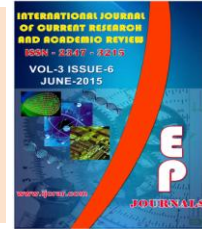




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Wound healing effect of topical Grape Seed extract (*Vitis Vinifera*) on Rat palatal mucosa

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A B S T R A C T

Wound healing is a process of re-establishing of the tissues physical integrity in the inside and outside of the body's structures and including the complex relationships between cells and different factors. Interest in the use of natural antioxidants, especially with plant origin in wound healing has been increased in recent years. The aim of this study was evaluation of Wound healing effect of topical Grape Seed extract (*Vitis Vinifera*) 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 Grape Seed extract (*Vitis Vinifera*) 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 reepithelialization rate was similar in different days in the test and placebo. The formation of granulation tissue was higher in the placebo. The presence of inflammatory cells involved in the healing process in the placebo group, from the second day to the fifth day had the upward trend and showed downward trend from sixth day. While in the test sample from the second to fourth day, the amount of inflammatory cells showed an upward trend and from fifth day, the downward trend was observed in these factors. The angiogenesis rate on different days was similar in both groups. In Overall, the thickness of the coating restored showed an upward trend, although was varied in different days and did not follow a regular ascending or descending pattern. 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 than placebo group had slower process. And at the end of eleven days, the result was almost the same. Because the sample testing, on the ninth day, the epithelial tissue was fully healed, Granulation tissue modeling and deposition of collagen fiber organization more in the sample tested was greater than placebo, overall it can be concluded that in this study, *Vitis vinifera* more effective than placebo in the treatment of wound healing is completely rats.

Introduction

Today, chronic wounds and non-healing wounds have turned into a health problem (1). The wound healing process occurs in 3 steps: inflammation, proliferation, and remodeling. Inflammation is the first step, which begins immediately after the injury, and is accompanied by deposition of inflammatory cells like monocytes and macrophages (2).

The proliferative phase is identified by angiogenesis, collagen aggregation, granulation tissue formation, epithelialization, and wound contraction (2).

Angiogenesis plays the main role in the wound healing procedure. Among many known growth factors, VEGF is the highest, most effective factor with long effect that stimulates angiogenesis in the wound (3).

VEGF is a homodimeric glycoprotein, which stimulates the migration and reproduction of endothelial cells, increases vascular permeability, and carries out the angiogenesis procedure (4).

Inflammation is part and parcel of an acute response, which causes neutrophil aggregation at the wound site. These cells play the key role in defense against bacteria and other pathogens and digestion and clearance of tissue slices at the site (5).

Oxidants are not produced at the wound site only by neutrophils; rather, they can also be produced by macrophages. Thus, the wound site is rich in reactive types of oxygen and nitrogen along with their derivatives (6-7).

Proanthocyanidins or tannins are a group of biologically active polyphenyl phenolic bioflavonoids produced by many plants. It is believed that proanthocyanidin and other

tannins facilitate the wound healing procedure (6, 7); however, it is not known how they take effect (8-9). The proanthocyanidin in grape seed can specifically induce production of VEGF in human keratinocytes (8, 9).

Lipid peroxidation is one of the main purposes of food product fermentation during the process of processing and storing them. Synthetic antioxidants like AAA are widely used as food additives to increase the shelf life of food. However, BHT and BHA not only have toxic and carcinogenic effects on man, but also improperly affect enzyme systems (10-11).

Therefore, interest in use of natural antioxidants, especially those with plant origin, has increased in recent years. Natural antioxidants can protect man against free radicals, which can cause some chronic diseases like cancer, cardiovascular diseases, and cataract. Antioxidant features in plant oils is due to their polyphenol contents (10-11).

Therefore, plants with high polyphenol contents play an important role as natural antioxidants. It has been well recognized that grape skin and its seed and stem are resources rich in polyphenol (10, 11).

These important compounds are an integral part of human diet, and can have protective effect against different types of cancer, arteriosclerosis, ischemia, and inflammatory diseases. Epidemiologic studies have demonstrated that use of drinks and foods rich in phenyl phenol like tea and grape juice is related to decrease in rate of death due to coronary artery diseases. Protective effects of vegetables, fruits, and drinks against coronary artery diseases and certain types of cancer are due to the flavonoid contents of these foods (12-14).

It has been demonstrated that these compounds containing phenyl phenol can apply their antiatherogenic advantages by controlling LDL oxidation. Presence of bioactive compounds in the grape, particularly phenyl phenolic products and synergistic effects between them, is related to this feature. The phenyl phenolic contents in the grape range from monomers to complex compounds of the tannin type (oligomers and polymers). The antioxidant compounds found in the grape that have been identified so far include phenyl phenolic acids, flavan-3-ols, flavonols, and antioxidants (12-14).

One of the most evident of these phenyl phenolic compounds is flavan-3-ols, catechin. Flavan-3-ols is mostly in the grape's seed and skin. The grape's phenyl phenolic acids and hydroxycinnamic acids are a form of tartaric acid esters found in the grape's skin and pulp. On the other hand, the flavanols in the white and red grape are only found in the grape's skin (12-14).

Epidemiologic studies demonstrate the relationship between moderate use of the red grape and decrease in the risk of coronary artery diseases. They have demonstrated that the polyphenyl phenolic compounds in the red grape eliminate endothelial contraction of vessels, activate nitric oxide synthase, control platelet aggregation, and prevent LDL cholesterol oxidation. The resveratrol polyphenyl phenolic compounds in the red grape are responsible for its useful cardiovascular effects (15-17).

The black grape has anticancer effects. It has been observed in several studies that the grape and its seed can have a significant controlling effect on tumor formation and development. For example, they have demonstrated that the grape seed oil

significantly prevents prostate tumor formation in man (18).

The antioxidant effect of the grape seed's proanthocyanidins is 20 times more than that of Vit-C and 50 times more than that of Vit-E. It was observed in a study that combined use of fish oil and vitis-vinifera has reinforcing effects on reduction of inflammation and reduction of tissue damage due to UC, and use of vitis-vinifera and fish oil can prevent colitis from recurring (19).

In view of the numerous benefits of vitis-vinifera mentioned and its anti-inflammatory effects and antioxidant properties and the lack of studies so far on local use of the substance in the oral mucosa, it was decided in this study that the substance should be locally used on palatal wounds in rats, so that by comparing its effect to that of placebo, a decision can be made about local use of this valuable substance to accelerate cleft palate healing in suffering children.

The purpose of this study is to examine local effect of vitis-vinifera on the trend of healing palatal area wounds in rats.

Materials and Methods

In an experimental study we conducted at ENT department of Tabriz University of Medical Sciences, we examined effect of local vitis-vinifera on the trend of healing palatal area wounds in rats by examining the results of local healing by phenytoin.

There were 20 rats including 10 in the test group and 10 in the control group. The animals were randomly and equally divided into the two test and control groups.

The study was performed on 20 male healthy rats weighing between 200 and 300

grams. The rats were kept in a standard environment with a temperature of 26±2 degrees centigrade.

No medication therapy was performed during the study. The rats were then randomly divided into two groups, where the first group contained 10 untreated rats (control group or group A), and the second group contained 10 rats treated by local vitis-vinifera (group B).

All the rats were anesthetized again every day, and after the wound sites were examined, the healing trends were macroscopically observed, and images were taken, the local product vitis-vinifera was applied once a day onto the wound sites in group B.

In group A, a placebo suitable to the local product grape seed with no effect on late or early wound healing was applied onto the wound sites. The rats in the two groups were executed on days 3, 5, 7, and 9 (two animals in each case), and tissue samples were taken of their palatal areas, and after providing tissue sections and dyeing them using H&E and Masson's trichrome methods, they were examined under an optical microscope and reported by a pathologist from a histopathological aspect. The pathological study included the study of the epithelialization tissue and the granulation tissue.

As for the epithelialization tissue, the cell strata were studied from the aspects of approximation of wound edges and formation of the integrity of the stratified squamous tissue.

The granulation tissue was examined from the aspects of hyperemia, edema, the amount, spread, and maturity of collagen fibers, whether or not these fibers are

parallel with the wound surface and the epithelium basement membrane, the amount, maturity, and spread of blood vessels formed and their directions as to whether they are parallel with or perpendicular to the wound surface and the epithelium basement membrane, infiltration of multi-core inflammatory cells and its intensity, and presence or absence of bacterial micro-colonies.

It should be mentioned that all the plan's steps were taken in the Single Blind form.

The microscopic study was conducted using OLYMPUS-CH30 optical microscope, and the histomorphometric calculations were performed using WF10X-18MM and PF10X lenses.

What is meant by "the length of the unhealed wound area" is the length of the part where no reepithelialization has been formed.

What is meant by the "inflammation intensity" factor is the rate of presence of polymorphonuclear and mononuclear cells present at the site for healing, not the inflammatory cells aggregating in surface parts of the injured epidermis, around the pseudo-stratified cylindrical tissue of the nasal cavity or inside the nasal cavity, due to wound infection. In order to count these cells, all cells in question were counted at a magnitude of 400 in three fields and in all 100 small squares, and their average was then recorded. For the "angiogenesis" factor, by which the rate of newly formed blood vessels is meant, blood cells were counted at a magnitude of 400 in three fields and in all 100 small squares, and their average was then recorded.

In order to measure "the epidermis healed area thickness," the factor was measured at

four points, and their average was then recorded.

Ethical considerations

In order to include the rats in the study, the permission of the ethical committee was obtained.

Results and Discussion

The following results were obtained from the examinations made of the samples under study in the daily examinations:

Examination of all the items under study, including the length of the unhealed wound area, the epidermis healed area thickness, the granulation tissue spread, the spread of the deposited collagen fibers, the inflammation intensity, angiogenesis, and post-surgery infection, on the days of examination for the two groups is displayed in Tables 1 to 4.

The results of Tables suggest that:

In view of the results observed in the present study, the amounts of reepithelialization on different days were similar in the placebo experimental sample. On the eleventh day of the study, in the experiment sample, the injured epithelium was completely healed, while in the placebo sample on that day, the injured epithelium healing still remained incomplete.

Formation of the granulation tissue began at a low rate on the second day of the study, and it was higher in the placebo sample until the third day of the study. On the fourth day, sudden, intense increase was observed in the experimental sample, and the trend continued until the eighth day, and after that, on day 11, it was again higher in the placebo

sample. The falling trend was observed in the factor from the ninth day.

The rate of presence of inflammatory cells involved in the healing process had a rising trend in the placebo sample from the second to the fourth day, and displayed the falling trend from the sixth day, while in the experimental sample, the rate of these inflammatory cells displayed a rising trend from the second to the fourth day, and the falling trend was observed in the factor from the fifth day. On the eleventh day, the factor was higher in the experimental sample than in the placebo sample.

The angiogenesis rate was also almost similar on different days of the study in both groups. The factor was maximized in both samples on the fifth day, and had falling trends in both samples from the sixth day on.

Collagen fiber deposition was observed at a low rate on the eleventh day of the study, which was higher in the experimental sample than in the placebo sample. On day twelve, no change was observed in the factor as compared to the day before in the experimental sample.

The healed epithelium thickness displayed an overall rising trend, although it fluctuated on different days, and did not follow a regular falling or rising trend. In view of the fact that there was only one sample in each group on each day, the fluctuation can be attributed to the individual characteristics of the animal. Usually in the general case, in the epidermis healing trend, the healed epidermis thickness is high in the first two to three weeks, and as the epidermis grows mature afterwards, its thickness also decreases a little, and then remains constant.

There was post-surgery infection in all samples with moderate to severe intensity, which can, of course, be considered as normal in this study in light of the location of the hard palate and its contact with food in the oral cavity.

Hard palate epithelium healing on the fifth day of the study is displayed in Figure 1, in which figure the experimental sample is 1B, and the placebo sample is 1A. New epidermis formation has begun on the second day (1), and has increased in both groups at good ratios on the fifth day. On the fifth day, the granulation tissue spread was higher in the experimental sample than in the placebo sample, while the number of polymorphonuclear and mononuclear cells involved in the healing procedure (the vertical arrows) was higher in the placebo group. The angiogenesis rate (the horizontal arrows) was almost the same in both samples.

Post-surgery infection on the fifth day of the study is displayed in Figure 2, in which figure the experimental sample is 2B, and the placebo sample is 2A. Inflammatory cell invasion (1) around the cylindrical pseudo-stratified epithelium of the nasal cavity (arrow) caused post-surgery infection to occur in all samples and on all days, though with different intensity. As clear from the figure, the intensity of post-surgery infection was higher in the placebo sample (severe infection) than in the experimental sample (moderate infection) on the fifth day of the study.

Hard palate epithelium healing on the eleventh day of the study is displayed in Figure 3, in which figure the experimental sample is 3B, and the placebo sample is 3A. On day eleven, the injured hard palate epithelium (1) was completely recovered in the experimental sample, while the healing

was performed incompletely and less quickly in the placebo sample.

The granulation tissue formed in the healed area of the hard palate epithelium on the eleventh day of the study is displayed in Figure 4, in which figure the experimental sample is 4B, and the placebo sample is 4A. In the granulation tissue formed, the number of polymorphonuclear and mononuclear cells involved in the healing procedure (the horizontal arrows) decreased more in the experimental sample than in the placebo sample, and more maturity occurred in newly formed vessels during the angiogenesis procedure on the eleventh day of the study (the vertical arrows in Figure A). As clear from the figure, vessels can be observed in the placebo sample that are not yet in the same direction as the epithelium is (the vertical arrows in Figure B).

Preservation of the skin's integrity is of particular importance in humans and animals, because it protects them against dehydration, bleeding, and invasion by microorganisms (19).

Loss of the epidermis and the dermis is called *wound*. The wound healing procedure begins with platelet aggregation and degranulation and formation of platelet-fibrin. Then in the inflammatory phase, multi-core leukocytes and macrophages are drawn to the wound site and discharge the cytokine and growth factors, fibroblasts and blood vessels from the surrounding dermis cause the formation of granulated tissue, and finally, reproduction of epidermis cells toward the wound edge until they reach the granulated tissue causes the wound to close (20-21).

Several local and global factors such as age (20, 22), use of medications, nutritional status, blood circulation status, tissue

hypoxia, use of local compounds, and wound dressing are also effective on the wound healing procedure (19-22).

Because as the healing time gets long, the wound site has the potential for bacterial infections, and formation of malformed scars is more likely, many physical, spiritual, and mental problems are imposed on the patient. Therefore, it has attracted the attention of researchers in medicine today to use factors that cause the wound healing time to decrease.

In a study conducted for investigation of GSE's local effects, after making two full-thickness wounds with a size of 16x8 millimeters on the dorsal part skin of the rats under study, one of the wounds was allowed to be secondarily healed, and 5.2 milligrams of saline or GSE was rubbed over the other wound. The rats were pursued for 5 days, and it was examined how the wounds were being recovered with digital photography and histological evaluation. It was concluded in this study that GSE accelerates wound healing procedure, and this was clearly observed within 1 day after wounds appeared. It was also found in the histological examination of wounds that cell density in the granulation tissue was clearly higher in the group where wounds were treated with GSE than wounds having received placebo (23).

In our study, formation of the granulation tissue began at a low rate on the second day of the study, and it was higher in the placebo sample until the third day of the study. On the fourth day, sudden, intense increase was observed in the experimental sample, and the trend continued until the eighth day, and after that, it was again higher in the placebo sample on day eleven. The falling trend was observed in the factor from the ninth day.

In another study, to examine effect of the local grape skin on the wound healing procedure, after making a full-thickness wound in the rats' backs, the rats were randomly placed in three groups. In group 1 (standard), the rats received the mupirocin ointment standard treatment. Group 2 (control) were treated with petroleum jelly, and group 3 (experimental) underwent treatment with the grape skin powder. It was observed in this study that the wound healing procedure has proceeded faster in the group having undergone treatment with the grape skin powder (24).

In our study, collagen fiber deposition was observed at a low rate on the eleventh day of the study, which was higher in the experimental sample than in the placebo sample. On day twelve, no change was observed in the factor as compared to the day before in the experimental sample.

In another study, to examine effects of GSE on wound healing in rabbits, a circular wound with dimensions of 20x20 millimeters was made in the rabbits' back skin. The rabbits were randomly placed in 7 groups. The negative control group received no treatment. The positive control group and the eucerin group received phenytoin cream (1%) and eucerin twice a day from the beginning of the study until wound healing completion. The treatment groups were treated twice a day with local GSE cream (2, 5, 10, and 70%) in eucerin base. To evaluate wound recovery percentage, the wound circumference was measured every day. Histological evaluations were performed on the seventh and fifteenth days. The results demonstrate statistically significant difference between the groups undergoing treatment with GSE and those undergoing treatment with eucerin on most days. The rabbits treated with 2% GSE had the best results; it was concluded that 2% GSE

administration can accelerate the wound healing procedure in rabbits (2).

In another study, to examine effects of oral GSE on full-thickness skin wound recovery made through surgery, 48 male rabbits were randomly placed in two groups. In one group, 260 mg/kg GSE was given orally for 20 days from a week before surgery. A circular full-thickness skin wound (with a diameter of 2.5 cm) was made in midline backs of all the rabbits while anesthetized, and the wounds were macroscopically and histologically evaluated on days 5, 10, 15, and 20 after surgery. In the group receiving GSE, wound healing, reepithelialization, granulation tissue formation, collagen aggregation, and angiogenesis occurred considerably faster, and the inflammatory reaction and the wound were removed within a shorter time (25).

In our study, the healed epithelium thickness displayed an overall rising trend, although it fluctuated on different days, and did not follow a regular falling or rising trend. In view of the fact that there was only one

sample in each group on each day, the fluctuation can be attributed to the individual characteristics of the animal. Usually in the general case, in the epidermis healing trend, the healed epidermis thickness is high in the first two to three weeks, and as the epidermis grows mature afterwards, its thickness also decreases a little, and then remains constant.

Conclusion

Granulation tissue formation, presence of inflammatory cells, and angiogenesis displayed a slower trend in the placebo sample than in the experimental sample. But in the experimental sample, on day 11, the epithelium had healed completely, the granulation tissue was more organized, and collagen fiber deposition was also higher in the experimental sample than in the placebo sample. In light of these results, it can generally be concluded that in this study, the effectiveness of green medication has been higher than that of placebo medication.

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	0	0	+3	+3	+3	+3	+2	+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	+2	+2	+3	+3	+3	+3	-	+3
The extent of deposition of collagen fibers	0	0	0	0	0	0	0	0	0	0	-	0
Inflammation	0	0	+1	+3	+3	+3	+3	+3	+3	+3	-	+3
Angiogenesis	0	0	+2	+2	+2	+2	+3	+2	+3	+3	-	+2
Wound infection	+2	+2	+3	+3	+2	+2	+2	+2	+2	+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	-	+1	+2	0	+1	0	-
The thickness of the healed epidermis	-	+2	+3	-	+3	+2	+2	+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	+2	-	+2	+2	+1	+1	+1	-
Angiogenesis	-	+2	+2	-	+2	+2	+1	+1	+1	-
Wound infection	-	+2	+1	-	+3	+1	+3	+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	13.5	0	0	0	0	Moderate	0
Second Day	13	13	29	6	0.8	Sever	0
Third Day	11	25	52	9	0.9	Moderate	0
Fourth Day	9	45	75	10	0.9	Moderate	0
Fifth Day	7.9	47	43	11	1.2	Moderate	0
Eight Day	3.5	45	39	6	1.2	Mild	0
Ninth Day	2.4	41	35	5	1.1	Sever	0
Eleventh Day	0	40	35	5	0.9	Sever	3
Twelfth Day	0	40	25	4	1.1	Moderate	3

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	0.8	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	0
Eleventh Day	3	44	25	4	1.1	Moderate	2

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

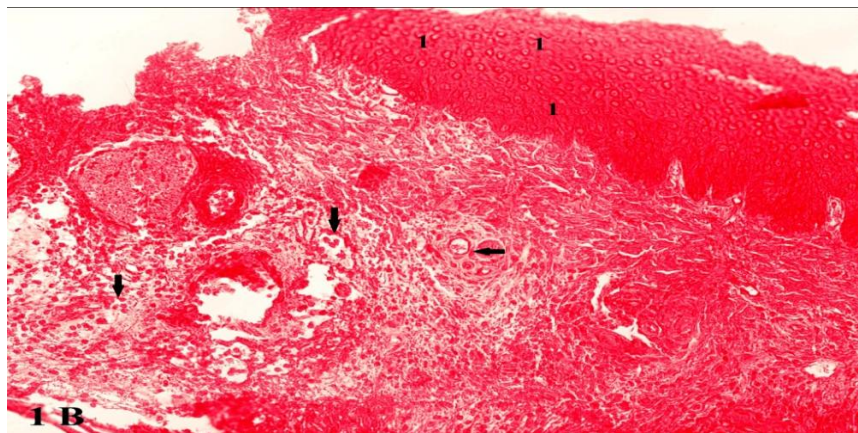
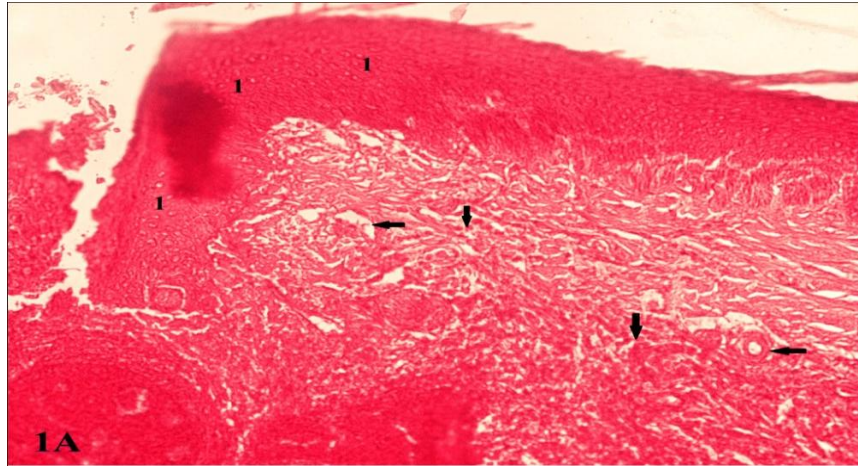
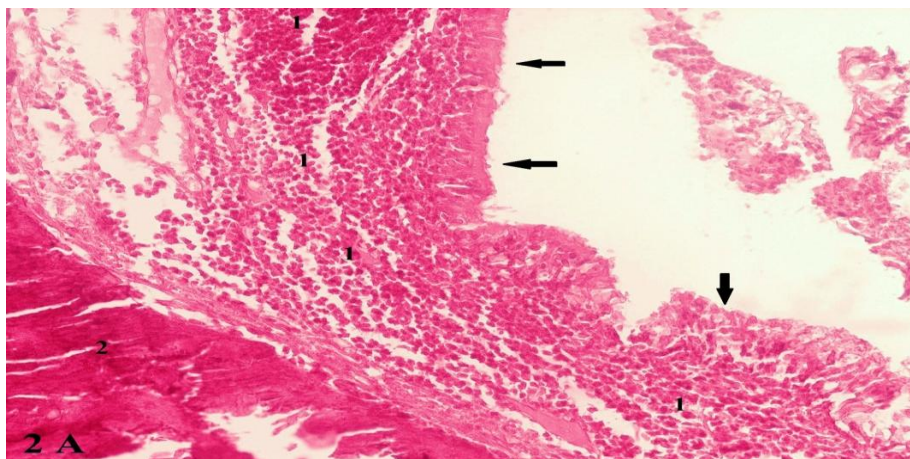


Figure.2 Post operative infection (the fifth day study)



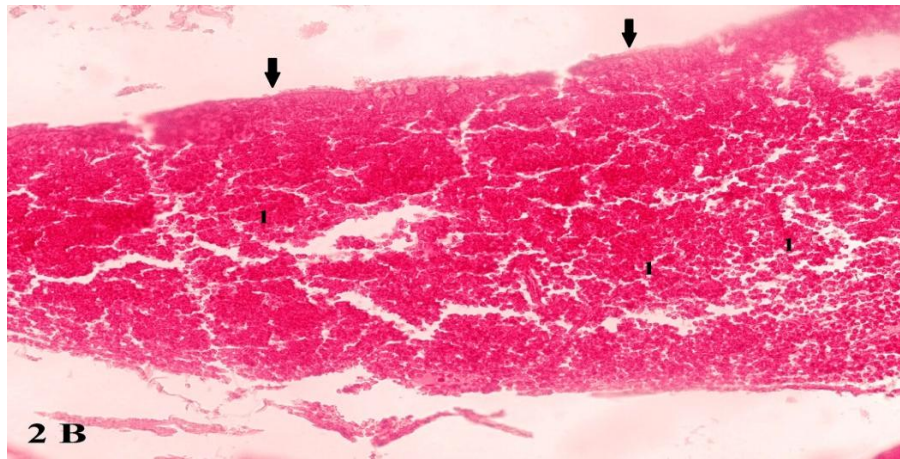


Figure.3 Healing of epithelial tissue (the ninth day study)

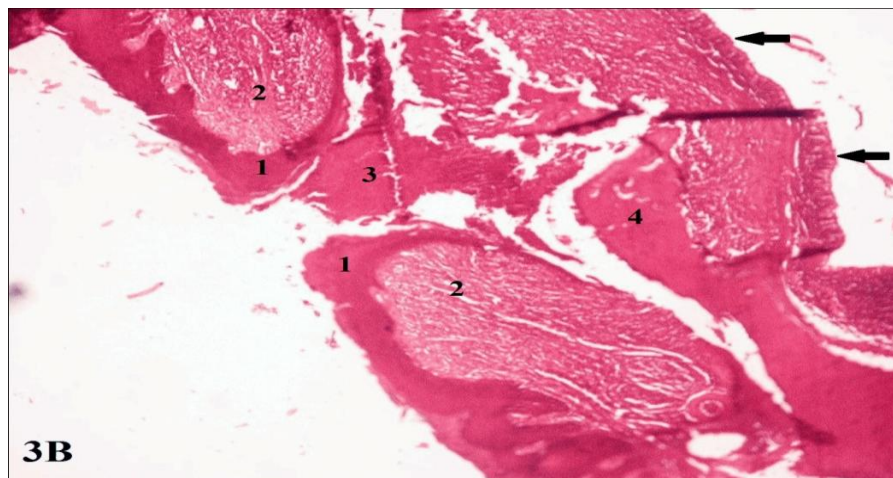
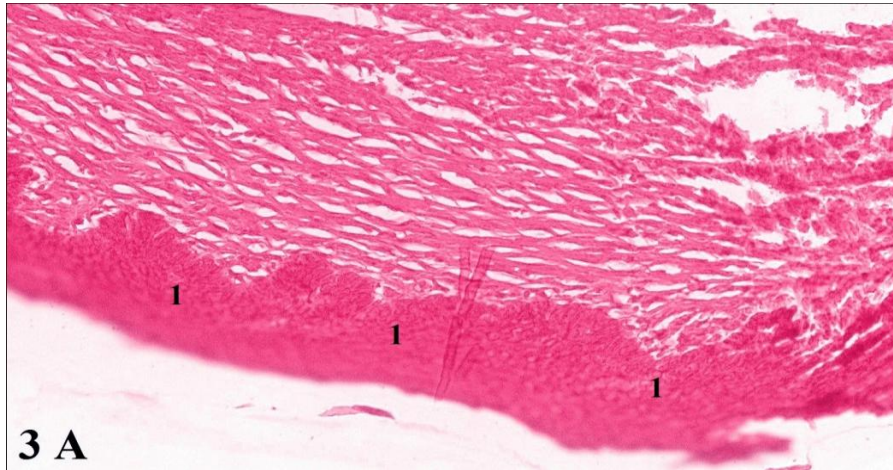
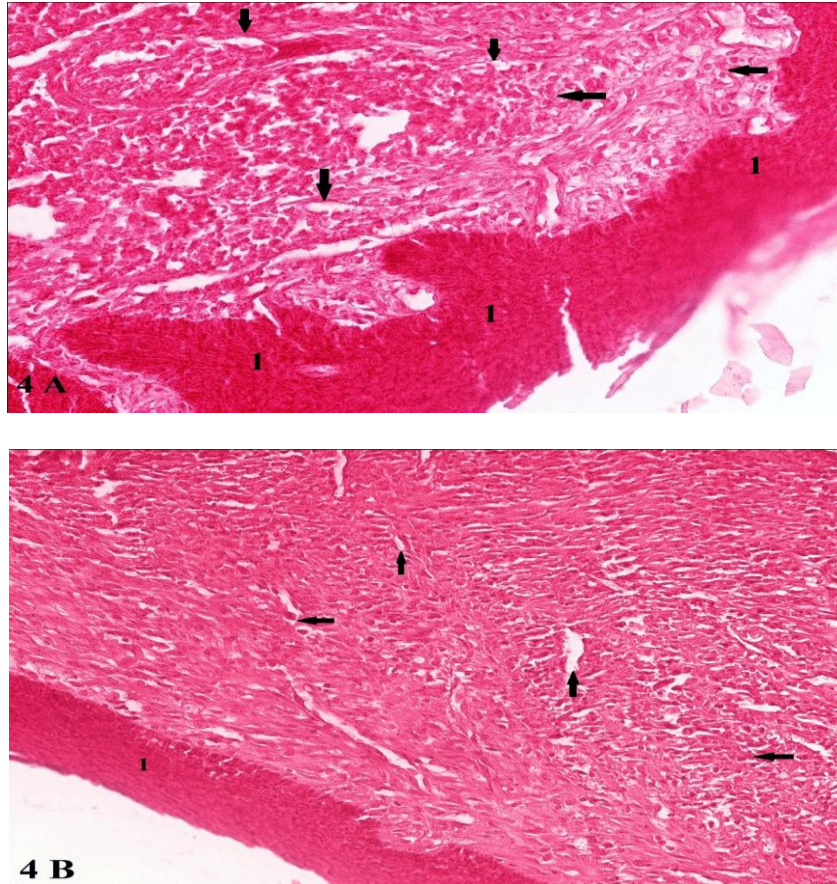


Figure.4 Granulation tissue in epithelial tissue (the eleventh day study)



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