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RESEARCH ARTICLE

BIOLOGICAL IMPACT OF MELATONIN ON THE HEALING OF ALBINO RATS' TONGUE ULCER

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Abstract

The pineal gland hormone melatonin is known to have both anti inflammatory and immunomodulatory effect. The present study assessed the effects of intraperitoneal injection of 10 mg/kg melatonin on the healing of induced tongue ulcer. 40 male albino rats were subjected to tongue ulcer using biopsy punch, group I (n=20), received daily 6% ethanol as vehicle. Group II (n=20), received daily melatonin. Specimens were taken after 1st, 4th, 10th day. The histological examination revealed prominent inflammatory cells infiltrations and increased new blood vessels formation. Ultrastructural studies showed marked increase in collagen fibers synthesis. Immunohistochemical results using α smooth muscle actin indicated significant difference between two groups after 4th days. Melatonin treatment improved the wound healing, both in terms of angiogenesis and orientation of collagen fibers. Considering our results, melatonin has powerful anti-inflammatory properties and has proven to be highly effective.

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Introduction:-

Oral mucosal wound or mouth ulcers are sores or open lesions in the mouth which are caused by various disorders⁽¹⁾. Wounds are not just physical problems due to blood loss or tissue damage, but they may threaten the individual survival by development of infection and sepsis due to invasion of micro-organisms or contaminants. Mucosal wounds occur frequently, and the healing of the mucosa is important in most surgical outcomes. Although wound healing in the oral mucosa is improved by sound surgical principles, yet it is also mediated by biologic processes beyond the surgeon's control⁽²⁾. It should also be noted that ulcers and/or erosions can be the final common manifestation, often clinically indistinguishable, of a wide and complex spectrum of conditions including traumatic lesions, infectious, vesiculo-bullous, neoplastic and gastrointestinal diseases⁽³⁾.

The process of wound healing takes place in three stages, the inflammatory, proliferative, and remodeling phases⁽⁴⁾. These phases consist of a sequence of events that ultimately leads to the re-establishment of tissue integrity and function. Persistence of inflammation during repair may lead to the development of chronic wounds that are of great concern to society. During the inflammatory phase, several enzyme systems are induced and growth factors are recruited, such as nitric oxide synthase (NOS), which help in the progression of healing and are critical for the repair process^(5,6).

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Platelets and fibroblasts, as well as macrophages and neutrophils, are important cellular elements of wound healing, engendering influential factors that affect processes of healing such as migration and proliferation. In addition, some specific proteins produced by the macrophages, such as growth factors, proteases, chemo attractants, and inhibitory factors, also play roles in the wound-healing process ⁽⁷⁾.

Immune system cells and their products (including cytokines and growth factors) stimulate wound healing, especially during the proliferative phase and angiogenesis. Fibroblasts serve as the source of the endothelial cells that generate neovascularization during the angiogenesis process ⁽⁸⁾.

Fibroblasts are the most abundant cellular components of connective tissue. They possess phenotypical heterogeneity and may be present in the form of smooth muscle cells or myofibroblasts (MFs). MFs are spindle-shaped cells with stress fibers and well developed fibronexus, and they display α -smooth muscle actin immunohistochemically and smooth muscle myofilaments ultrastructurally. MFs play pivotal roles not only by synthesizing and producing extracellular matrix components, such as other connective tissue cells, but also are involved in force production. ⁽⁹⁾

MFs generate forces in two ways. Initially, actin filaments present within the cell form a fibronexus by connecting intracellular actin and extracellular fibronectin fibrils using integrins. Integrins mediate the reorganisation and contraction of collagen matrices with the help of fibroblasts. Later, MFs connect to each other through gap junctions to form a "multicellular contractile unit". They again exert a force on the ECM by implicating the use of this unit. Both mechanisms exert a high level of tractional forces for wound closure ^(10,11).

Wound healing in the oral cavity essentially occurs without scarring and is faster than skin healing. Fibroblasts in the oral mesenchyme possess a unique phenotypical character by constitutively expressing elevated α -SMA levels, along with a higher capacity to contract collagen gel and a higher replicative potential than dermal fibroblasts, ultimately leading to a "scar-free" healing process. Factors, such as epidermal growth factor, vascular endothelial growth factor, bFGF, and insulin-like growth factor, present in saliva and crevicular fluid are responsible for wound healing in the oral cavity ⁽¹²⁾.

The pineal gland hormone, melatonin (N-acetyl-5-methoxytryptamine) has a variety of physiological, immunological and biochemical functions. It is a direct endogenous free-radical scavenger and indirectly exerts chemoprotective, immunostimulatory and myelostimulatory effects ⁽¹³⁾.

Melatonin is capable of entering the oral cavity by diffusing into the saliva from blood. As the majority of the melatonin remains bound to serum albumin, the amount of melatonin in saliva is approximately one third of that present in the blood ⁽¹⁴⁾.

Melatonin is an anti-inflammatory agent known to reduce several hallmarks of inflammation. Melatonin down-regulates a variety of pro-inflammatory cytokines such as interleukin (IL)-1b, IL-6 and tumour necrosis factor- α by preventing the translocation of nuclear factor kappa B to the nucleus and its binding to DNA ^(15,16). In addition, melatonin has been shown to inhibit the production of adhesion molecules that promote the sticking of leukocytes to endothelial cells and by reduces oxidative stress ⁽¹⁵⁾. We therefore investigate if melatonin administration would improve the course of wound healing in albino rats' tongue ulcers

Materials and Methods:-

This study was undertaken in the animal house of Faculty of Pharmacy of Mansoura University, based on an ethical approved protocol. 40 albino rats of average weight (150-200gm) were used in this study. The animals were fed a standard diet and free access to water.

The animals were divided into two groups

Group I: twenty rats were subjected to tongue ulcer and received daily 6% ethanol saline as a vehicle. Group II: twenty rats were subjected to tongue ulcer and received daily 10 mg/kg BW melatonin through intraperitoneal injection (IP).

Melatonin preparation:-

Pineal indole melatonin (Sigma, St. Louis, MO, USA) was freshly dissolved in saline containing 6% ethanol (total volume of 1 ml/kg). Daily IP injections of 10 mg/kg BW were administered using a 1-mL syringe with a 25-gauge needle. To avoid interfering with the daily circadian rhythm, melatonin and saline were administered at 8:00 and 9:00 am⁽¹⁷⁾.

Ulcer induction:-

All surgical procedures were performed under general anesthesia, by intramuscular administration of 0.1 ml of ketamine hydrochloride (SIGMATEC Company) combined with 0.05 ml of xylazine hydrochloride (ADWIA Company), per 100 g body weight of the animal. After anesthesia, the lingual mucosa was antiseptically cleaned with 2% chlorhexidine then a surgical mucosal wound was made by using biopsy punch (Acu-Punch, Acuderm Inc., Ft. Lauderdale, FL, USA) to ensure that all ulcers will have the same size. The ulcer was circular in shape about 4mm in diameter and 2mm in depth.

Specimens' preparation:-

After ulcer induction both groups, specimens were obtained from each animal after one day, 4 days and 10 days respectively; tongue from each animal were removed and immediately fixed with 10% formalin solution and become ready for histological, and immunohistochemical for alpha smooth muscle actin. Specimens were put in glutaraldehyde fixative (4%) solution for electron microscope preparation.

Computer Assisted digital image analysis (Digital morphometric study):-

Slides of IHC (alpha smooth muscle actin) were photographed using Olympus[®] digital camera installed on Olympus[®] microscope with 1/2 X photo adaptor, using 40 X objective. The result images were analyzed on Intel[®] Core I3[®] based computer using VideoTest Morphology[®] software (Russia) with a specific built-in routine for stain quantification, results were expressed as integrated density.

Statistical Analysis:-

Data was analyzed using Statistical Package for Social Science software computer program version 17 (SPSS, Inc., Chicago, IL, USA). Quantitative data was presented in mean and standard deviation. Student's t-test was used for comparing means of the two groups. P value less than 0.05 was considered statistically significant.

Results:-

Clinical Observations:-

Three rats were excluded from our study; 2 rats from group I were died after one day of ulcer induction, one rats from group II was died after one week of ulcer induction. All these rats were compensated by others.

Light microscopic results:-

Haematoxylin and Eosin Stain:-

After one day of ulcer induction:-

The specimens of group I revealed discontinuity of the epithelium and the underlying connective tissue, the granulation tissue showed inflammatory cell infiltration (Figs. 1A&1a). While group II section showed granulation tissue formation, inflammatory cell infiltration and dilated blood vessels. Mitotic figures appeared in the epithelium which representing the beginning of the epithelial proliferation (Figs. 1B&1b)

After four days of ulcer induction:-

The sections of group I showed slight proliferation of the covering epithelium and formation of the granulation tissue with dense inflammatory cell infiltration (Figs. 2A, 2a). While in group II : The sections showed the beginning of the healing process via proliferation of the epithelium. The granulation tissue appeared with fibroblasts, collagen fibers, inflammatory cell infiltrations (lymphocytes and macrophage appeared) and newly formed blood vessels (Figs. 2 B, 2b).

After ten days of ulcer induction:-

Group (I) showed the healing process appeared by prominent proliferation of the covering epithelium to close the ulcer margin as well as the connective tissue stroma that showed fibroblasts, collagen bundles, newly formed blood vessels and few inflammatory cells (Figs. 3 A, 3a).

Group (II) showed complete reepithelialization of the ulcerative area with normal stratification, reorganization of the

epithelium and the underlying connective tissue (Figs. 3 B, 3b).

Immunohistochemical stain results:

The immunohistochemical positive results were detected as brown deposits using alpha smooth muscle actin stain (Figs. 4).

Statistical results: using Paired Student's t-Test. There were no significant difference after one and ten days, while after four days, there were significant difference in the mean of α smooth actin of group I, II (Table 1).

Transmission electron microscope results

After one day; group I: There was little infiltration of the granulation tissue with neutrophils with widening of the intercellular spaces between the cells of the mucosa (Figs. 5, A). Group II: There was heavy infiltration of the granulation tissue with inflammatory cells (neutrophils) with normal intercellular spaces between the cells of the mucosa (Figs. 5, B)

After four days; group I: the sections showed irregular shaped fibroblasts and loosely arranged collagen bundles (Figs. 5 ,C).. Group II: the fibroblasts cells appeared regular spindle shaped with more closely packed collagen bundles (Figs. 5, D)

After 10 ten days; group I: the collagen fibers are loosely aligned (Figs.5,E) while in group II there were more better aligned collagen fibers (Figs. 5,F).

Discussion:-

In this study, a model of wound healing has a clinical condition that is frequently encountered in traumatic oral ulcers. An experimental time period of 10 days was chosen because most oral wounds even if infected, would show complete healing by the end of this time period. The wounds of this study were all made under the same experimental conditions and were standardized for size, depth and site. The choice of male rats also cancelled the effect of sex hormones on wound healing.

Melatonin, a hormone secreted mainly by pineal gland has been found to have antioxidant and anti-inflammatory properties in the oral cavity where it reaches through saliva⁽¹⁸⁾. The histological study demonstrates that melatonin accelerates the process of normal wound healing by interfering and modulating key biological processes involved in driving the wound healing response including inflammation, angiogenesis and collagen synthesis in rats. the healing process appeared by prominent proliferation of the covering epithelium to close the ulcer margins as well as the connective tissue stroma that showed fibroblasts, collagen bundles, newly formed blood vessels and few inflammatory cell. The present study reported the beneficial effects of melatonin on wound healing, which comes in accordance with Soybir et al., who investigated the role of melatonin in an experimental wound healing model and detected a higher number of macrophages, fibroblasts, neovascularizations, and higher collagen density in treated animals. They determined that exogenous melatonin has positive effects on the angiogenic phase of wound healing⁽¹⁹⁾.

Angiogenesis occurs during the proliferative phase of healing which is an important part of the wound healing process⁽²⁰⁾. Endothelial cells proliferate and migrate during new blood vessel formation and new vessels provide nutrients and oxygen to the newly formed tissue. These events are known to be initiated and driven by various growth factors especially VEGF165, the biologically active and most potent angiogenic protein known⁽²¹⁾. During normal wound healing, new blood vessel formation is initiated on day 3, peaks at day 7 and is essential for the formation of granulation tissue⁽²²⁾. In the present study, the angiogenic process peaked after fourth day in the melatonin treated wounds, implying that granulation tissue formation was accelerated. This results on the effect of melatonin on angiogenesis in wounded tissue is supported by Pugazhenth et al., that melatonin treatment accelerate the angiogenic process, increasing the formation of new blood vessels and elevating the level of vascular endothelial growth factor protein expression during granulation tissue formation⁽²³⁾.

The role of growth factors in the wound-healing process has been demonstrated. Platelet derived growth factor (PDGF) and TGF are well known growth factors secreted during wound healing by the α granules of platelets, macrophages, and fibroblasts⁽²⁴⁾. Both showed an increase in the content of collagen in the early phase of wound healing. Growth factors bind to the target with their specific cellular surface receptors and induce the cells to migrate, to divide, and to produce the other elements that influence the wound-healing process⁽²⁵⁾. Melatonin induces

the production of interleukin-1, tumor necrosis factor (TNF)- α cytokines, and transforming growth factor (TGF). In addition, melatonin is an immunomodulator and a neuroendocrine hormone, and stimulates both monocyte cytokine and fibroblast proliferation, which influence angiogenesis⁽²⁶⁻²⁸⁾.

Melatonin mainly exerts antioxidant effects by interacting with melatonin receptor 1 (MT1) and melatonin receptor 2 (MT2) receptors on cells⁽²⁹⁻³⁰⁾. Perhaps, a potent anti-inflammatory property of melatonin is linked to its ability to act as a scavenger of exogenous and endogenous reactive oxygen species (ROS) and reactive nitrogen species (RNS)⁽³¹⁾.

During wound healing, fibroblasts differentiate into myofibroblasts, which generate large traction forces for wound closure and tissue remodeling. One of the best-characterized markers of myofibroblast phenotype is α -SMA⁽³²⁾.

In our study α -SMA results peaks after 4th days, with significant difference in melatonin treated groups. In agreement with our results Cornelissen et al., found that normally no α SMA was found until 4 days post wounding and the number of positive cells increase from 4 to 8 days post wounding, at 8 or 9 days the majority of the granulation tissue fibroblasts were stained for α SMA and at about 12 days the proportion of α -SMA positive cells are steadily decreased till 22 post wounding where they found no single positive myofibroblast in the newly formed connective tissue⁽³³⁾.

The favorable effects of exogenous melatonin on wound healing were detected using ultrastructural study. Fibroblasts were denser with higher proliferation and more closely packed collagen fibers. Mechanisms associated with melatonin that may accelerate wound healing were presently investigated. One mechanism was TGF- β associated collagen synthesis. Melatonin has been shown to induce production of TGF- β ,⁽¹⁹⁾ which plays a significant role in promoting collagen synthesis and healing the wound, also the direct action of pineal hormone on the myofibroblasts of the scar could be responsible for melatonin-induced augmentation of collagen level⁽³⁴⁾. Moreover, this effect is dependent on the activation of melatonin membrane receptors on the cells synthesizing collagen⁽³⁵⁾.

Melatonin affects the activity and the levels of cellular mRNA of antioxidant enzymes including superoxide dismutase, glutathione peroxidase, and glutathione reductase⁽³⁶⁾. It may stimulate and regulate gene transcription of these enzymes via its receptors⁽³⁷⁾. Melatonin can cross all biological membranes, and thus it can indicate protective effects against oxidative stress. Melatonin reaches the nucleus of the cell and protects essential intracellular structures, including DNA, from oxidative damage⁽³⁸⁾.

Another investigation of the same authors revealed that collagen accumulation in the intact skin is under the control of the pineal gland. However, it was also reported that the effect of melatonin depends on the time of the application, so that morning injections increase the level of collagen in a wound⁽³⁹⁾. Hence, in the current experiment, melatonin injections were administered in the morning, between 8:00 am and 9:00 am for regular collagen production during wound healing. Previous studies showed that dosage and duration of the exogenous melatonin administration is variable and depends on the purpose of usage. The suggested dose required to exert antioxidant properties was considered

10mg/kg BW, in animal experiments⁽⁴⁰⁾. However Bulbulla et al. observed that subcutaneous melatonin application decreased collagen synthesis and epithelium proliferation and indicated undesirable effects on incision and anastomotic wound healing in normal and pinealectomized rats⁽⁴¹⁾.

Our study demonstrates that the administration of melatonin leads to significantly improved wound healing, the mechanism by which melatonin acts in this process, may be secondary to growth factors. Further research is required to establish in greater detail the effects of melatonin on the wound-healing process.

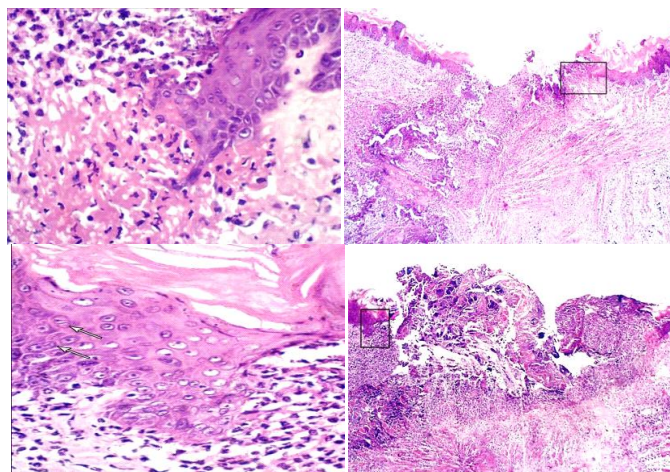


Fig 1:- Group I: A, a: showing discontinuity of the epithelium and the underlying connective tissue. The granulation tissue appears with inflammatory cell infiltration. Group II: B, b: showing the granulation tissue appears with inflammatory cell infiltration with different form of mitotic figure (arrow) (H&E, x100, x400)

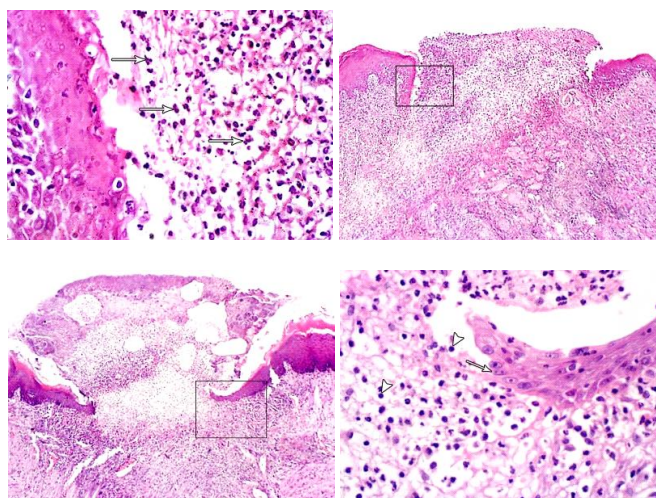


Fig 2:- Group I: A,a showing slight proliferation of the covering epithelium and formation of granulation tissues with heavy inflammatory cell infiltrations (white arrows). Group II: B,b showing beginning of the process via proliferation of the epithelium. The granulation tissue consists of fibroblasts, blood vessels, and inflammatory cell infiltration (arrow heads) and the epithelium shows mitotic figures (white arrow) (H & E stain, x100, 400).

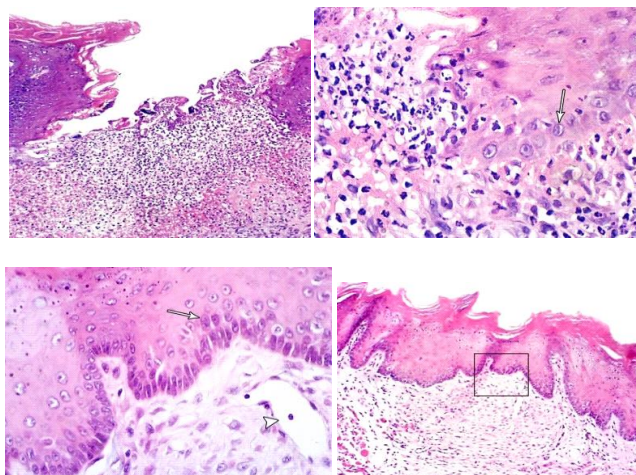
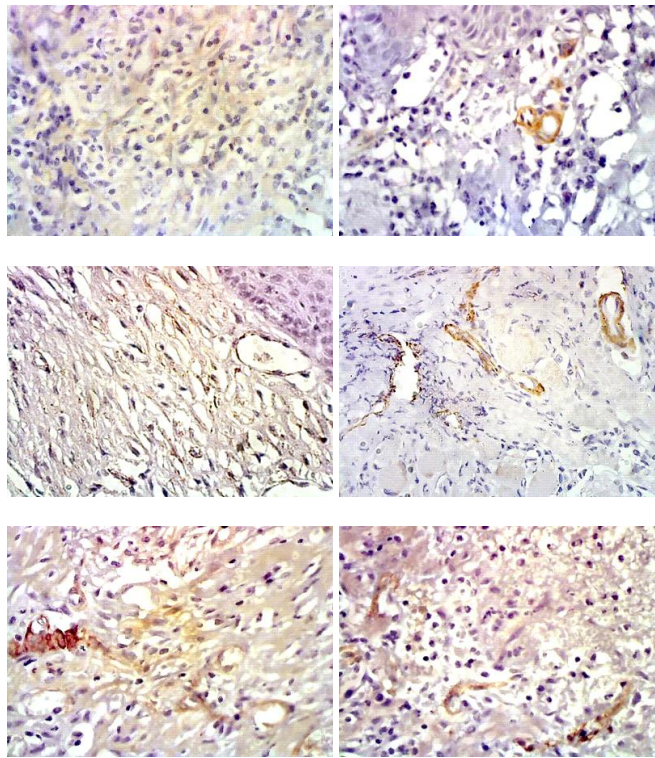


Fig 3:- Group I: A,a showing proliferation of the epithelium with different mitotic figure (arrow) and inflammatory cell infiltration. Group II: B,b showing complete reepithelialization (arrow) of the ulcer with proper architecture of collagen bundles in the lamina propria and dilated blood vessels (arrow head) (H & E stain, x100, 400).



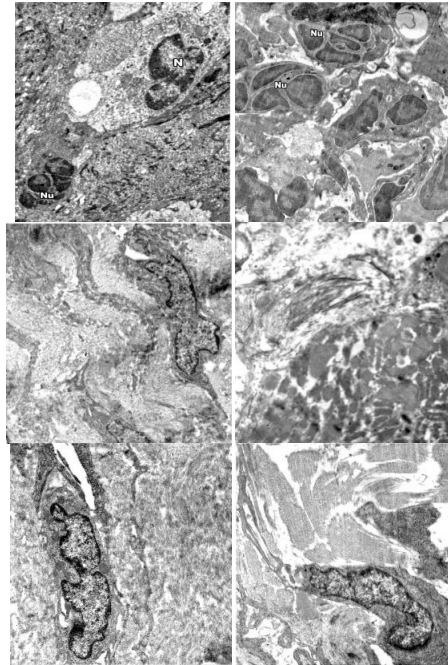
Figs. 4:- Group I (a) after 1 day, showing expression of α -SMA in the wall of blood vessels only. (b) After 4 days, positive immunoreactive at the granulation tissues. (c) After 10 days expression of α -SMA in fibroblasts and newly formed blood vessels. Group II (d) After 1 day, showing expression of α -SMA in the wall of blood vessels and very weak reaction in the lamina propria. (e) After 4 days, increased expression of α -SMA in the granulation tissues. (f) After 10 days, increased expression of α -SMA in the wall of blood vessels and lamina propria (IHC stain, α -SMA, x400)

Table 1:- difference in the mean of α smooth actin of group I, II using Paired Student's t-Test:

	Group I		Group II		P
	Mean	\pm SD	Mean	\pm SD	
Day1(10*5)	210854.3	61123.39	222756.23	65369.27	0.67
Day4	4708992.000	1472369.	7219015.000	1918931.	0.006
Day10	1647870.000	436412.	1889056.000	574152.	0.3

Not significance ($p > 0.05$)

Figure 4:- Line chart represents the mean differences between group (I, II) at 4th days.



Figs 5:- Transmission electron micrograph showing (A) Group I, after 1 day, showing neutrophils(Nu) and abnormal configuration of the nuclei of the cell of the epithelium. (B) Group II, after 1 day, showing heavy infiltration with neutrophils (A,B x 11700). (C) Group I, after 4 day showing loosely packed collagen fibers and irregularly arranged. (D) Group II, after 4 day showing regular spindle shaped fibroblasts with more packed and better aligned collagen fibers (C,D x17500). (E) Group I, after 10 day. The fibroblast was irregular in shape and collagen fibers were loosely packed. (F) Group II, after 10 day, showing spindle shaped fibroblast and densely packed collagen fibers (E,F x14600).

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