

Effect of Pomegranate Extracts on the Tissue Covering the Surgically Created Bone Defect on Rabbit'S Mandible

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Abstract

Pomegranate is a medicinal herbal that has been reported to promote healing of periodontal tissue due to its antioxidant and anti-inflammatory mechanism of action. The objective of this study is to evaluate the histological effects of potential effects of pomegranate on the tissue covering surgically created bone defect. 24 rabbits were used in this study; 12 rabbits were treated with 0.3g of pomegranate extracts powder by inserting into surgically created bone defect, while the other 12 rabbits were used as baseline control group. The surgical process was performed locally through horizontal incision, 2mm below marginal gingiva, circular bone defect created into rabbit's mandible, 3mm in diameter between the lower two central incisors. Tissue biopsies were obtained from both experimental and control groups at time intervals of 1, 3, 7, and 14 days post operation. Histological analysis of the tissue showed a less severity acute inflammatory reaction, 1 day post operation, in experimental group comparing with the control group, with presence of collagen fibers. While, 3 days post operation, microscopic examination of the experimental showed that acute inflammation reaction changed to chronic by increasing number of macrophages and fibroblasts cells, with re-epithelization, increased number of congested capillaries, and epithelial hyperplasia compared with control group that still filled with acute inflammatory cells. Microscopic section showed dense fibrous tissue capsule separated the incised tissue from healthy one, numerous dilated capillaries, with no inflammatory reaction, 7 days post operation, compared with control group that still showed mild inflammation with delicate fibrous tissue. Under light microscope, 14 days post operation. The experimental group showed that endothelial cells are large swollen and form large and small capillaries, fibrous tissue not fully covered the incised area compared with control group, that observed with thin flat epithelial covering the incised area. The results of this study revealed that Pomegranate had rapid wound healing effects, thus the local insertion of this material into the surgical region can be used successfully in periodontal disease treatment and therapy.

Keywords: Pomegranate extracts, bone defect, rabbit mandible, histology.

1. Introduction

Pomegranate (*punica granatum* L, Punicaceae), is an edible fruit cultivated in many countries, including the United States and consumed around the world. It is well documented that the edible part of pomegranate is rich in compounds that possess antioxidant and anti-inflammatory activities (Khan *et al.*, 2008). The periodontium is the set of adjacent structures to the teeth that provides them with support and protection. These structures are: Gingiva, cementum, alveolar bone and periodontal ligament (Clarke, 2001). In the only study which is available for the evaluating the effects of pomegranate on gingivitis, Pereira and Sampaio showed a significant reduction of gingival bleeding after using a dentifrice containing pomegranate extract (Pereira and Sampaio, 2003). Toklu *et al.* (2009) showed that pomegranate peel extract (PPE) supplementation reduced oxidative damage in the ileal tissues, probably by a mechanism that is associated with the decreased production of reactive oxygen metabolites and enhancement of antioxidant mechanisms. (Toklu *et al.*, 2009).

Aim of the study:

This study was designed to evaluate the histological effects of pomegranate extracts powder inserted in surgically created bone defect in rabbit's mandible.

2. Animals and methods

2.1 Animals:-The study was carried out in Hawler Medical University, College of Dentistry, Department of Periodontics,

Twenty four male rabbits (*Oryctolagus cuniculus*) were used in this study. Their age was (5-6 month) and their weight was (1.5-2kg). These animals were kept in animal house at the same temperature (25-30°C) and they were given the same type of food (green vegetable and free water). The rabbits were left to acclimate for seven days before starting the experiment.

2.2 Study design and tissue samples:- The (24) rabbits were divided into two groups (each 12 rabbits); experimental group, in which, 0.3g. Pomegranate extract powder inserted into surgically created bone defect, while the baseline control group (12 rabbits) surgically created bone defect was irrigated with normal saline ;

1- Pomegranate extracts powder groups were subdivided into (4) groups (each 3 rabbits); D1, D2, D3, and D4

(Table 2-1). At the time of surgery, the rabbits were weighted and anesthetized with subcutaneous injection behind the neck using a combination of (35mg/kg) Ketamine-HCL and (0.5mg/kg) Xialazine 2% (Nelson *et al.*, 1989) with few drops of 0.2% lidocaine as local anesthesia at the area of surgery in order to decrease bleeding and pain during surgical procedure. They were exposed to surgically created bone defect between roots of two central incisors on rabbit's mandible with insertion of 0.3g of pomegranate extract powder into the defect region. The tissue samples were collected after sacrificed the animals at time intervals of 1 day (group D1), 3 days (group D2), 7 days (group D3), and 14 days (group D4), post operation for histological analysis.

2- Blood clot filled the surgically created bone defect groups also were subdivided into (4) groups (each 3 rabbits); E1, E2, E3, and E4 (Table 2-1). All the (12) rabbits were exposed to surgically created bone defect between roots of two central incisors on rabbit's mandible with irrigation of normal saline (0.9% NaCl) into the defect region. The tissue samples were collected after sacrificed the animals at time intervals of 1 day (group E1), 3 days (group E2), 7 days (group E3), and 14 days (group E4), post operation for histological analysis.

Table (2-1):- The different groups of rabbits and the time intervals post operation.

Rabbit groups	No. of rabbits	Surgical bone defect filled with	Tissue sample after time intervals (days)
D1	3	POM Powder	1
D2	3	POM Powder	3
D3	3	POM Powder	7
D4	3	POM Powder	14
E1	3	Blood clot	1
E2	3	Blood clot	3
E3	3	Blood clot	7
E4	3	Blood clot	14

POM: Pomegranate

2.3 Method

2.3.1 Surgical procedure:-

Periodontal horizontal incision of 2 cm in length extend between the distal surface of the lower right central incisor and the distal surface of the lower left central incisor, and was carried along the labial surface of lower anterior central incisors and at the level of 2 mm below the free (marginal) gingiva by using a scalpel blade No.15. The full thickness flap was reflected by periosteal elevator to expose the underlying osseous bone. A circular defect of 3 mm in diameter was made in the bone and between roots of the two central incisors using a surgical round bur with conventional straight hand piece with low-speed rotary engine device (Costich *et al.*, 1964), The surgical procedure was irrigated by 2ml of normal saline (0.9% NaCl), and the defect region filled with blood as a control group, while 3g concentration of Pomegranate extract powder was inserted into the surgically created bone defect area, and considered as an experimental group. The incision was sutured by one stitch with (5/0) black silk in order to close the wound and replaced into the same previous position. The area of surgical site was pressed with sterilized gauze to reduce bleeding that may happen after the operation. No any local or systemic antibiotic was given postoperatively. The animal was placed in sterilized clean dry bed until recover. The surgical site was carefully observed clinically for any swelling, bleeding. The suture was removed after (7) days.

2.3.2 Preparation of histological Specimens (biopsies):

At the end of each experimental work, these animals were sacrificed from each surgical groups in the following interval of times (1, 3, 7, and 14 days).The excision block of two lower central incisors with gingival tissue and underlying alveolar bone specimens were excised from lower anterior region includes the surgical region and the cutting was made 5mm at least away from either sides of the surgical site. The gingival tissue was separated from underlying bone and divided by sharp scalpel into two parts (facial and lingual). The specimens were immediately fixed in 10% freshly prepared neutral buffered formalin for 3 day. The biopsies prepared in the histological laboratory by serial sagittal sectioning of the block by microtome, 4µm thickness sections were mounted on clean glass slides for routine hematoxylin and eosin staining (H&E).

2.3.3 Histological examination: The histological sections were analyzed at different magnification (40X, 100X, 400X) under a digital biological microscope, change in the incised tissue that covering the surgically created bone defect, in both control and experimental group will be recorded.

3. Results

3.1 Control group

Figure (1) showed the histological preparation of the soft tissue covering the defect (oral mucosa), one day of the surgically created bone defect. Edema with remnant of sutured material was observed at the site of surgical incision. There was an obvious discontinuously and break of oral mucosa with presence of hemorrhage

and congested blood vessels surround by a great number of acute inflammatory cells mainly polymorph nuclear neutrophil (PMNL). With increasing the power of magnification, extravasated red blood cells, macrophage cells, eosinophil cells, giant cells, and different acute inflammatory cells were examined.

3.2 Experimental group:

After one day of insertion of Pomegranate extract powder into the surgically created bone defect (figure 2), microscopic examination for the soft tissue covering the bone defect, showed acute inflammatory reaction with a large number of inflammatory cells invading the surgical area with the presence of coagulated materials surrounded by thin fibrous tissue. In the lower border of incision (underlining lamina propria), microscopic examination also show invading of inflammatory cells along the collagen fibers, with increasing the power, different types of acute inflammatory cells were examined such as plasma cells, macrophages cells, eosinophil cells, neutrophil cells, and congested capillaries.

3.3 Three days after surgery:

3.3.1 Control group :

After 3 days of surgically created bone defect, low power of light microscope (figure 3) shows that the area of the surgical incision still filled with a lot of inflammatory cells but slightly less than the previous group (1st day control after surgery), while the number of congested blood vessels in the area was markedly higher than the 1st day control group. Using higher magnification, the section show different types of acute inflammatory cells such as macrophages, eosinophil, neutrophil, surrounded by fibrous tissue and spread congested capillaries.

3.3.2 Experimental group :

Figure (4) shows that the area of incision still filled with remnant of material, large number of inflammatory cells, which tend to change to chronic inflammatory cells (increase macrophage cells, eosinophil cells, and decrease neutrophil cells). The inflamed area is seen to be completely separated from underlining normal connective tissue by a basophilic slightly homogenized area which close to the appearance of extracellular material of cartilage (glycoaminoglycans).

Under light microscope the section shows the epithelial hyperplasia, increase in collagen fibers within and around the nodular area, with many congested capillaries, monocytes cell, few eosinophil, and active fibroblasts. Other animal, still acute inflammation, with acute inflammatory cells like eosinophil cells, monocyte cell, and macrophage cells with cytoplasmic inclusion bodies.

3.3 Seven days after surgery:

3.3.1 Control group:

Figure (5) shows that the surgical incision filled by delicate granulation tissue with numerous capillaries. The surrounding epithelium is hyperplastic. The underneath connective tissue contain a central homogenized pinkish material with mixed inflammatory cells associated with eosinophiles and few giant cells near the remnant of suture material (dark aggregation in between cells). At the periphery of the inflammatory reaction there was dense fibrous capsule (fibroblast and collagen fibers) separate it from normal healthy connective tissue.

3.3.2 Experimental group:

Figure (6) shows the surgical incision filled by dense granulation tissue. The surrounding epithelium is also hyperplastic. The under lying connective tissue is heavily infiltrated by mixed inflammatory cells that also associated with eosinophiles, but no giant cells. There are numerous dilated and congested capillaries that may containing the tested material and show swelling of their endothelial wall. At the periphery there is dense fibrous capsule separate it from normal healthy connective tissue.

3.4 Fourteen days after surgery:

3.4.1 Control group:

The surface covered by flat stratified squamous epithelia, still there is chronic cells and eosinophiles infiltrate just beneath the epithelia and the site of suture material with foreign body giant cells adjacent to it. The underlying tissue consists of oriented spindles of active fibroblast and collagen fibers run in parallel to the epithelial surface (Figure 7).

3.4.2 Experimental group: Under microscope sections showed that the surface was still not fully covered by epithelia, and there are chronic inflammatory cells and eosinophiles in the granulation tissue (Figure 8). The underlying tissue consists of bundles of active fibroblast and collagen fibers less organized than in control group. The macrophages showed epitheloid features, endothelial cells are large swollen and form large and small capillaries.

4. Discussion

4.1 Wound healing process

It was reported that the damage caused by reactive oxygen species (ROS), which were produced during the inflammatory stage of the wound-healing process delayed wound-healing (Shukla *et al.*, 1999). Therefore, strong antioxidant activity of the extract, could be attributed as one of the mechanisms of *Buddleja globosa* extract in

the wound-healing process (Yamasaki *et al.*, 1994).

Indeed, the results of the present study showed that the tensile strength of animals treated with Pomegranate extract powder was significantly greater than those of the control group. The increase in tensile strength of treated wounds may be due to increase in collagen concentration and stabilization of the fibers (Udupa *et al.*, 1995). The results obtained in this study were similar to those obtained by studying the effect of Aloe vera on collagen characteristics in healing dermal wounds in rats (Chithra *et al.*, 1998). Also, a similar effect has been observed with the ethanolic extract of *Centella asiatica* on the rat dermal wound healing (Suguna *et al.*, 1996).

4.2 Histopathological survey of the wound healing process

Animals were sacrificed on the 1st, 3rd, 7th, and 14th days after surgically bone defect creation, tissue samples (only the soft tissue covering the surgical created bone defect) were evaluated for the following histological criteria: the extent of re-epithelization, the degree of granulation tissue formation, and for collagenisation, prominent vascularity and inflammation considered as an indication for immature granulation tissue. Contrary, the presence of well formed and oriented collagen fibers, along with scarce inflammation cells and inconspicuous blood vessels were considered as an indication for the maturity of the generation or healing of the surgical incision. This evaluation of the histological criteria for wound healing was resemble to that obtained by (Hayouni *et al.*, 2011), which demonstrated in vivo healing potential on dermal wounds.

Section of soft tissue samples, which covering the surgically created bone defect, were collected on various days, examined for inflammatory cellular infiltration, neo-vascularization, epithelial regeneration and matrix organization.

One day after treating the animals with pomegranate extracts powder, the experimental group showed very close profiles when compared to the control group. These results were in agreement with the results that obtained by (Socorro-Ferreira *et al.*, 2011), which showed hemorrhage, edema and an intense inflammatory infiltrate with predominance of polymorph nuclear neutrophil (PMNL) adjacent to the material, after one day of treatment.

The result of this study indicated that pomegranate extract powder behaved toward wound healing of rabbit periodontal. A strong acute inflammatory reaction was detected, in the histological analysis, characterized by hemorrhage, edema and an intense inflammatory infiltrate, predominantly neutrophilic, adjacent to the material. In the subsequent periods was verified granulation tissue externally at this infiltrate and an area of degradation of pomegranate extracts powder, associated with neutrophilic and macrophagic cells. The present research was in agreement with these studies that found an intense acute inflammatory response in the test group of CHX chip (Adriana *et al.*, 2011). Even in a low concentration, pomegranate extract solution has no serious toxic effects on gingival fibroblasts and may affect wound healing, disagreement with CHX in low concentration (Mariotti and Rumpf, 1999).

Three days after treating the animals with pomegranate, the sections showed increased number of chronic inflammatory cells infiltration (decrease neutrophil), with epithelial hyperplasia in experimental group, unlike the control group, where still in acute stage, and increase in collagen fibers in experimental animals treated with the pomegranate extracts powder than in control, a well advanced organization of granulation tissue and on-going epithelization lining of the inflammatory area was observed in experimental group, with Pomegranate extracts powder than the control group. Under light microscope, the sections show presences of macrophages in both control and experimental group. This result was agreement with (Shalini *et al.*, 2010), who showed significant decrease (p-value < 0.05) in epithelization period and significant increase in percentage wound contraction in excision wound model in pomegranate aqueous extracts treated animals as compared to control. These results were disagreement with (Hayouni *et al.*, 2011) study, in which showed increase in cellular infiltration in animals, after 4 days of topically treating with the pomegranate ointment comparing with control.

Seven day after treatment, the histological examination of the experimental animals showed that the surgical incision appear filled by dense granulation tissue, with no giant cell, and no edema in animal experimental group, while the surgical incision appear filled by delicate granulation tissue, with presence of giant cell, and edema in control. Therefore regeneration was much faster in the experimental animals than in the control one. In both groups, numerous dilated and congested capillaries, with dense fibrous capsules were present at this period. These results were in disagreement with the results that obtained by (Adriana *et al.*, 2011), in which the microscopic examination showed edema and necrotic area close to the chlorhexidine (CHX) material in the skin healing at the same period.

Fourteen days after treatment, Experimental animals showed very close profiles when compared to the control group. Under microscope sections show that the surface is still not fully covered by epithelia, in experimental group, while in control the surface appear covered by flat stratified squamous epithelia without retepegs formation, still there is chronic cells and eosinophiles infiltrate just beneath the epithelia. In addition, in the lamina propria, maturation and organization of the collagen fibers was similar. The lamina propria was cellular with proliferation of bundles of active fibroblasts, while a spindle of active fibroblasts and collagen fibers run in parallel to the epithelial surface, with macrophages cells, this result were disagreement with (Faria

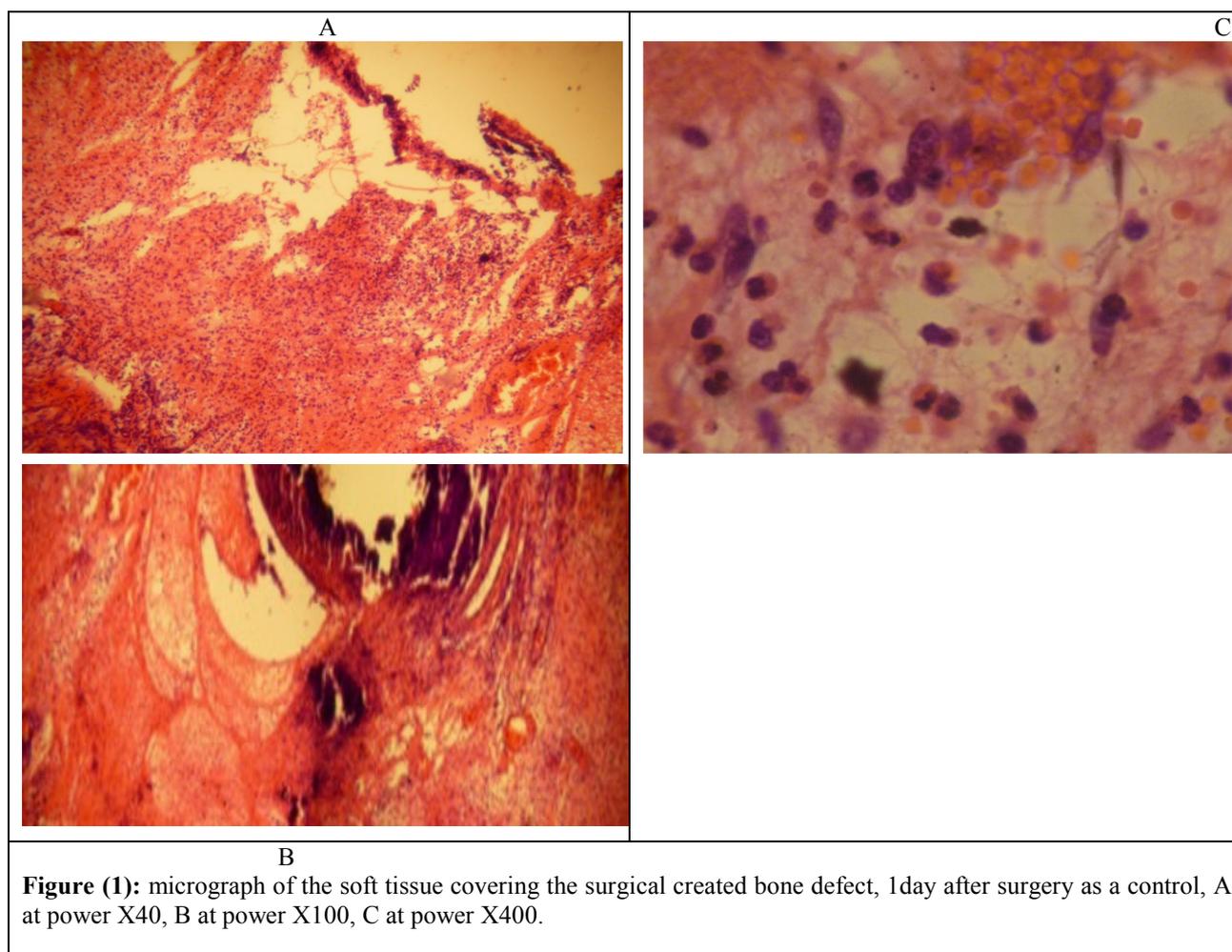
et al., 2007).

Prominent capillary-large sized and dilated blood vessels were seen with swelling in the endothelial cells in site of experimental group than in control, where these results were agreement with (Adriana *et al.*, 2011), where the light microscopic examination showed the degradation lacunas still appear filled with macrophage and neutrophil cells, 14 days, experimental animals.

These results were in agreement with that obtained by (Hayouni *et al.*, 2011), who indicated that on day 12, the histological examination showed that the original tissue regeneration was much faster in the skin wound treated with extract ointment or cetrimide cream without any edema, congestion or inflammatory disorders compared with control group at the same period.

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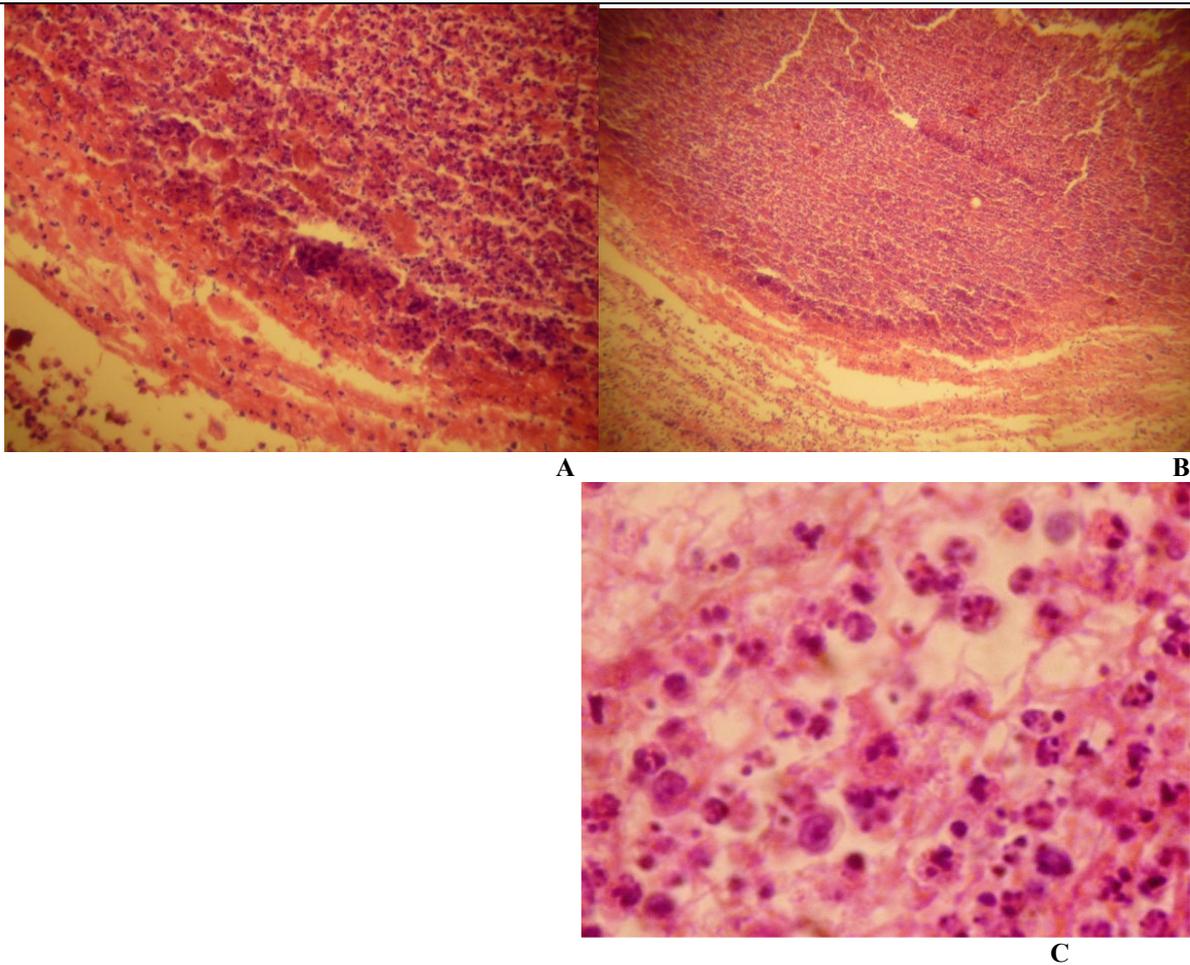
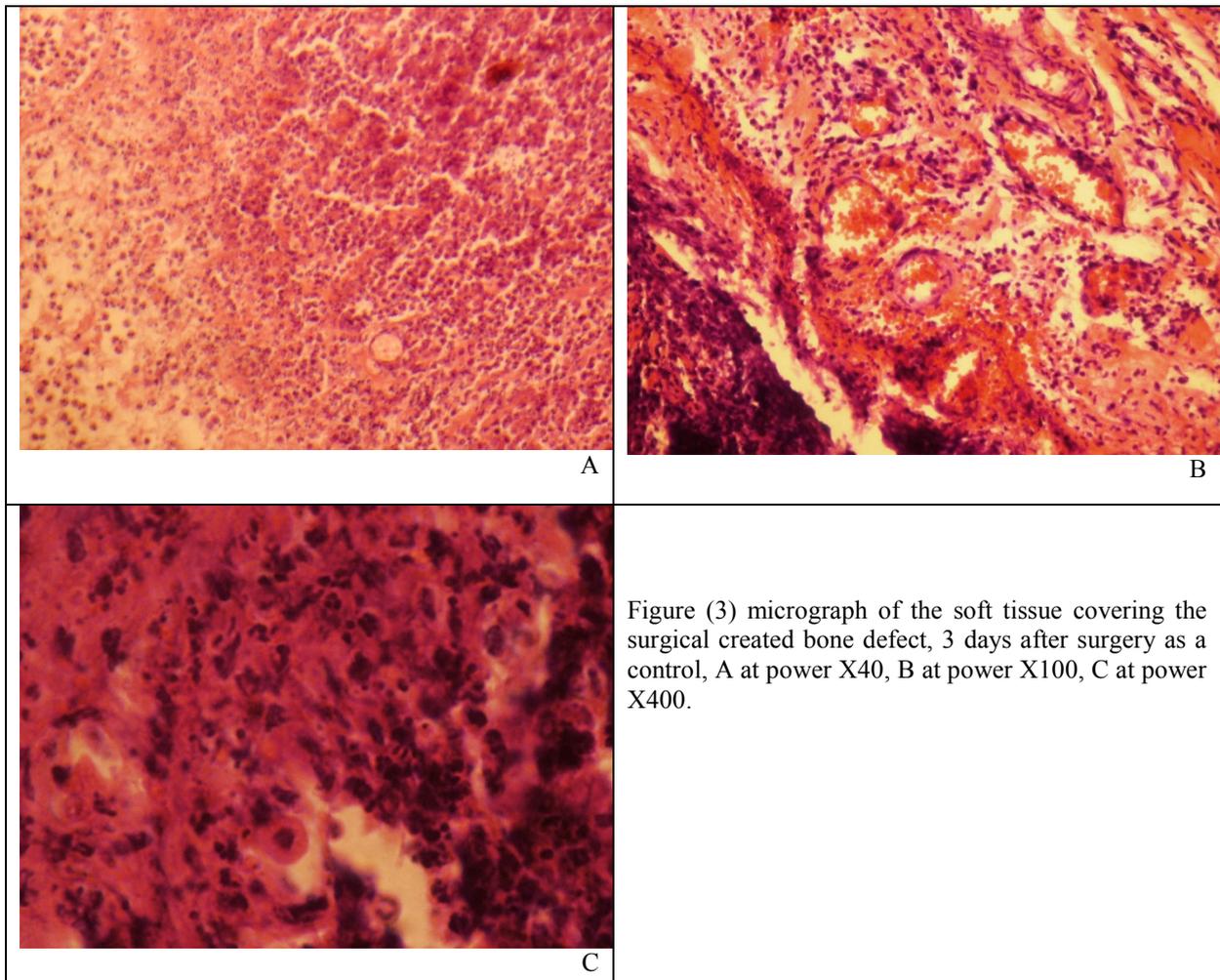
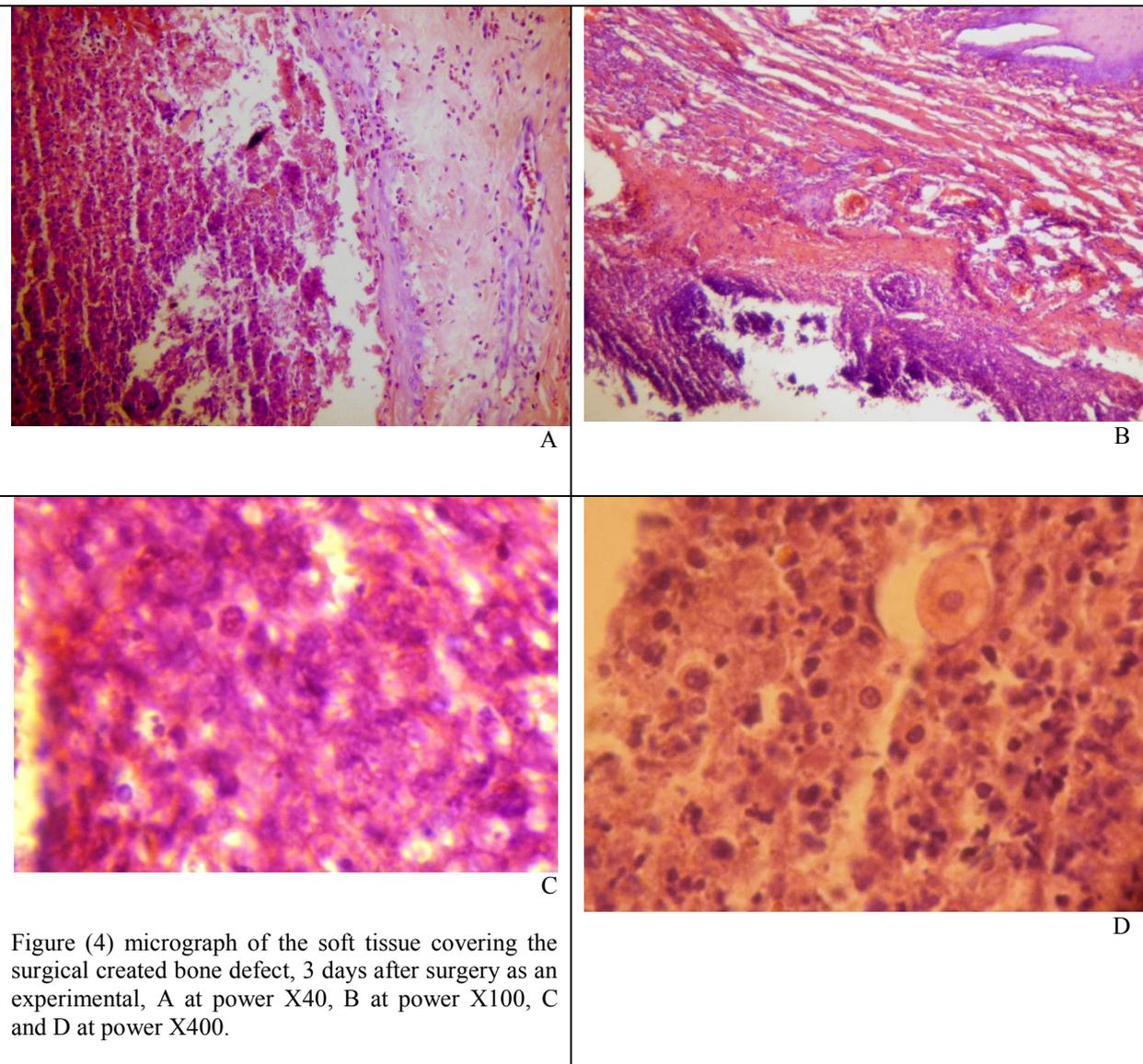


Figure (2): micrograph of the soft tissue covering the surgical created bone defect, 1day after surgery as an experimental, A at power X40, B at power X100, C at power X400.





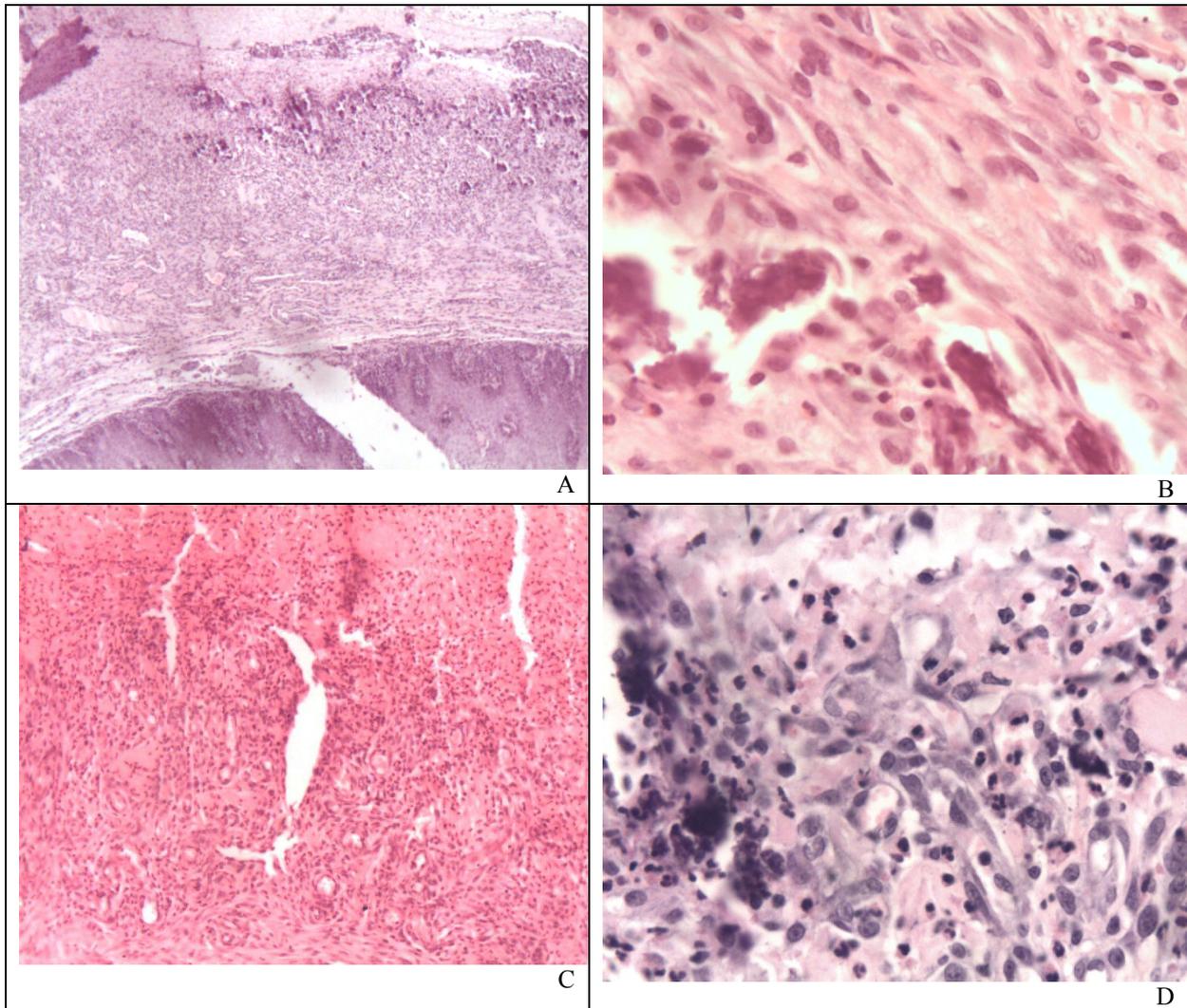
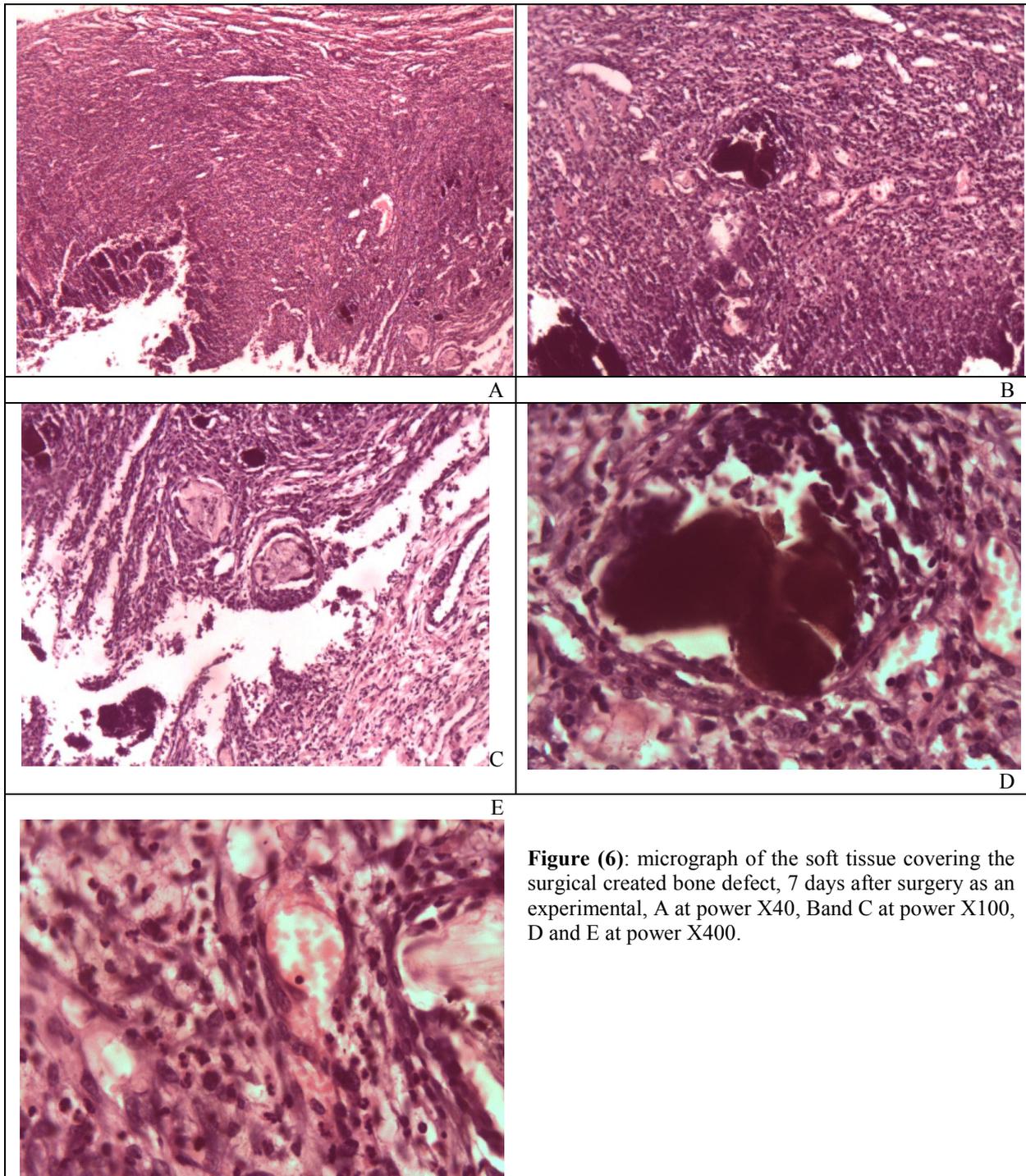


Figure (5) micrograph of the soft tissue covering the surgical created bone defect, 7 days after surgery as a control, A at power X40, C at power X100, D and B at power X400.



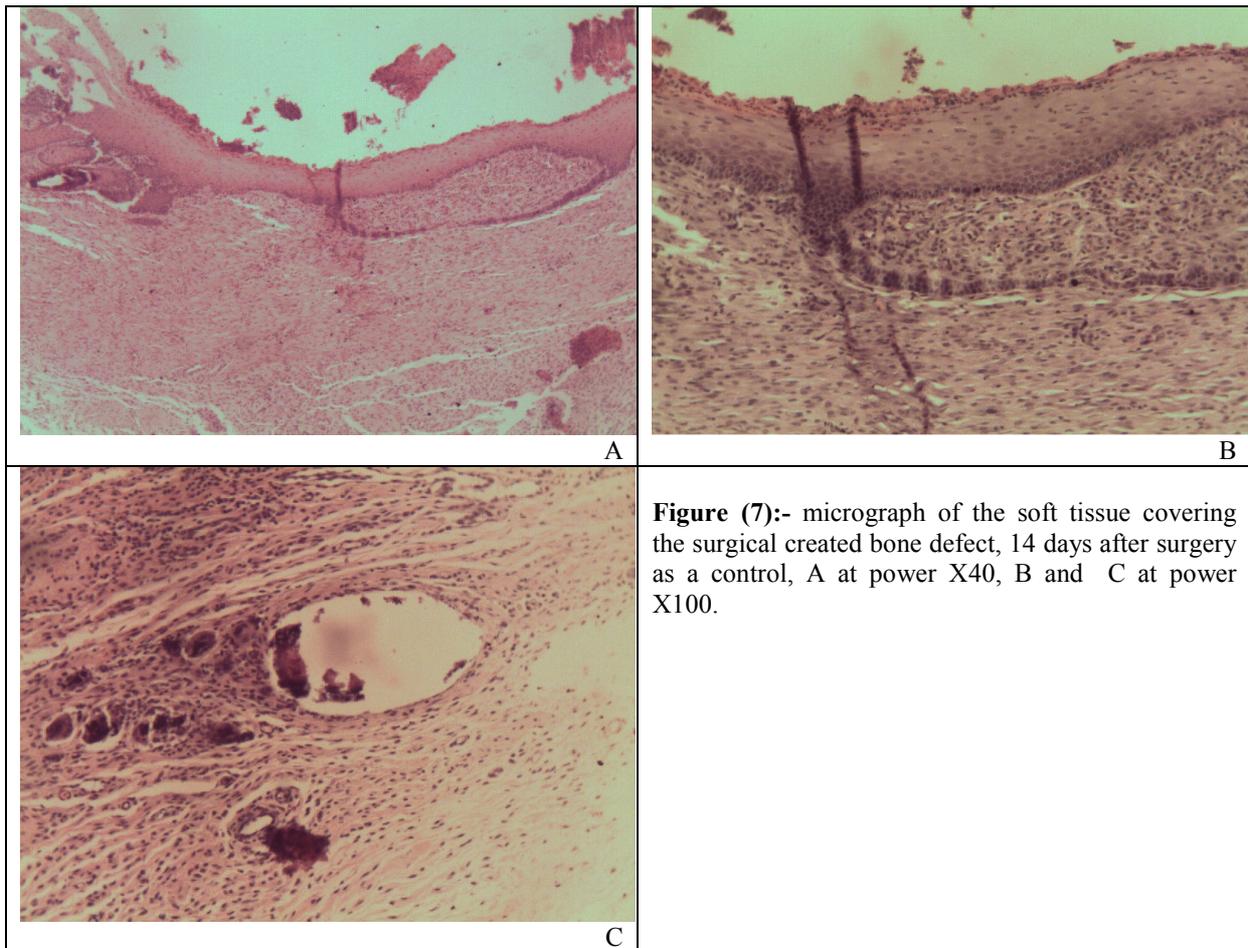


Figure (7):- micrograph of the soft tissue covering the surgical created bone defect, 14 days after surgery as a control, A at power X40, B and C at power X100.

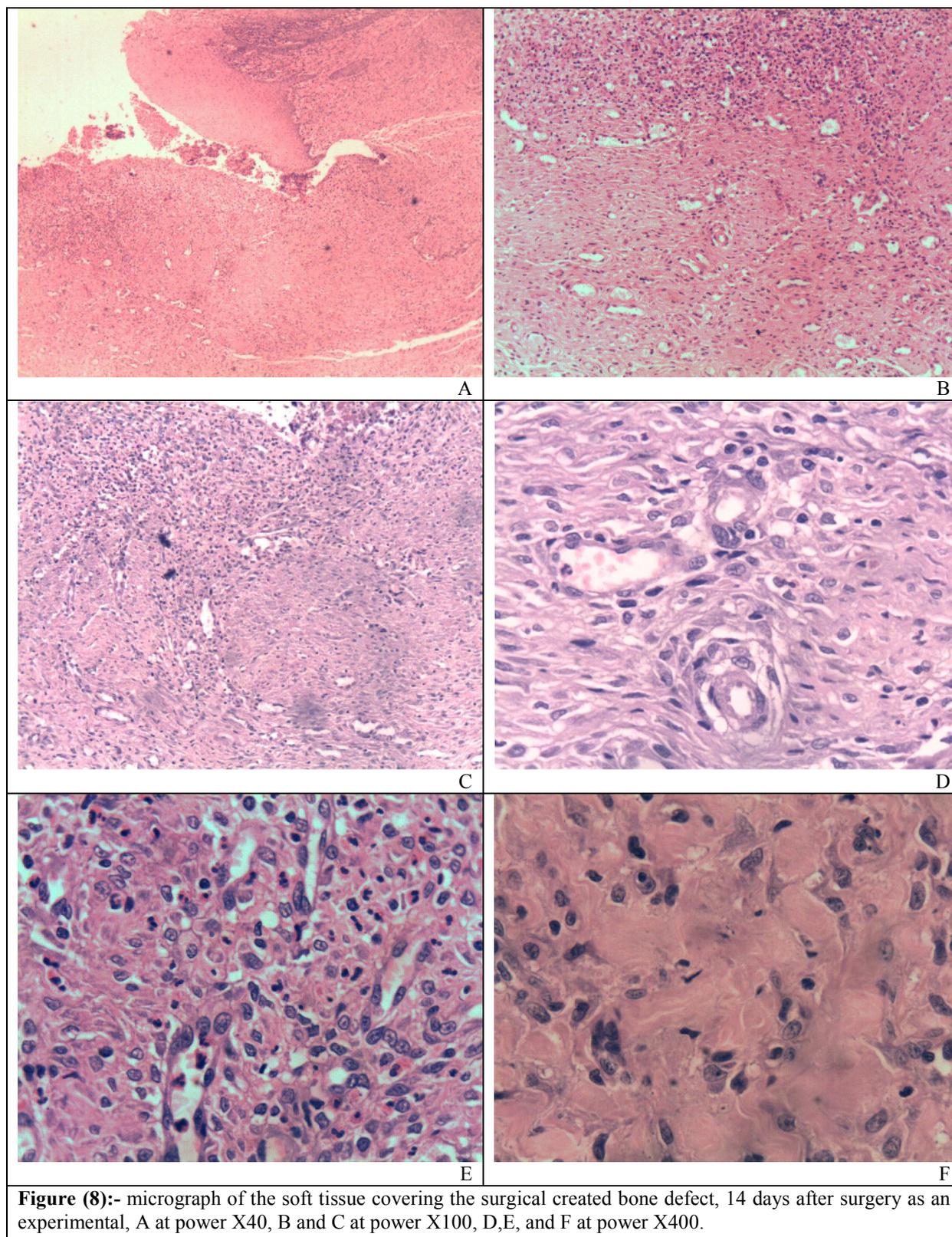


Figure (8):- micrograph of the soft tissue covering the surgical created bone defect, 14 days after surgery as an experimental, A at power X40, B and C at power X100, D,E, and F at power X400.