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Wound healing effect of topical Phenytoin on Rat palatal mucosa

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A B S T R A C T

Today, chronic wounds and non-remitting ulcers have become a health problem. Wound healing process involves several processes, including inflammatory response, regeneration of the epidermis, wrinkles, and wounds and eventually builds connective tissue and remodeling. The aim of this study was to evaluate the wound healing effect of topical Phenytoin on Rat palatal mucosa. In an experimental study that performed in the Drug Applied Research Center of Tabriz University of Medical Sciences, by examining the results of local restorative Phenytoin, wound healing effect of topical Phenytoin on Rat palatal mucosa were evaluated. In this study, 20 rats, which included 10 rats in the test group, 10 rats in the control group, were studied. The most of reepithelialization rate was occurred from forth to fifth day that more in test sample than placebo. On the ninth day, in the test samples, the damaged epithelium fully restored, while in the placebo group, until the eleventh day, hilling of damaged epithelium still remained incomplete. The formation of granulation tissue as low as started from the second day until eighth day have been rising trend and from ninth day, showed a downward trend that more in placebo samples than teat samples. The presence of inflammatory cells involved in the healing process was more in placebo samples than test samples. The angiogenesis rate in different days, in comparison with placebo was more in placebo samples than test samples. The thickness of the healed tissue showed an upward trend in all samples. Post operative wound infection was found in all samples with moderate to severe intensity. The formation of granulation tissue, the presence of inflammatory cells and angiogenesis in the test sample compared to placebo had a slower trend and at the end of eleven days, the result was almost the same. Because the sample testing, on the ninth day, lining was fully healed, granulation tissue deposition of collagen fiber organization more and more tests on samples from the placebo, Overall it can be concluded that in this study, Phenytoin more effective than placebo in the treatment of wound healing is completely rats.

Introduction

Today, chronic wounds and unremitting wounds have become major health problems. The healing process includes inflammatory responses, regeneration of epidermis, wound shrinkage, and formation of connective tissues and re-modeling. Proper wound treatment facilitates the healing process and prevents infection and chronicity of the wound (1).

So far, different methods have been employed to reduce the length of the wound healing process and one of these methods is the administration of Phenytoin. Diphenylhydantoin, which is known as Phenytoin, was for the first time introduced as an oral anti-seizure drug in 1937 (1). This medicine affects the motor cortex of the brain similar to barbiturates and leaves its anti-seizure influence by inhibiting the activity of sodium channels for the long time (2).

In 1939 it was found out that Phenytoin can lead to gingival hyperplasia in patients who use the drug for a long time. It was also indicated that this medicine positively contributes to the healing of wounds and formation of granulation tissues. Consequently, the idea of using this medicine for the treatment of wounds was formed (3).

Although the mechanism of the effect of Phenytoin on wound healing is not known yet, the resulting hyperplasia seems to be caused by an increase in the growth factors of connective tissues, B1 transforming growth factor, platelet-driven growth factors, type II fibroblast growth factors, and epidermal growth factor (4, 5).

It has been reported that when Phenytoin is applied on the skin and mucosal surfaces it considerably contributes to the healing

process. It was also indicated that Phenytoin is useful for the treatment of acute and chronic lesions with different etiologies such as decubitus ulcers, surgical wounds, pressure ulcers, diabetic wounds, traumatic injuries, scorch, war and bullet wounds, venous stasis ulcers, abscess, epidermolysis bullosa simplex, aphthous ulcers, and oral lichen planus (6-8).

The best way for topical application of Phenytoin is not known yet but in the study by Modagheh et al. the performance of four different types of Phenytoin was compared on the rats. It was also reported that pure Phenytoin powder yields the best results (9).

Phenytoin powder is applied on single-layer thin wounds, but powders obtained from Phenytoin capsules can lead to the emergence of a white scar on the wound, which can be cured by mixing Phenytoin with NaCl 0.9% (10).

It is not recommended to use the Phenytoin syrup due to the additional compounds that are present in the syrup. The injectable form of Phenytoin also is not recommended for topical use as it has a high PH (11).

The side effects of topical Phenytoin are very rare and only a temporary burning sensation has been reported. The resulting sensation can be addressed with the use of pure Phenytoin powder.

Hypertrophic granulation tissue was observed in 10-36% of patients studied in two studies. This complication can be cured by stopping the treatment (12-13).

The systemic absorption of topic Phenytoin is not significant and in most studies the blood concentration of Phenytoin was insignificant.

Not only has topical Phenytoin slight side effects and gives insignificant systematic absorption, but also is a safe, effective, available and cheap medicine (6).

This study was an effort to explain the negative effect of topical Phenytoin on mouth ulcers in the rats. Since no study has been so far conducted on the effect of topical Phenytoin on the palatal region, findings of this study can prove the necessity of using this medicine on human models.

Since so far no study has been conducted on the topical administration of this medicine on palatal mucosa, this study was carried out to examine the topical application of this drug on the palatal ulcers. A comparison was also made between the results of the topical administration of this medicine and a placebo after treatment of cleft palate in child patients. The objective of this study was to examine the effect of topical Phenytoin on the healing of palatal ulcers in rats.

Materials and Methods

In an experimental study which was carried out in the Medicinal Applied Research Center of Tabriz University of Medical Sciences the reconstructive results of topical Phenytoin were examined to investigate the effect of topical Phenytoin on the healing of palatal wound in rats.

A total of 20 rats were selected and 10 rats were classified in the experimental (test) group and 10 were put in the control group. The rats were randomly but equally divided into the experimental and control groups.

This research was carried out on 20 healthy male rats with a weight ranging from 200 to 300 grams. The rats were kept in a standard

environment with a temperature of $22 \pm 2^{\circ}\text{C}$.

In order to include the rats into the study the required permission was obtained from the ethical committee and then all of the rats were anaesthetized using a mix of intramuscular ketamine hydrochloride (20 mg/kg) and xylazine (5 mg/kg). Next a full-thickness round wound was created on the mucoperiosteum area and on the hard palate midline exactly on the anterior portion of the second ruga of the hard palate and in front of the rats' molar teeth. The wounds had equal shapes and sizes.

No medical treatment was employed in the course of this study. Afterwards, the rats were randomly divided into two groups: the first group included 10 untreated rats (the placebo group or Group A) and the second group included 10 rats who received topical Phenytoin (Group B).

All of the rats were exposed to mild anesthesia and topical Phenytoin (mucoadhesive gel) was applied once a day on the wounds of patients in group B after examining the wound, making an microscopic observation of the healing process, and taking images. In group A, placebo was applied to the wound exactly the same as the aforementioned topical product of Phenytoin. The placebo, however, had no effect.

The rats in the two groups were demolished on days 3, 5, 7 and 9 (each day two rats were killed) and tissue samples were obtained from their palates. After obtaining the tissue samples and staining them using the H&E and Masson's-Trichrome methods, histopathological studies were carried out on the samples by two pathologists using optical microscopes. The results were reported subsequently. The histopathological

examinations included assays of the epithelial tissue and granulation tissue of the samples. The epithelial tissues were studied for the connection of wound edges and formation of integrated stratified squamous tissues in cellular strata. The granulation tissues of samples were examined for the following factors: hyperemia; edema; the extent and maturity of collagen fibers; parallel or non-parallel arrangement of the fibers, the wound surface, and the base membrane of the epithelial tissue; the maturity and extent of the resulting arteries; and the orientation of the arteries (such as parallel or perpendicular to the wound surface and the base membrane of the epithelial tissue); penetration of multinuclear inflammatory cells and the degree of penetration; and presence or absence of bacterial micro-colonies.

It is worth mentioning that all of the histological studies of this research were single blind examination.

Ethical considerations

A written permission of the ethical committee and an ethical code were obtained to include the rats in the study.

Results and Discussion

Daily examination of the study samples revealed the following results.

On day 2, bacterial micro-colonies were observed on the infection of the damaged epidermis of the experimental samples. These colonies were not observed in other samples.

On day 11, in addition to the definition and grading of the post-operative infections in the experimental samples, a severe infection caused by the penetration of an external

object into the space between the granulation tissue and periosteum of the nasal cavity bone was also observed.

From day 5 of the test process, a decreasing trend was observed in the tissue edema and hyperemia. In all cases the healing of epidermis was natural with a row of basal cells and three to four rows of barbed cells.

From day 8, a decreasing trend was observed in the inflammation and angiogenesis factors. Moreover, the resulting vessels also seemed mature from the eighth day onward.

On day 11 and 12 of the test, slight deposition of collagen fibers was observed. The fibers were immature and perpendicular to the tissues.

The healing of the epithelial tissues of the hard palate on the fifth day is shown in Figure -1. The experimental and placebo (control) samples shown in this figure are the 1B and 1A samples, respectively.

Formation of a new epidermis started on day 2 (1) and the epidermis of the two groups had grown adequately on day 5. On the fifth day, a slight difference was observed between the untreated areas (the diagonal line) in the experimental sample as compared to the placebo sample.

The post-operative infection on the fifth day of the test is shown in Figure 2. The experimental and placebo samples shown in this image are the 2B and 2A samples, respectively. The invasion of the surroundings of the pseudo-stratified cylindrical epithelial tissue (the arrow) of the nasal bone by inflammatory cells in all samples and on all days (with varying severities) led to the emergence of post-operative infections. As seen in this figure,

on the fifth day of the study the severity of post-operative infections in the placebo and experimental samples was almost equal (both groups developed severe infections).

Figure 3 shows the healing of the hard palate epithelial tissue on the ninth day of the study. The experimental and placebo samples used in this figure are the 3B and 3A samples, respectively. On day 9, the damaged epithelia tissue of the hard palate (1) in the experimental sample was reconstructed completely whereas the healing in the placebo sample was incomplete (3) and took place at a lower speed.

The granulation tissue formed on the healed region of the hard palate epithelial tissue on day 11 is shown in Figure 4. The experimental and placebo samples shown in this figure are the 4B and 4A samples, respectively. In the experimental sample the number of polymorphonuclear and mononuclear cells involved in the healing process (horizontal arrows) in the resulting granulation tissue declined more than the placebo sample and more maturity was observed in the newly developed vessels (vertical arrows in Figure 1) on day 11 during angiogenesis. As seen in this figure, there are arteries in the placebo sample which lie in a direction other than that of the epithelial tissue.

According to the results of this research, the highest level of re-epithelization occurred from day 4 to day 5 of the research. Moreover, the aforementioned level of re-epithelization was higher in the experimental sample than the placebo sample. On day 9 of the research, the damaged epithelium of the experimental sample was fully repaired whereas the repair of epithelium in the placebo sample was not complete on day 11.

Formation of granulation tissue started slowly from day 2 of the study and showed an ascending trend until the eighth day. From day 9 onward the formation of this tissue declined. The level of this factor was higher in the placebo sample than the experimental sample on different examination days.

The number of the inflammatory cells involved in the reconstruction process was higher in the placebo sample than the experimental sample on different days. From day 2 to day 5 an increasing trend was observed in the number of these inflammatory cells and from day 6 onward the number of these cells declined. On day 11, the number of inflammatory cells in both groups was almost the same.

The level of angiogenesis in the placebo sample was higher than the experimental sample on different days. This factor also increased from day 2 to day 5 and declined from day 6. On day 11 the level of angiogenesis was almost the same in both groups.

Deposition of collagen fibers started slowly on day 8 and showed a gradual growing trend until day 12. The level of this factor in the experimental sample was higher than the placebo sample.

The thickness of the repaired epithelial tissue showed a growing trend in all samples although fluctuations were observed day by day. Therefore, the thickness of the repaired epithelial tissue did not follow a regular ascending or descending trend. Since only one sample from each group was studied per day the fluctuations can be ascribed to the individual characteristics of the animals. Generally the thickness of the repaired epidermis is high in the first two or three weeks of healing and then it declines

slightly and eventually reaches a constant level as the epidermis matures.

Post-operative infection was observed in all samples with moderate to severe intensity. However, considering the location of the hard palate and its exposure to food in the oral cavity the infections were considered natural in this study.

In previous studies the effects of powder, gelatin, creamy and ointment Phenytoin on the wound healing process were studied separately and different results about the acceleration of wound reconstruction were reported. For instance, an increase in the inflammation time was reported with powder Phenytoin (14). Acceleration of the angiogenesis process using powder Phenytoin was also reported (14). However, application of powder Phenytoin accelerates the formation of granulation tissues but does not make a significant change in the angiogenesis process (15).

Shafer et al. stated that Phenytoin can only contribute to the strength and cohesion of wounds (16). In a study, 20 patients with sore throats who had been treated for 12 weeks previously but had responded poorly to the treatments (or showed no response at all) were treated by Phenytoin in two weeks (17).

The healing effects of topical Phenytoin in combination with normal saline were studied on 100 patients. After 4 weeks, the average reduction in the size of ulcer in the Phenytoin group and control group was 72% and 55%, respectively. The use of Phenytoin for the treatment of gluteal abscess was examined by Lodha et al. The control group received eusol and urea solution. In addition, the levels of healing in the Phenytoin and control groups were 10 and 20 days, respectively (18). Phenytoin was also used

for the treatment of diabetic foot wounds and the treatment completed in 21 days. However, sterile bands were used on the control samples and the treatment was completed in 45 days (19). Phenytoin reduces the wound healing process (19).

A study was conducted on 30 patients who were receiving Phenytoin. The patients were classified into the control and experimental groups to be monitored for the reduction in the depth and surface area of their wounds within three weeks (20). In another study a comparison was made between the wound healing effects of topic Phenytoin and Triple antibiotic ointment. Reconstruction progress was observed in all wounds but the Phenytoin group recovered more rapidly. The expected granulation tissue developed in one to two weeks in the experimental group but it took 6 to 21 days for the control group to demonstrate these tissues (21). In a study in Iraq it was reported that topical application of Phenytoin contributes to the treatment of external ulcers caused by war injuries. It was also reported that administration of this medicine leads to a rapid release from pain, a reduction in wound secretions and a decline in microbial pollutions (22). Consumption of powder Phenytoin also increases angiogenesis, the growth of hair follicle, and tensile strength of tissues (14).

Researchers have reported different findings about the outcomes of using powder, gelatin, creamy and ointment Phenytoin (with different percentages) for the treatment of wounds (23).

Sinson et al. reported that consumption of Phenytoin increases fibroblast activity and leads to the accumulation of collagens (24). Results of various studies have shown that due to the effects of high concentrations of Phenytoin on the fibroblasts of human skin

(in a long term treatment), the proliferation of fibroblasts declines (25). Another study that was carried out with a culture medium showed that Phenytoin cannot stimulate the proliferation of dermal skin fibroblast and human epidermal keratinocytes (26).

Robino reported the positive effect of powder Phenytoin on wounds and introduced this product as a factor that accelerates the formation of granulation tissues (15). El-Zayat believed that consumption of Phenytoin results in an increase in the thickness of the granulation tissue (26).

In the research by El-Nahas et al. the topical effect of Phenytoin (2% powder) on the reconstruction of diabetic foot ulcer was examined in patients with persistent neuropathy. It was concluded that Phenytoin accelerates the repair of wounds (27).

In a systematic review of 14 clinical trial articles the effect of topical Phenytoin on the treatment of pleurisy, chronic wounds and diabetic foot ulcers was studied. However, very limited reasons for the application of this medicine to burning and chemical wounds were provided (28).

Subbanna et al. studied 28 patients with stage II bedsores by randomly treating half of them with Phenytoin and the other half with normal saline for 15 days every day. They later compared the effects of the two treatments by measuring the wound sizes, scores, and volumes. Although according to the measurements of serum Phenytoin levels the systemic absorption of Phenytoin was very slight and application of Phenytoin solution to the wound dressing seemed safe and risk-free, the examinations of the trend of wound healing using Phenytoin (as compared to normal saline) revealed no significant statistical difference (29).

Moreover, in the study by Chan et al. the speed of wound healing in diabetic rats which were dressed using Phenytoin was higher than the rats in the control group (30). The contribution of Phenytoin to the healing of fraction was also studied by Mathew et al. and results of their radiographic and histological studies reflect the positive effect of Phenytoin on the healing of fraction in both groups (31).

In the study by Shams al-Dini the positive effect of Phenytoin on the treatment of ulcers on male rats was higher than samples in the control group, but no significant statistical difference was observed between the results of the two groups (32).

The first clinical trial for examining the effect of oral Phenytoin on periodontal wounds was carried out by Shapiro et al. They reported acceleration of wound healing and a reduction in the pain and inflammation of the wound area with Phenytoin (33). Since then numerous studies have been carried out on the effect of Phenytoin on the acceleration of skin wounds, bed sore, diabetic foot ulcer, burn wounds, and war wounds. The findings of all the studies stressed the positive effect of Phenytoin on the acceleration of wound healing.

In the current study the epidermis was healed naturally in all patients and included one row of basal cells and three to four rows of barbed cells.

In the following three different studies a comparison was made between administration of topical Phenytoin and placebo on chronic leg ulcers.

Simpson et al. reported a slight reduction in the wound area in patients of the Phenytoin group as compared to the placebo group (34).

Carneiro and Nyawawa reported a drastic increase in the generation of healthy granulation tissues in the Phenytoin group as compared to the placebo group (35).

Oluwatosin et al. made a comparison between the effects of Phenytoin and honey and reported a considerable difference between the progress of healing in patients receiving Phenytoin within 4 weeks and patients receiving honey (36).

In this research, the highest level of re-epithelization was observed from day 4 to day 5 of the study. The level of re-epithelization in the experimental group was higher than the placebo group. In addition, formation of granulation tissue started from the second day of research and increased until the eighth day. From day 9 the formation of granulation tissue declined. The level of this factor on different days of experimentation was higher in the placebo group than the test group.

In a study by Huseyin Kosgar in 2009 the effect of *Arnebia Densiflora* extract on the palatal wounds of 48 rats was examined and it was found out that the aforementioned extract can contribute to the treatment of palatal wounds (37).

In the present study, the number of inflammatory cells involved in the healing process was higher in the placebo sample than the experimental sample on different study days. From day two to day five, the number of the inflammatory cells showed a growing trend and from day six a descending trend was observed in this factor. On day 11 the number of inflammatory cells in the samples of the two groups was almost the same.

In another study by Al-Mashhandane the effect of oral Phenytoin on the treatment of buccal ulcer in 20 rabbits was examined and

it was concluded that systematic Phenytoin postpones the healing of oral ulcers on the buccal area of rabbits (38).

Shaw carried out a study in which a scar with a diameter of 1 mm was created in the soft palate of 6 rats and histological recovery of the palates of all of the rats was observed after 7 days. Considering the anatomic and histological similarities between the palatal muscles of rats and humans the muscular generation after development of wounds was examined (39).

In the present study, the thickness of the re-epithelized tissues showed an ascending trend in sum. It was indicated that since there were day-by-day fluctuations in the results the trend did not follow a regular ascending and descending path. Since only one sample from each group was studied per day the fluctuations can be ascribed to the individual characteristics of the animals. Generally the thickness of the repaired epidermis is high in the first two or three weeks of healing and then it declines slightly and eventually reaches a constant level as the epidermis matures.

Conclusion

Formation of granulation tissues, presence of inflammatory cells and angiogenesis followed a slower trend in the experimental sample than the placebo sample. At the end of the 11 days a similar result was obtained in this regard. Since the epithelial tissue of the experimental sample was fully healed on day nine, it can be said that the granulation tissue was more organized. Moreover the deposition of collagen fibers in the experimental sample was higher than the placebo sample. Therefore, it can be concluded that in this study, the effect of Phenytoin on the treatment of palatal wounds in rats was higher than that of placebo.

Table.1 Findings of study (First day to Sixth Day)

	Days											
	First Day		Second Day		Third Day		Fourth Day		Fifth Day		Sixth Day	
	Case	Control	Case	Control	Case	Control	Case	Control	Case	Control	Case	Control
Not healed wound Length	+3	+3	+3	+3	+3	+3	+3	+3	+2	+2	-	+2
The thickness of the healed epidermis	0	0	+2	+1	+2	+1	+2	+1	+2	+2	-	+2
The extent of granulation tissue	0	0	+1	+2	+1	+2	+1	+2	+2	+3	-	+3
The extent of deposition of collagen fibers	0	0	0	0	0	0	0	0	0	0	-	0
Inflammation	0	0	+2	+3	+3	+3	+3	+3	+2	+3	-	+3
Angiogenesis	0	0	+1	+2	+2	+2	+2	+2	+2	+3	-	+2
Wound infection	+2	+2	+3	+3	+2	+2	+2	+2	+3	+3	-	+2

Table.2 Findings of study (Seventh Day to Twelfth Day)

	Days									
	Seventh Day		Eight Day		Ninth Day		Eleventh Day		Twelfth Day	
	Case	Control	Case	Control	Case	Control	Case	Control	Case	Control
Not healed wound Length	-	+2	-	+1	0	+2	0	+1	0	-
The thickness of the healed epidermis	-	+2	-	+3	+2	+2	+3	+3	+3	-
The extent of granulation tissue	-	+3	-	+3	+3	+3	+3	+3	+3	-
The extent of deposition of collagen fibers	-	0	-	0	0	0	+1	+1	+1	-
Inflammation	-	+3	-	+1	+1	+2	+1	+1	+1	-
Angiogenesis	-	+2	-	+1	+1	+2	+1	+1	+1	-
Wound infection	-	+2	-	+1	+3	+1	+2	+2	+2	-

Table.3 Findings of study in case group

	Not healed wound Length	The extent of granulation tissue	Inflammation	Angiogenesis	The thickness of the healed epidermis	Wound infection	The extent of deposition of collagen fibers
First Day	15	0	0	0	0	Moderate	0
Second Day	13.2	22	40	5	1	Sever	0
Third Day	11.5	23	49	7	1	Moderate	0
Fourth Day	11.2	24	58	9	0.9	Moderate	0
Fifth Day	8.5	28	38	6	0.8	Sever	0
Eight Day	3.2	54	29	5	1.1	Mild	3
Ninth Day	0	44	27	5	0.8	Moderate	3
Eleventh Day	0	42	27	4	1.3	Moderate	4
Twelfth Day	0	41	27	4	1.2	Moderate	4

Table.4 Findings of study in control group

	Not healed wound Length	The extent of granulation tissue	Inflammation	Angiogenesis	The thickness of the healed epidermis	Wound infection	The extent of deposition of collagen fibers
First Day	14	0	0	0	0	Moderate	0
Second Day	12.8	29	48	8	0.7	Sever	0
Third Day	12	34	53	9	0.9	Moderate	0
Fourth Day	11	35	64	10	0.8	Moderate	0
Fifth Day	9.4	36	67	11	0.9	Sever	0
Sixth Day	8.5	37	59	10	0.9	Moderate	0
Seventh Day	8.1	39	55	9	1	Moderate	0
Ninth Day	6.4	51	37	6	1	Mild	1
Eleventh Day	3	44	25	4	1.1	Moderate	2

Figure.1 Healing of epithelial tissue (the fifth day study)

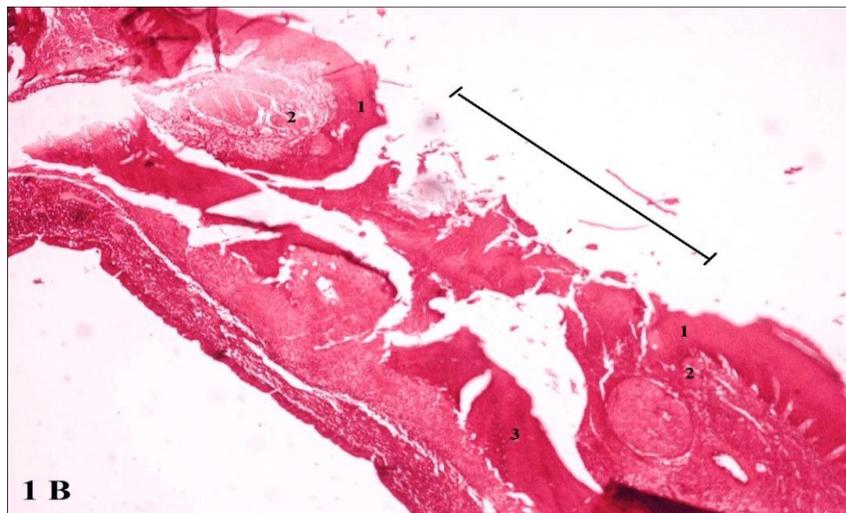
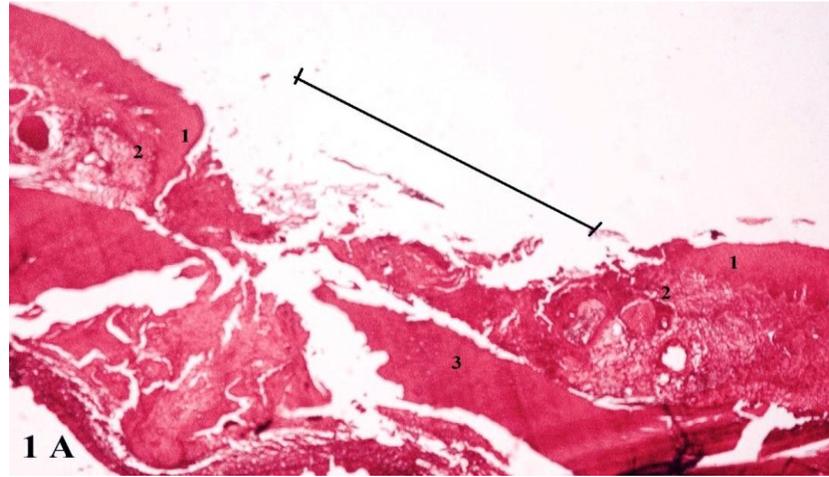
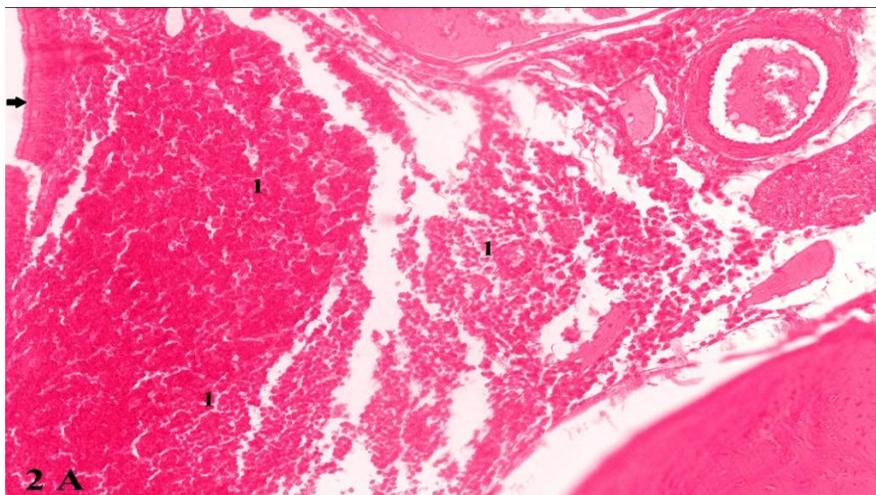


Figure.2 Post operative infection (the fifth day study)



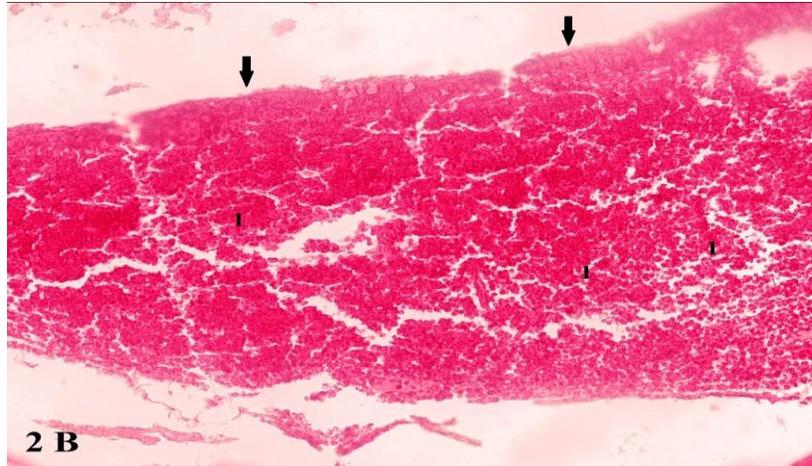


Figure.3 Healing of epithelial tissue (the ninth day study)

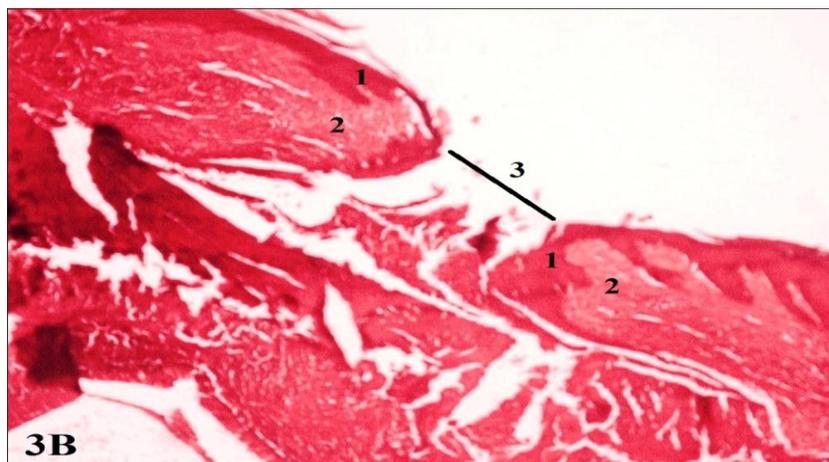
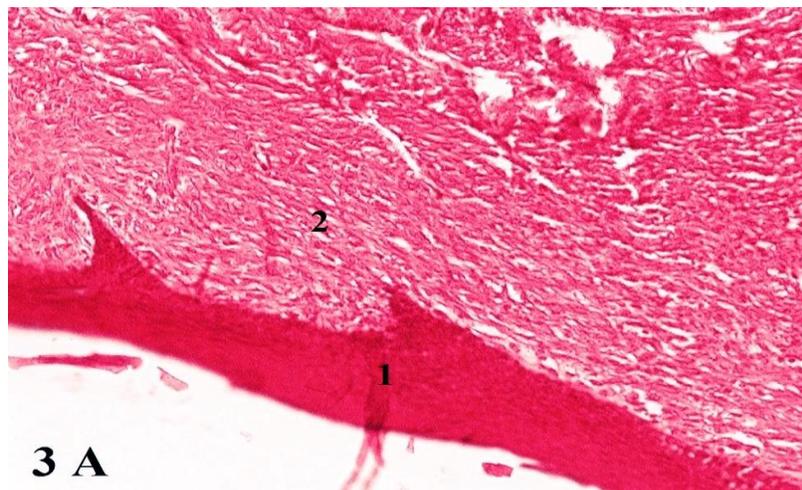
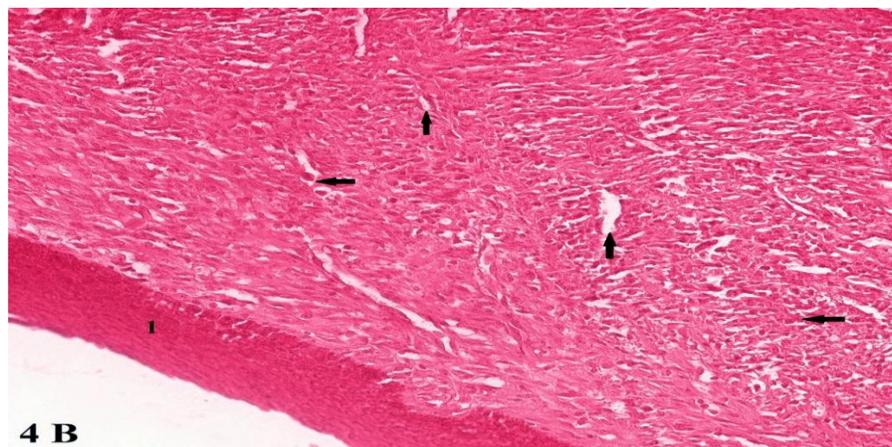
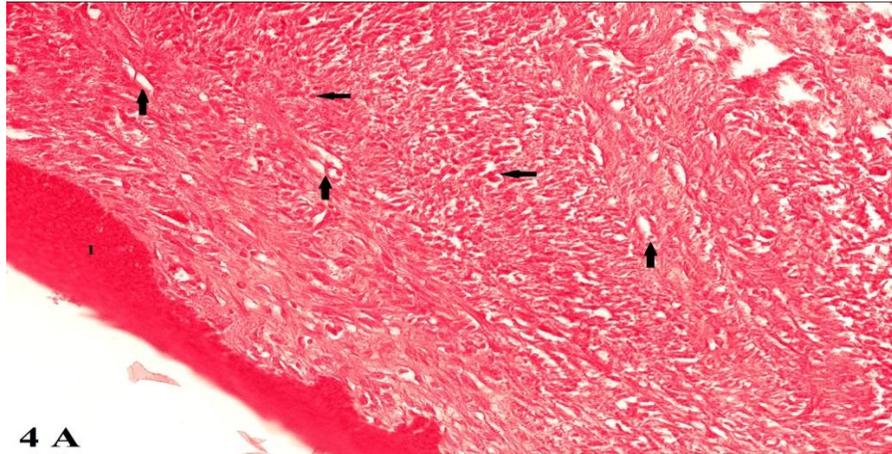


Figure.4 Granulation tissue in epithelial tissue (the eleventh day study)



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