -tocopherol that finally leads to reduced inflammation [26].

In this study, AV treated subgroup IIB showed re-epithelization of the ulcerated area in the day nine. This result was coinciding with a study which reported that Wistar rats treated with AV at ten days after surgically induced skin wound showed re-epithelization of the whole wound area compared to the untreated group [27]. Coelho et al. (2015) [28] documented that AV treated group showed re-epithelization that covered the whole ulcerated area on rats' tongue at ten days because of high expression of transforming growth factors beta

(TGF- $\beta$ ) and basic fibroblast growth factor (bFGF) which motivate epithelization.

The current study revealed apparently minimal degeneration areas in AV subgroup IIB compared with untreated ulcerated subgroup IB at day nine which might be attributed to collagen fibers deposition. This is according to the study of Atiba et al. (2011) [29] where collagen deposition was increased in rats given AV after skin wounds. Khan et al. (2013) [30] documented that mannose-6-phosphate which is present in AV gel had been shown to increase macrophage activity and therefore stimulated fibroblast activity and collagen synthesis.

In the present study, after three days, histological samples of AgNPs treated subgroup IIIA showed healing of the ulcerated area and revealed scant inflammatory cells in the LP. These findings were in agreement with a study reporting that treatment with AgNPs after three days of ulcer induction lessened the number of inflammatory cells due to inhibition of bacterial replication and reduction of pro-inflammatory cytokines especially TNF- $\alpha$ , so decreased the production of destructive matrix MMPs which in turn reduced wound inflammation [31]. It has also been reported that re-epithelization occurred at day three post-wounding after topical application of AgNPs on skin wound in rats model [32].

In the present study, after nine days, H&E results of AgNPs treated subgroup IIIB showed formation of epithelial layer with relatively normal thickness. This result was in agreement with a previous study which revealed that the AgNPs group required a total period of about eight days for complete re-epithelization and healing, resembling normal skin after inducing excision wound in the dorsal region in rat [33]. The investigators suggested that AgNPs could increase the rate of wound closure through the promotion of proliferation and migration of keratinocytes from the edge to the center of the wound and trigger the differentiation and maturation of keratinocyte. AgNPs also could stimulate differentiation of fibroblasts into myofibroblasts, thereby promoting wound contraction and accelerating healing [34].

Anti-PCNA antibody was used in the present study to detect proliferating cells as it is considered valuable for assessing cell proliferation throughout wound healing [35].

In the current study, immunohistochemical results of untreated ulcerated subgroups IA showed few numbers of positive nuclei in cells in the epithelium of ulcer margins and connective tissue (C.T) at three days. This result was parallel to a study reporting few positively stained cells in the epithelium and C.T at three days in acetic acid induced gastric ulcer in rats of untreated group [36].

The immuno-expression in the untreated ulcerated subgroup IB revealed moderate level of anti-PCNA immuno-positivity cells expression in the epithelium of ulcer margins and C.T at nine days. This result was in agreement with Beckert et al. (2004) [37] who revealed mild anti-PCNA immuno-staining in epithelial and C.T cells in untreated group of induced gastric ulcer in rats after seven days.

In the present study, the statistical analysis results of area fraction (AF) % of PCNA immuno-positivity showed that the ulcerated untreated group showed low area fraction % and there was no significant difference between untreated ulcerated subgroups (IA&IB) at three and nine days. This is in accordance with Rashed et al. (2019) [38] who found that there was no statistical significant difference in PCNA immuno-positivity in untreated oral cheek ulcer in rat on day three and ten. An earlier explanation has been reported by Jainu et al. (2010) [39] that acetic acid used in gastric ulcer induction was accompanied by reduced PCNA immunohistochemical expression.

In the present work, the immunoexpression in AV subgroup IIA revealed anti-PCNA immunopositivity cells in the epithelium of ulcer margins and low expression in C.T cells on third day post ulceration. This result is in parallel with Moriyama et al. (2016) [40] who documented that Ki-67 proliferation marker showed low immunoexpression in cells at three days after wound induction in rabbits.

Anti-PCNA immuno-expression in AV subgroup IIB showed apparently increase in cells nuclei of the epithelium and C.T at nine days post-ulceration compared with AV

subgroup IIA. This result was in parallel with a study investigating the effect of AV on cell proliferation in skin of mice after skin incision wound by keratin 1/10 proliferation marker. An increase in expression of keratin 1/10 proliferation marker was found in AV group at day eight after injury [41].

In the present study, the statistical analysis results of AF% of anti-PCNA positive cells showed that there was no significant difference between AV group and untreated subgroup at three and nine days. This result was in agreement with a study which reported that there was no significant difference between AV treated group and untreated group in skin wound healing of rats on day five and day ten. The investigators explained that this could be expected at the early stage of repair and that the significant difference was found at day 15 [42].

In the current study, anti-PCNA immunoreaction in AgNPs subgroup IIIA revealed many positive nuclei in epithelial and some positive C.T cells in the healed area on third day of treatment. This finding coincided with the results of Saad et al., (2015) [43] who demonstrated that anti-PCNA immunopositive cells increased in number in ulcerated labial mucosa of rat model treated with AgNPs compared with untreated ulcerated group at three days.

In the present study, AgNPs subgroup IIIB showed many anti-PCNA immunopositive cells in the epithelium and also in many C.T cells at nine days post-ulceration. This finding was in parallel with a study which reported that strong anti-PCNA immunoreaction detected in cells of dermal and epidermal layer of skin wound in rat model at seven days by overexpression of TGF- $\beta$ 1 [34].

The immunohistochemical statistical analysis of the ongoing research revealed significant increasing in anti-PCNA immunopositive AF % in AgNPs group compared to untreated group at three and nine days. This result was in parallel with a study documenting a significant increase in proliferation of the cells in the dorsal excisional wound of rat started from the third day and sustained till ten days from wounding [44].

In the current study, there was highly significant increase in AF% in AgNPs subgroups at nine days compared to three days, although there was almost complete restoration of histological picture of rat's labial mucosa at nine days. This result could be explained by Zuccari et al. (2008) [45] who stated that PCNA has a long half-life and therefore it may continue to be detected by anti-PCNA for a while after completion of cell division.

## Conclusions

Histological, immunohistochemical and statistical results of the present study suggested that topical application of AV and AgNPs helped in accelerating healing of chemically induced ulcer, best results were shown with AgNPs.

## Conflict of interest

There is no conflict of interest to declare.

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