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Research Article

Local Effect of Insulin on Healing of Extraction Socket (Experimental Study)

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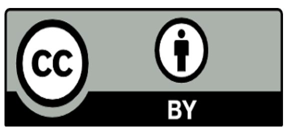

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Abstract: The present study aims to investigate the effect of Insulin on the healing of the dental socket of rabbits. Evaluating oral mucosa healing and bone regeneration of the healing process during three intervals: 3-, 10-, and 30-day post-extraction**. Materials and Methods**: Twelve male albino rabbits were used in this study, randomly divided into three sub-groups (N=4) for histological and immunohistochemical evaluation at 3-, 10-, and 30-days post-extraction, and compared to the controls. The rabbits were euthanized at the corresponding time interval; each rabbit represented a control and a treated sample. Bilateral maxillary first premolar extraction (treated side and control side) was performed, followed by treatment injection into the socket. A gel foam cylindrical insert of (3*5 mm) dimensions was placed in both sockets. For the treated groups, a standardized dose of 10 IU in 0.1 ml from a solution of 100 IU/ml concentration of Insulin; in each corresponding group was injected into the gel foam in the socket of the treated side, while for the control side, was kept plain without injection. A secondary post-extraction standardized dose was split equally and injected buccally and palatally around the gingival margins of the studied socket on the 2nd, 4th, and 6th-day post-extraction only for the treated side. For each study interval, rabbits in each group were euthanized, and then specimens from the oral mucosa and maxillary alveolar bone from the extraction sockets with sufficient margins were immediately harvested and arranged for laboratory examination. Assessment of inflammatory response, granulation tissue formation, re-epithelialization, bone healing, and the expression of CD34 and Ki67 was performed. Non-parametric statistical analysis was performed, and significance was recorded at $p \leq 0.05$. **Results**: Histological evaluation results revealed a significantly reduced inflammation response, reduced granulation tissue formation, higher and quicker re-epithelialization, and enhanced bone formation for the treated group when compared to the control at certain time intervals within the total period of the study. The Immunohistochemistry results revealed a significantly increased expression of CD34 and Ki67 markers in the treated group as compared to the control groups at certain study intervals. **Conclusions**: Within the limits of the present study, local application of Insulin demonstrated a positive effect on the healing process of oral mucosa as well as bone regeneration of the dental socket. The local injection of Insulin would be a suitable candidate to be used in the enhancement of post-extraction healing.

Keywords: Healing; Insulin; Tooth socket

INTRODUCTION

Oral surgical practice commonly encounters wounds involving the oral mucosa and jawbones. Tooth extraction is the most common surgical procedure performed by dentists; dental extraction (also referred to as exodontia) is the removal of teeth from their sockets in the alveolar bone [(1)]. The wound healing after tooth extraction, represented by dental socket healing, involves the healing process in soft tissues; connective tissue, gingival epithelium, and the hard tissue of the alveolar bone [(2)]. The healing process involves interaction between various cells and growth factors [(3)]. Therefore, improving the microenvironment of the tooth extraction sockets in early healing can promote healing and induce bone tissue regeneration. The healing stages occur in a regular order at a specific time, although the type of wound, microbial, physiological, and immunological factors can affect the duration of each stage.

A wide range of therapies for promoting wound healing have been suggested, such as the topical application of insulin, metformin, adiponectin, growth factors, negative pressure-assisted wound closure oxidized regenerated cellulose/collagen, hyaluronic acid conjugated with glycidyl methacrylate or gelatin dressings, honey, aloe vera, stem cells, synthetic drugs, Low-level laser therapy.[(4,5)]

Insulin (INS) is a peptide hormone that mainly allows glucose to enter cells and maintain the normal glucose level in the bloodstream. Also, it plays multiple functions in our body, such as the control of inflammation, increased cell differentiation, lipid and protein biosynthesis, etc., in addition, it controls glucose levels in the blood through glucose metabolism [(6)]. Also, INS has some anabolic effects, including glycogen synthesis, fatty acid synthesis, and protein synthesis in tissues, which can be effective in the wound-healing process; recently, the beneficial effect of topical INS on wounds has also been observed and described as a potent contributor to wound healing management [(7,8)]. It stimulates keratinocyte (migration, proliferation, and differentiation), and fibroblast proliferation, modulating the release of inflammatory cytokines (9). It also increases the amount of collagen at the wound site. Topical application of INS may improve wound healing even in healthy animals, reduce the duration of the inflammatory phase, and improve wound re-epithelialization. [(10)] The positive effects of INS on bone strength are direct or indirect (i.e., via glucose control, anti-inflammatory, and antioxidant effects). Therefore, INS does not affect bone microarchitecture/BMD in humans [(11)] or animals [(12)]. The physiological concentration of INS can stimulate the proliferation and differentiation of osteoblasts, and its application to the local tooth extraction nests of diabetic rats can promote bone wound healing. In addition, the topical application of INS in bone defects induces new bone formation and the healing of the defect.[(13)]

The present study aims to investigate the effect of Insulin on the healing of the dental socket of rabbits. Evaluating oral mucosa healing and bone regeneration of the healing process during three intervals: 3-, 10-, and 30-days post-extraction.

MATERIALS AND METHODS

Sampling and study design

A randomized controlled in vivo study design was used. The total sample consisted of 12 animals, 6-7 months old male albino rabbits with a weight averaging (1.5-2) kg were used in this study. The rabbits were physically healthy and were adapted pre-experimentally for two weeks. They were housed in a suitable place to simulate a natural environment possible. They were kept under the same standard conditions of good ventilation, humidity (60–70%), temperature (20 ± 2 °C), and a 12-hour light/dark cycle. The rabbits had free access to tap water and an adequate stable diet of 85% of the following; 30% corn, 34% wheat, 25% soybean, 10% animal protein, 0.5% lima, and 0.5% sodium chloride and leafy green vegetables 15%. [14] All the rabbits were continuously monitored by a veterinarian and the researcher with daily body weight monitoring and regular checking for oral health and physical activity. The rabbits were randomly assigned to three experimental groups according to the time intervals (rabbits were euthanized at 3, 10, and 30 days) post-extraction. Each group consisted of four rabbits (N=4), and each rabbit within the group represented a control and treated sample, as the jaw has a treated side (right) and a control side (left) to minimize any bias. Each group was identified by coding colors on the animal's tail.

An insulin solution of 100 IU/ml concentration, the total volume of 10 ml that contains 1000 IU of (Actrapid®, Novo nordisk, Denmark), as seen in Figure (1). The extraction socket was filled with a cylindrical gel foam (Gel foam Roeko, Coltene, Germany) as detailed below.

Figure (1): Photograph of the Insulin solution used in the study, Actrapid®, (Novo nordisk, Denmark).

Surgical Procedure:

-General Anaesthesia

One to four hours before the initiation of the study (pre-operative), rabbits were food-deprived, while they were water fasted for 1 hour before the administration of anesthesia. Animals were anesthetized by intramuscular injection in the rabbit's thigh muscle of a mixture of Ketamine hydrochloride 500 mg/10ml (Hameln pharmaceuticals gmbh, Germany) anesthetic solution and Xylazine base 1000 mg/50ml (Interchemie werken, Venray, Holland) muscle relaxant. Each dose was calculated according to the weight of each rabbit as follows: Ketamine 50 mg/Kg in combination with Xylazine base 10 mg/kg.[15]

-Surgical procedure

Surgical procedures were performed under a standardized protocol and approved by the ethical committee of the University of Mosul / College of Dentistry / Department of Oral and Maxillofacial Surgery under ethical approval number (UoM.Dent/ A.L.83/ 21). After the administration of anesthesia, each rabbit was laid on its lateral side on the surgical board, covered with a sterile towel, exposing the head only. The rabbit's mouth was opened using a rabbit mouth gag (Starlabs, UK). The surgery was performed through bilateral extraction of upper first premolars using rabbit extraction forceps (Starlabs, UK). This results in two sockets per rabbit, providing a treated side and a control side. After the extraction, a simple curation of the socket was done, followed by irrigation with 2 mL sterile normal saline (Gibco, UK) to prepare the socket for the insert. A cylindrical gel foam (Gel foam Roeko, Coltene, Germany) insert of (3*5 mm) dimensions was placed in both sockets (treated and control). For the treated groups; a standardized dose of INS 10 IU in 0.1 ml from a solution of 100 IU/ml concentration; for each corresponding group was injected into the gel foam whilst in the socket of the treated side. The control side was kept plain without any INS injection into the gel foam. A secondary post-extraction standardized dose from the same treatment agent was split equally and injected buccally and palatally around the gingival margins of the studied socket on the 2nd, 4th, and 6th-day post-extraction for the treated side only (19). The surgical procedure is detailed in Figure (2).

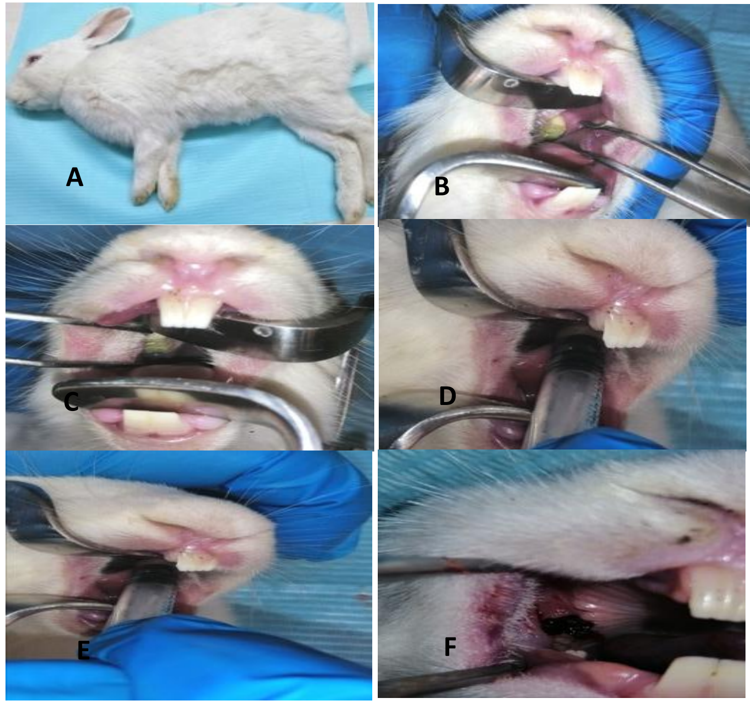

Figure (2): Photographs of the steps of the surgical procedure: (A) anesthetized rabbit, (B-C) tooth extraction, (D-E) gel foam insertion and local insulin injection, (F) final socket.

Postoperative Procedures:

-Postoperative animal care

Intramuscular injection of Oxytetracycline (Vilsan Pharmaceuticals, Turkey) 50 mg/kg dose was administered immediately postoperatively and continued for two days. The operated rabbits were caged in special cages separately until full recovery from the anaesthesia was achieved. 48 hours long postoperative observation for the operated rabbits was maintained by a veterinarian, monitoring their feeding and physical activity .

-Specimen collection and preparation

After authorization of rabbit at each subsequent study interval, specimens from the oral mucosa and maxillary alveolar bone of the extracted tooth socket at the operated areas with sufficient margins (for both treated and control sides) were immediately harvested and directly preserved in 10% freshly prepared buffer formalin (UK) in glass tubes with identification labels for (48 hours) period for tissue fixation, then, immersed in formic acid (Duda Energy LLC, USA) for decalcification. The decalcification process for the bone requires 21 days to remove calcium and minerals from the bone altogether. This process was done by immersing the samples in a

mixture solution composed of 50 ml of Hydrochloric acid (NEECOR, India), 50 ml of 99% Formic acid (Duda Energy LLC, USA), and 900 ml distilled water (Awamedica, India). [16] After bone decalcification, the specimens were removed from the solution rinsed thoroughly with distilled water, and stored ready for histological analysis.

-Histological preparation and staining

The specimens (soft tissue and bone) were prepared before embedding, firstly each specimen was sectioned into 2-3 mm thickness sections and then these sections were placed into cassettes. The cassettes were then immersed into a series of gradually increased ethyl alcohol (Schar lab S.L, Spain) concentrations of (10%, 20%, 30%, 50%, 70%, 90%, and 100%) to ensure complete dehydration. After that, xylene (Medex for chemicals, UK) was replaced by paraffin, and specimens were embedded and labeled in paraffin wax blocks. The specimens were then frozen for 24 hours and then sliced into 4 microns of tissue thickness in series using a microtome (Leedo, China). A ribbon of sliced tissue with wax at the incision level was then taken to a 50ºC water bath for de-waxing, then each slice was collected on a labeled glass slide (Citoplus, China). The obtained slides were then, stained with hematoxylin and eosin stains (Thermo Scientific, UK), and mounted with DPX (Iso-com Ltd., UK) for further microscopical examination.[17]

-Immunohistochemistry preparation and staining

Paraffin wax (UK) embedded oral mucosal specimen blocks were sectioned and mounted on slides as mentioned above. The slides were then heated at 60ºC for 1 hour in an oven and then left to cool for 30 min at room temperature. After cooling the slides were deparaffinized in xylene (Medex for chemicals, UK) and rehydrated in graded alcohol concentrations of 100%, 95%, and 70% respectively for 5 mins in each concentration, then transferred and rinsed twice with phosphate-buffered saline (PBS) (Gibco, UK). Endogenous peroxidase activity was inhibited by the placement of the slides in a 3% hydrogen peroxide solution (Thermo, UK) to reduce nonspecific background staining, followed by proper washing in PBS for 5 mins. The heated sections were kept in a pressure pot containing sodium citrate buffer (pH 6.0) for up to 3 minutes. The slides were then boiled for epitopes (antigens) retrieval and optimization of staining consistency, then the slides were left to cool. Then the slides were washed three times with PBS and incubated with the primary antibody solutions for (CD34, Ki67) using a mouse monoclonal antibody against (CD34, Ki67) antigen (Dako, Denmark). After that, the slides were incubated in a moisture chamber for 60–90 min followed by two gentle rinsing with PBS. Once completed the slides were

incubated for 10 min with an appropriate secondary antibody according to the manufacturer's recommended protocol at room temperature then washed with PBS three times and prepared to receive the chromogenic solution. The slides were then incubated with DAB tetrahydrochloride solution, which is a peroxide-compatible chromogen, for 5 minutes at room temperature as a substrate chromogen solution to produce a brown color, followed by twice rinsing with distilled water for 5 minutes each. Finally, sections were counterstained with Mayer's haematoxylin solution for 3–5 min, and dehydrated via placement for 3 min each in: 70%, 95%, 100% ethanol, and xylene, twice in each solution. Then the slides were mounted by one drop of an aqueous mounting medium (per mount) and covered with a coverslip for microscopical analysis.[18]

Histometric analysis and evaluation of oral mucosa and alveolar bone healing:
-Histometric analysis and evaluation of Hematoxylin and Eosin-stained oral mucosa and alveolar bone:

The extracted tooth socket healing was evaluated histologically via means of a semi-quantitative scale, measuring the intensity of inflammatory response, amount of granulation tissue formation within the socket, wound re-epithelialization, whether partial or complete, regular, mature or not, and bone healing. Three examiners were recruited to examine the slides: the researcher and two experienced histopathologists, who both recorded their score readings independently and blindly; the final score was recorded as the mean value of the readings of the three examiners. Histopathological estimation was conducted according to the following scoring criteria, which are explained in Tables (1, 2, 3, and 4). The histometric evaluation was done using a light microscope (Optica, Italy) at power magnification of X10 and X40.

Table (1): The criteria for inflammatory response intensity scoring [19].

Score	Observed histopathological response
0- Nil	No inflammatory cells are seen in the field of the operation at (X10).
1- Mild/Scanty	Presence of inflammatory cells in few numbers, in less than half of the field at (X10).
2- Moderate	Presence of inflammatory cells in more than half of the field at (X10).
3- sever/Marked	Presence of inflammatory cells in huge numbers, more than 3/4 of the field at (X10).

Table (2): The criteria for granulation tissue formation scoring [20,21].

Score	Observed histopathological response
0- Nil	Absence of granulation tissue formation in the field of operation at (X10).
1- Mild/Scanty	Scanty amount of granulation tissue formation, less than half of the field of operation at(X10).
2- Moderate	Moderate amount of granulation tissue formation, more than half of the field of operation at(X10).
3- profound/Marked	Profound amount of granulation tissue formation, more than 3/4 of the field of operation at(X10).

Table (3): The criteria for re-epithelization scoring [22]

Score	Observed histopathological response
0	Re-epithelialization at the edge of the wound only.
1	Re-epithelialization covering less than half of the wound.
2	Re-epithelialization covering more than half of the wound.
3	Re-epithelialization covering the entire wound with irregular thickness.
4	Re-epithelialization covering the entire wound with normal thickness.

Table (4): The criteria for histological analysis of bone regeneration scoring [23]

Score	Observed histopathological response
0	No newly formed vessels. None to a very minimal number of fibroblasts. No osteoid (bone matrix). No bone cells.
1	Few newly formed vessels. Few numbers of fibroblasts. Evidence of osteoid presence (bone matrix). Evidence of bone cells.
2	Moderate amount of newly formed vessels. Predominant number of fibroblasts. Moderate osteoid (bone matrix). Moderate bone cells.
3	Extensive amount of newly formed vessels. Fewer number of fibroblasts. Dense and highly organized osteoid (bone matrix). Extensive bone cells.

-Histometric analysis and evaluation of immunoreactive socket tissue sections against CD34 and Ki67 epitopes :

The angiogenesis process of the socket (wound) healing was evaluated on a semi-quantitative scale measuring the intensity of CD34 immunoreaction expression in the operating field under a light microscope (Optica, Italy) at X10 magnification. The specific protein molecules of CD34 are presented mainly in endothelial cells. Positively marked cells (immunoreactive cells against CD34 antigen) in the wound area were

counted and expressed, counting cells were performed according to the documented procedure, and cells with any degree of staining were scored positively [24] .

Ki67 is a well-established marker for cellular proliferation during wound repair. [26] The immunostaining of Ki67 appeared as a brown/yellow nuclear stain, the expression of Ki67 is considered positive when there is a brown/yellow granule in the nuclei of the cell or the cytomembrane beside the cell nuclei. All stained nuclei were counted as positive, irrespective of the staining intensity [26]. All slides for the immune staining were evaluated using a light microscope (Optica, Italy) at (X10) magnification to identify the wound tissue response .

For both markers, the slides were examined by three different examiners: the researcher and two experienced histopathologists, using special scoring criteria detailed in Table (5). The final scores were reported as the mean of the three examiners.

Table (5): The criteria for immunoreaction expression scoring [27]

Grade	Observed histopathological response
0 Nil	No immunoreactive cells in the field of operation at (X10) magnification.
1 Mild	Presence of immunoreactive cells in few numbers in less than half of the field of operation at (X10) magnification.
2 Moderate	Presence of immunoreactive cells in more than half of the field of operation at (X10) magnification.
3 Marked	Presence of immunoreactive cells in large numbers in more than 3/4 of the field of operation at (X10) magnification.

Statistical analysis:

The Statistical analysis was done by using the Sigma Plot software program; data were expressed as mean ± standard error. The scores are the descriptive expression of the inflammation response, granulation tissue, re-epithelialization, bone formation, and CD34, Ki67 expression for each of the study groups. The collected scores were analyzed statistically by the Mann-Whitney U test for all histological and IHC variables of group comparisons, whereas the comparisons of the time intervals were statistically analyzed using the Kruskal-Wallis test. The statistically significant difference was considered at $p \leq 0.05$.

RESULTS

Histological analysis:

The histological analysis results of the study were assessed at three post-extraction time intervals (3, 10, and 30 days). The microscopical analysis of histological sections for these time intervals for both control and treated groups is presented in Figure (3).

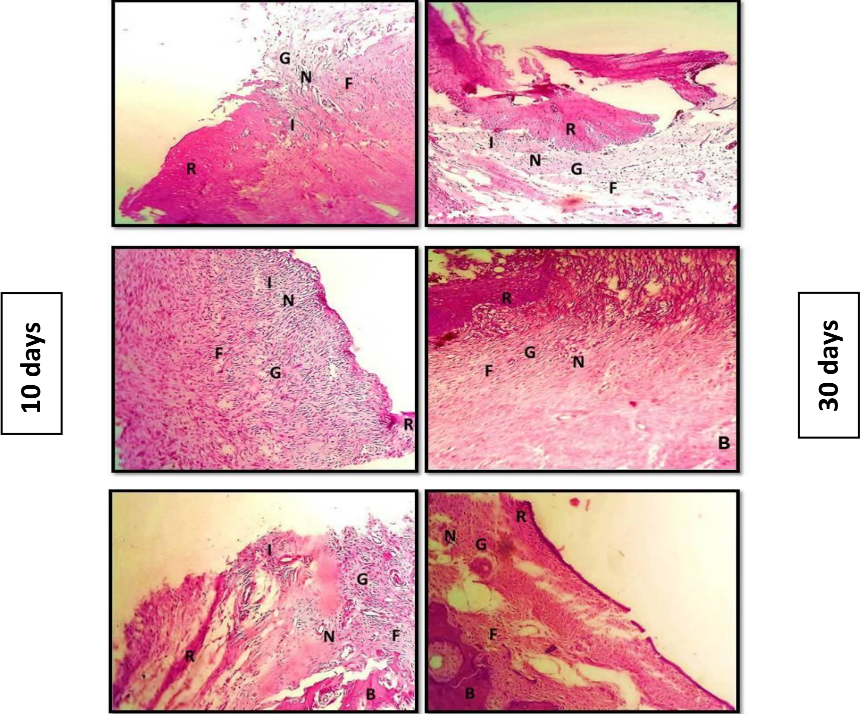

Figure (3): Photomicrographs of the oral mucosa and maxillary alveolar bone (left: control, right: treated) at the 3 (A-B), 10 (C-D), and 30 (E-F) days post-extraction. (G) = granulation tissue, (N) = new vasculature, (F) = fibrous connective tissue, (I) = inflammatory response, (R) = re-epithelialization formed, and (B): new bone formation at the edge of the socket. Magnification 10X, H&E stain.

Post-operative histological findings at three days period

Sections of oral mucosa and maxillary alveolar bone of the control group that was obtained on the third day post-extraction showed marked infiltration by inflammatory cells and the presence of the profound amount of granulation tissue with re-epithelialization seen covered less than half of the area at the wound surface. Bone sections showed none to very minimal numbers of fibroblasts, and no newly formed vessels, osteoids, or bone. The oral mucosa sections of the treated group showed moderate infiltration by inflammatory cells which was less than that for the control group, presence of a profound amount of granulation tissue with re-epithelialization seen covering more than half of the area at the wound surface. The bone sections showed a statistically significant reduction in the inflammatory response and a significant increase in re-epithelialization scoring in treated group specimens was shown as compared with the control group, while there was no statistically significant difference between the two group specimens in granulation tissue formation and bone formation, as detailed in Table 6. The mean values of the histological findings of oral mucosa and maxillary alveolar bone socket healing estimation are demonstrated in Figure (4).

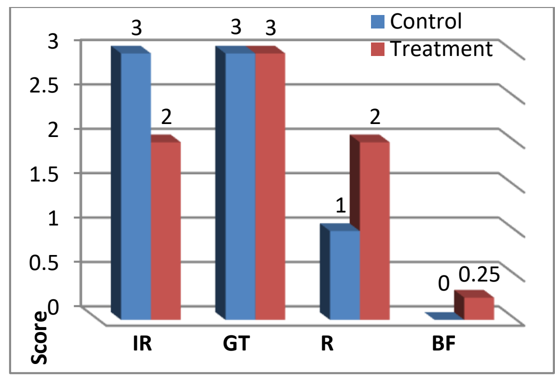

Figure (4): Means of histological evaluation scores for inflammatory response (IR), granulation tissue (GT), re-epithelialization (R), and bone formation (BF), at 3 days post-extraction period, for control and treatment groups.

Post-operative histological findings at ten days period

Sections of oral mucosa and maxillary alveolar bone of the control group that were obtained on the 10th day post-extraction showed marked infiltration by inflammatory cells and the presence of a profound amount of granulation tissue with re-epithelialization seen covering less than half of the area at the wound surface. The bone sections showed no to a very minimal number of fibroblasts and exhibited no newly formed vessels, osteoid, or bone. The oral mucosa sections of the treated group showed moderate infiltration by inflammatory cells, which was less than that for the control group. A moderate amount of granulation tissue with re-epithelialization was seen covering more than half of the area at the wound surface. The bone sections showed few newly formed vessels, fewer number of fibroblasts, and evidence of osteoid (bone matrix) and bone formation .

A statistically significant reduction in inflammatory response and granulation tissue formation, and a significant increase in re-epithelialization and bone formation scoring in treated group specimens were shown as compared with the control group, as detailed in Table (6). The mean values of the histological findings of oral mucosa and maxillary alveolar bone socket healing estimation are demonstrated in Figure (5).

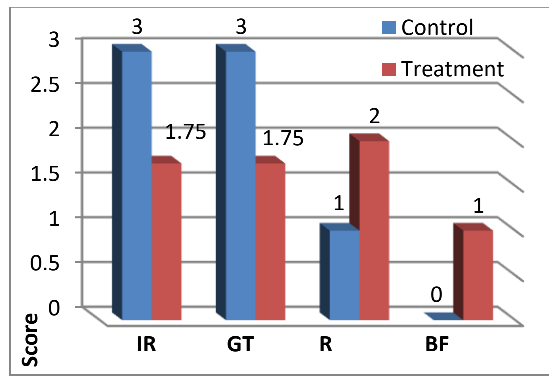

Figure (5): Means of histological evaluation scores for inflammatory response (IR), granulation tissue (GT), re-epithelialization (R), and bone formation (BF), at 10 days post-extraction period, for control and treatment groups.

Post-operative histological findings at thirty days period

Sections of oral mucosa and maxillary alveolar bone of the control group that were obtained at this period showed moderate infiltration by inflammatory cells, moderate amount of granulation tissue with re-epithelialization was seen covering less than half of the area at the wound surface. The bone sections showed a few newly formed vessels, a few number of fibroblasts, and evidence of osteoid (bone matrix) and bone formation. The oral mucosa sections of the treated group showed no inflammatory cells seen in the operation field, with a scanty amount of granulation tissue; the re-epithelialization was seen covering more than half of the area at the wound surface. The bone sections of the treated group showed a predominant number of fibroblasts and moderate newly formed vessels, with osteoid, bone cells. A statistically significant reduction in inflammatory response and granulation tissue formation, and a significant increase in re-epithelialization and bone formation scoring were observed in treated group specimens as compared with the control group, as detailed in Table (6). The mean values of the histological findings of oral mucosa and maxillary alveolar bone socket healing estimation are demonstrated in Figure (6).

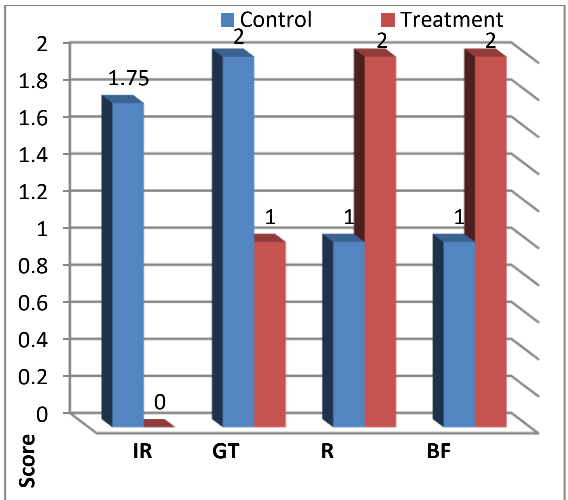

Figure (6): Means of histological evaluation scores for inflammatory response (IR), granulation tissue (GT), re-epithelialization (R), and bone formation (BF), at 30 days post-extraction period, for control and treatment groups.

Table (6): Statistical analysis of oral mucosa and bone healing evaluation comparing between control and treated group.

Parameter	Groups / Intervals	Control	INS Treatment	*p*-value (N=4)
I.R	**3 days**	3.00 ± 0.00 Aa	2.00 ± 0.00 Ba	0.029
	10 days	3.00 ± 0.00 Aa	1.75 ± 0.25 Ba	0.029
	30 days	1.75 ± 0.25 Ab	0.00 ± 0.00 Bb	0.029
	***p*-value (N=4)**	0.015	0.011	
G. T	**3 days**	3.00 ± 0.00 Aa	3.00 ± 0.00 Aa	1.00
	10 days	3.00 ± 0.00 Aa	1.75 ± 0.25 Bb	0.029
	30 days	2.00 ± 0.00 Ab	1.00 ± 0.00 Bb	0.029
	***p*-value (N=4)**	0.015	0.001	
R.	**3 days**	1.00 ± 0.00 Ba	2.00 ± 0.00 Aa	0.029
	10 days	1.00 ± 0.00 Ba	2.00 ± 0.00 Aa	0.029
	30 days	1.00 ± 0.00 Ba	2.00 ± 0.00 Aa	0.029
	***p*-value (N=4)**	1.00	1.00	
B.F.	**3 days**	0.00 ± 0.00 Ab	0.25 ± 0.25 Ab	0.686
	10 days	0.00 ± 0.00 Bb	1.00 ± 0.00 Ab	0.029
	30 days	1.00 ± 0.00 Ba	2.00 ± 0.00 Aa	0.029
	***p*-value (N=4)**	0.040	0.001	

Data expressed as Mean ± Standard error (N. Total specimens (rabbits) = 4). The Capital letters refer to differences between groups at $p \leq 0.05$. The Small letters refer to differences between post-extraction intervals at $p \leq 0.05$. (I.R.) = inflammatory response, (G.T.) = granulation tissue, (R) = re-epithelialization, (B.F.) = bone formation.

Immunohistochemistry analysis

The immunohistochemistry analysis results of the study were assessed at three post-extraction time intervals (3, 10 and 30 days). The microscopical imaging of immunohistochemistry sections for these time intervals for both control and treated groups are presented in Figures (7 and 8).

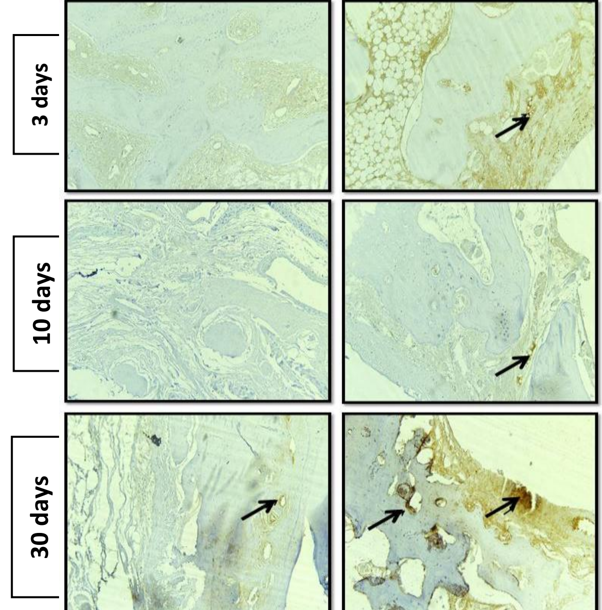

Figure (7): Photomicrographs of the oral mucosa and maxillary alveolar bone (left: control, right: treated) at the 3 (A-B), 10 (C-D), and 30 (E-F) days post-extraction stained with immunohistochemistry technique for CD34 biomarker. Black arrows mark the positively stained cells (immunoreactive against CD34) presented as dark brown color. 10X magnification.

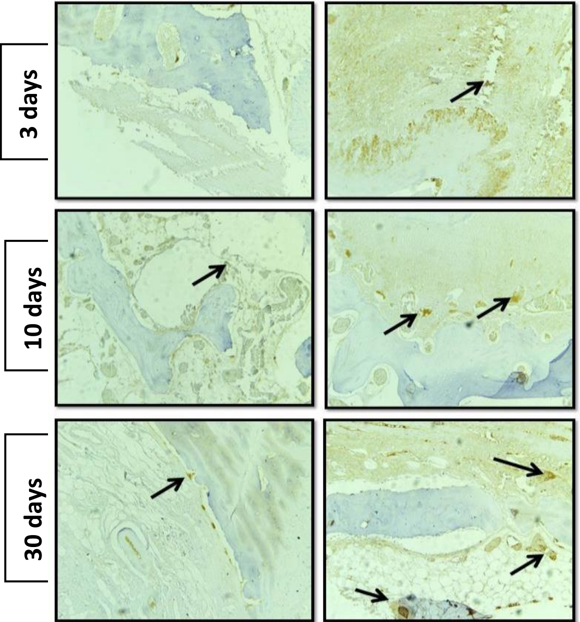

Figure (8): Photomicrographs of the oral mucosa and maxillary alveolar bone (left: control, right: treated) at the 3 (A-B), 10 (C-D), and 30 (E-F) days post-extraction stained with immunohistochemistry technique for Ki67 biomarker. Black arrows mark the positively stained cells (immunoreactive against Ki67) presented as dark brown color. 10X magnification.

Three days post-extraction period, histological sections of oral mucosa and maxillary alveolar bone of control group specimens demonstrated no expression of positively stained cells in the field of operation. While the treated group specimens showed mild positively stained cells present in few numbers for both antibodies.

Statistically, a significant increase expression scoring of (Ki67) and a non-significant increase expression scoring of (CD34) was observed in treated groups as compared to control groups, as seen in Figure (9 and Table 7) for CD34, Figure (10 and Table 8) for Ki67 .

Ten days post-extraction period, the histological sections of control group specimens demonstrated no expression of positively stained cells, while the sections of the treated group specimens showed mild positively stained cells present in few numbers for (CD34 antibody). Nevertheless, mild positively stained cells were observed in the histological sections of control group specimens, and moderate positively stained cells were seen in the field of operation of treated group specimens for (Ki67 antibody). Statistically, a significant increase in the expression scoring of (CD34) and a non-significant increase expression scoring of (Ki67) was observed in treated groups as compared to control groups, as seen in Figure (9 and Table 7) for CD34, Figure (10 and Table 8) for Ki67 .

Thirty days post-extraction period, the histological sections of control group specimens showed moderate positively stained cells, also marked positively stained cells were seen in the field of operation of histological sections of treated group specimens for (CD34 antibody). Likewise, mild positively stained cells were detected in the histological sections of control group specimens, while histological sections of treated group specimens showed moderate positively stained cells present for (Ki67antibody). Statistically, a significant increase in the expression scoring of both antibodies was observed in treated groups as compared to control groups, as seen in Figure 9 and Table 7 for CD34, Figure 10, and Table 8 for Ki67.

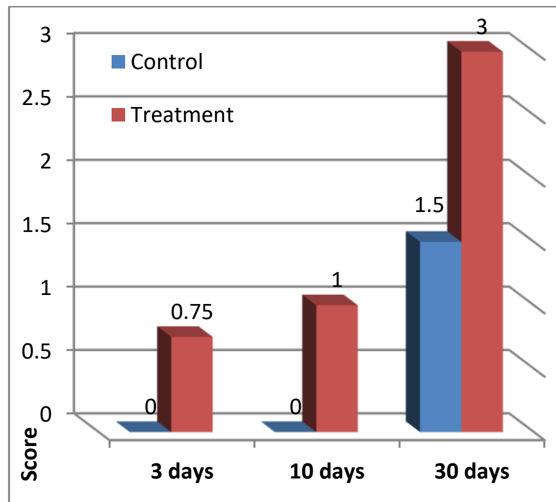

Figure (9): IHC analysis of the CD34 shows the mean scores for both control and INS-treated groups at 3-, 10-, and 30-days post-extraction periods.

Table (7): Statistical analysis of the CD34 expression scores at the healing site for control and INS treatment groups.

Groups / Intervals	Control	INS Treatment	*p*-value (N=4)
3 days	0.00 ± 0.00 Ab	0.75 ± 0.25 Ab	0.104
10 days	0.00 ± 0.00 Bb	1.00 ± 0.00 Ab	0.029
30 days	1.50 ± 0.28 Ba	3.00 ± 0.00 Aa	0.029
***p*-value (N=4)**	0.015	0.011	

Data expressed as Mean ± Standard error (N. Total specimens (rabbits) = 4). The capital letters refer to differences between groups at p≤0.05. The small letters refer to differences between post-extraction time intervals at p≤0.05.

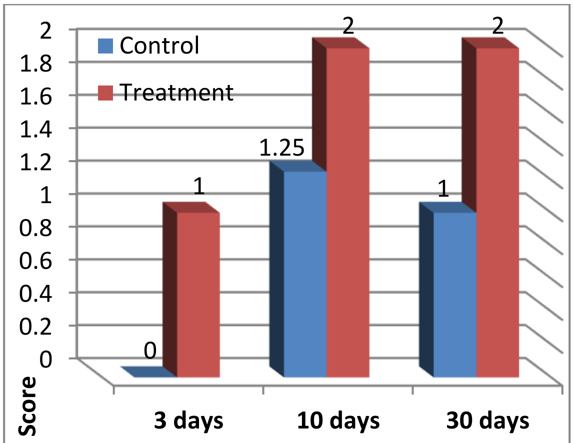

Figure (10): IHC analysis of the Ki67 shows the mean scores for both control and INS-treated groups at 3-, 10-, and 30-days post-extraction periods.

Table (8): Statistical analysis of the Ki67 expression scores at the healing site for control and INS treatment groups.

Groups / Intervals	Control	INS Treatment	*p*-value (N=4)
3 days	0.00 ± 0.00 Bb	1.00 ± 0.00 Ab	0.029
10 days	1.25± 0.25 Aa	2.00 ± 0.00 Aa	0.114
30 days	1.00 ± 0.00 Ba	3.00 ± 0.00 Aa	0.029
***p*-value (N=4)**	0.011	<0.001	

Data expressed as Mean ± Standard error (N. Total specimens (rabbits) = 4). The capital letters refer to differences between groups at p≤0.05. The small letters refer to differences between post-extraction time intervals at p≤0.05.

DISCUSSION

The present study was designed to evaluate the oral mucosa and alveolar bone healing responses to the local application of INS into the extracted tooth socket of rabbit models. The role of topical INS in wound healing has been reported in the

literature since the 1970s. However, until the late 1990s it got greater attention with the development of more advanced materials for the long-term release of bioactive Insulin [28]. The outcome of our study suggested that the INS solution when administered locally has a positive effect on the healing process of oral mucosa and alveolar bone including (hemostasis, inflammatory response, granulation tissue proliferation, and bone remodeling phases).

Inflammatory response:

Insulin is a peptide hormone and growth factor that can stimulate wound healing through cell migration. [29] This hormone improves the infiltration of macrophages and chemo-like monocytes, which stimulates phagocytosis and reduces necrotic tissue at the wound site. It can increase the transforming growth factor- β production which accelerates the migration of the epithelial cells to the wound, INS has a great influence on the inflammatory response during the acute phase of inflammation and it prevents the activation of the inflammatory factors.[30]

In the present study, wounds treated with INS showed moderate inflammation in the wound area during the early phase of healing at 3 days post-extraction, while during the other intervals of treatment 10 and 30 days, it showed significantly reduced inflammatory response when compared with control specimens. All oral mucosa and alveolar bone specimens treated with INS exhibited significantly reduced inflammatory cell infiltrate means than that recorded in the control specimens at all study intervals. These findings are in agreement with Mirhoseini et al., concluded that INS treatment diminished chronic inflammation factors such as macrophages after 3 days, it accelerates wound healing by reducing the inflammation during the early phase in comparison with control specimens [31]. Our results also match with Macedo et al. study, which found that INS can reduce wound inflammation during the healing process.(4)

Granulation Tissue and Re-epithelialization

Our results showed that the local use of INS stimulates the development of new micro-vessels, associated with a significant reduction in the generation of new connective tissue during the wound healing as compared to control specimens throughout all study time intervals except for 3 days post-extraction intervals, in which non-significant changes were observed as compared to the control specimens. These findings follow Martinez-Jimenez et al. concluded that local INS stimulates the development of new micro-vessels but is not associated with the generation of new connective tissue during wound healing in non-diabetic patients, showed that

neovascularization is critical for successful wound healing [32]. The new blood vessel generation increases blood flow to the damaged tissue, thus providing the oxygen and nutrients required by newly synthesized granulation tissue for collagen deposition and wound epithelialization. The formation of new blood vessels is necessary to improve the growth of granulation tissue and sustain the newly formed granulation tissue. Nevertheless, once an abundant collagen matrix has been deposited in the wound, the fibroblast will stop producing collagen; these findings are in agreement with our results.

Ehterami et al. findings are also in agreement with the present study results in that they studied the effect of wound dressing loaded with insulin-chitosan nanoparticles on the healing of cutaneous wounds in rats. They found that INS successfully enhances the proliferation of mouse fibroblasts. [33] However, there are differences between our study and the mentioned study in that they studied cutaneous wounds, not soft tissue and bone healing of the extracted socket as in our study. In contrast, Azevedo et al. recommendations disagree with our data as they found that INS has no effect on collagen deposition in non-diabetic rats. (10) However, there are many differences between the mentioned study and the current one, regarding study unit, study design, material dose, and duration of study .

Significantly higher and quicker re-epithelialization was observed in INS-treated specimens as compared to control specimens at all intervals of the present study. This is consistent with Besson et al., who reported that the effect of INS stimulates re-epithelialization of skin wound healing in rats when observed at 4, 7, 10, and 14 days [34]. Similarly, Martinez-Jimenez et al. reported that INS application to excision wounds in mice leads to accelerated re-epithelialization by stimulating angiogenesis and by promoting a higher level of maturation of the healing tissue (32). Abianeh et al. reported similar results as they found that sub-dermal injection of INS significantly improves the re-epithelialization at the wound site in a clinical study.[35]

Bone Formation:

The present study results confirm that the local use of INS results in a significantly higher bone formation, and it promotes newly formed bone in the treated specimens when compared to control specimens after 10- and 30-day post-extraction. These findings coincide with previous studies that showed that INS or insulin-like growth factor-1 (IGF-1) has an important role in osteogenesis [36]. As the receptors of insulin and IGF-1 are widely distributed on the surface of osteogenic cells and display a high activity in the differentiation of osteoblasts [37]. While for the osteoblast, the INS and/or IGF-1 regulate the activity of RUNX2, as an osteoblast-specific transcription factor

plays an important role in osteocyte differentiation and bone formation. [38] Furthermore, INS binding receptors of osteoblasts promote the uptake of glucose for bone formation, and binding of the receptors of osteoclasts increases the secretion of osteocalcin, thus regulating bone resorption and mineralization. The synthesis of extracellular collagen, the differentiation of osteoblast, and the induction of osteoclasts depend on the regulation of INS in vivo.[39]

CD34 and Ki67 Bio-Markers Expression:

All oral mucosal and alveolar bone specimens treated with INS demonstrated higher expression of CD34 than the control group at all study intervals, with significant differences at 10- and 30-days post-extraction intervals. Besson et al. results are in agreement with this finding; they reported that INS stimulates neovascularization of skin wound healing (34). The present study results are also in agreement with Thomas et al., who suggested that INS stimulates endothelial cells' proliferation, leading to faster neovascularization and enhanced formation of granulation tissue [40]. Topical INS application rapidly induces up-regulation of the INS signaling-related proteins in wound areas following injury. However, these studies did not analyze CD34 expression as they investigated the new blood vessel formation only, but these give an indication about the effect of INS on neovascularization, these are an agreement with the results of the current study .

The Ki67 results demonstrated significantly higher expression of Ki67 in the INS-treated groups when compared to the control group at all study intervals, with significant differences at 3- and 30-days post-extraction intervals. These findings are in agreement with Macedo et al., who found that INS has been shown to have the ability to promote tissue neogenesis, as well as promote collagen deposition and maturation (4). Neogenesis is generally investigated through investigating the expression of Ki67, which has been performed in the current study; it shows a significant association between cell proliferation and increased Ki67 expression.

CONCLUSIONS

Within the limits of the present study;

- Local application of INS demonstrated a positive effect on the healing process of the oral mucosa.
- Local application of INS demonstrated a positive effect on the bone regeneration process of the dental socket.
- Insulin would be a suitable candidate to be used to enhance the post-extraction healing .

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Ethical statement: All the experiments were approved by the Committee of Ethics of the University of Mosul/College of Dentistry, (UoM.Dent/A.L.83/21).

Conflict of interest

The authors declare that there are no conflicts of interest regarding the publication of this manuscript

REFERENCES

1. Abd Elrahman, S.M., Younes, S.A. and Kawana, K.Y. Effect of Nigella Sativa on Socket Healing in Rabbits. Alexandria Dental Journal, 2019; 44, 60-64.
2. Hassan, M.A. and AL-Ghaban NM. Histological Evaluation of the Effect of Local Application of Grape Seed Oil on Healing Process of Extracted Tooth Socket in Rabbits. Diyala Journal of Medicine, 2019; 17, 2.
3. Liu, X., Zhang, X., Tian, X., Chen, J. and He, Y. Effect of Osteogenic Inducer Sustained Release System on Bone Remodeling after Tooth Extraction. Acts Universitatis Medicinalis Anhui,2021; 56 (5): 823-827.
4. Macedo, A.S., Mendes, F., Filipe, P., Reis, S. and Fonte, P. Nanocarrier-Mediated Topical Insulin Delivery for Wound Healing. Materials,2021;14, 4257.
5. Yang, S., Li, Y., Liu, C., Wu, Y., Wan, Z. and Shen, D. Pathogenesis and Treatment of Wound Healing in Patients with Diabetes after Tooth Extraction. Front. Endocrinol.,2022; 13 (94953), 5.
6. Kaur, P. and Choudhury, D. Insulin Promotes Wound Healing by Inactivating Nfkβ P50/P65 and Activating Protein and Lipid Biosynthesis and Alternating Pro/Anti-Inflammatory Cytokines Dynamics. BioMol Concepts,2019; 10: 11-24.
7. Wang, J. and Xu, J. Effects of Topical Insulin on Wound Healing: A Review of Animal and Human Evidences. Diabetes Metab Syndr Obes,2020; 13: 719-727.
8. David, R. and Rajkumar, N.A. Comparative Study to Assess the Effectiveness of Topical Insulin Dressing Vs Normal Saline Dressing on Wound Healing of Diabetic Foot Ulcer. Int. J. Res Pharm. Sci, 2020; 11, 134-138.
9. AbdelKader, DH, Osman MA, Elgizawy SA, Faheem AM, and McCarron PA. The role of insulin in wound healing process: Mechanism of action and pharmaceutical applications. J. Anal. Pharm. Res. 2018; 2: 1–6.
10. Azevedo, F., Pessoa, A., Moreira, G., Dos, S., M, Liberti, E, Araujo, E, Carvalho, C, Saad, M, Lima and M, H. Effect of Topical Insulin on Second-Degree Burns in Diabetic Rats. Biol. Res Nurs,2016; 18 (2), 181-192.

11. De Waard, E., Driessen, J., and De Jong, J. The Association between Insulin Use and Volumetric Bone Mineral Density, Bone Micro-Architecture and Bone Strength of the Distal Radius in Patients with Type 2 Diabetes - the Maastricht Study. Bone, 2017; 101, 156-161.
12. Nyman, J.S., Kalaitzoglou, E., Clay Bunn, R., Uppuganti, S., Thrailkill, K.M. and Fowlkes, J.L. Preserving and Restoring Bone with Continuous Insulin Infusion Therapy in a Mouse Model of Type 1 Diabetes. Bone Rep,2017; 7: 1-8.
13. Mahmoud, H.M., Sheriff, H., Ismael, A., and Zaki, A. Effect of Topical Insulin Administration on Bone Defect Healing in Diabetic Rats. Braz Dent SciApr/Jun,2020; 23, 2.
14. Mapara, M., Thomas, B.S. and Bhat, K. Rabbit as an Animal Model for Experimental Research. Dent Res J, 2012, 9(1): 111-118.
15. Pandian, S. Ketamine-Xylazine anaesthesia in rabbits. Indian Vet. J.2005; 82(4): 388–389.
16. Callis, G. and Sterchi, D. Decalcification of Bone: Literature Review and Practical Study of Various Decalcifying Agents. Methods, and Their Effects on Bone Histology. Journal of Histotechnology, 1998; 21 (1), 49-58.
17. Yousif, M.Q., Qasem, S.a.M.Q. and Qasem, S.A. Tissue Processing and Staining for Histological Analyses, Academic Press Elsevier Inc. San Diego United States.2016.
18. Pity, I.S. and Jalal, J.A. (2013). Expression of Ki-67 and P53 in Oral Squamous Epithelial Abnormalities. Medical Journal of Babylon, 10 (1), 85-99.
19. Mohammed saleh. Immediate augmentation of extraction socket using bone substitute prepared from Iraqi gypsum rock (an experimental study) on dogs. PhD thesis. Collage of dentistry, university of Mosul. Mosul, Iraq. 2014.
20. Lucaciu, O., Gheban, D., Sorițau, O., Băciuţ, M., Câmpian, R.S. and Băciuţ, G. Comparative Assessment of Bone Regeneration by Histometry and a Histological Scoring System. The Romanian Journal of Laboratory Medicine,2015; 23 (1): 31-45.
21. Gupta, A. and Kumar, P. Assessment of the Histological State of the Healing Wound. Plast Aesthet Res, 2015 (2), 239-242.
22. Sinha, U. K., and Gallagher, L.A. Effects of steel scalpel ultrasonic scalpel, CO2 laser, and monopolar and bipolar electrosurgery on wound healing in guinea pig oral mucosa. Laryngoscope. 2003; 113(2): 228-236.
23. Alemi, H., Asghari, A., Abedi, G., Akbarzadeh, A. and Mortazavi, P. Effect of a Nanocomposite Containing Ostrich Eggshell on Calvarium Healing in the Rabbit: A Pathologic Study. J Hellenic Vet Med Soc, 2019; 70 (4):1757-1770.
24. Pity, I.S., and Ibrahim, S.N. Cellular Proliferation in Oral Mucosal Atypia. I J of Sci & Eng Res. (2013). 4(2): 1–4.

25. Alsaegh, M.A., Altaie, A.M. and Zhu, S. p63 expression And Its Relation to Epithelial Cells Proliferation in Dentigerous Cyst Odontogenic Keratocyst, and Ameloblastoma. Pathol Oncol Res, 2020; 26 (2): 1175-1182.
26. Kitson, S., Sivalingam, V.N., and Bolton, J. Ki-67 in Endometrial Cancer: Scoring Optimization and Prognostic Relevance for Window Studies. Official journal of the United States and Canadian academy of pathology, 2017; 30: 459-468.
27. Fedchenko, N. and Reifenrath, J. Different Approaches for Interpretation and Reporting of Immunohistochemistry Analysis Results in the Bone Tissue -- a Review. Diagnostic Pathology, 2014; 9 (221), 1-12.
28. Quitério, M., Simões, S., Ascenso, A., Carvalheiro, M., Leandro, A.P., Correia, I., Viana, A.S., Faísca, P., Ascensão, L., and Molpeceres, J. Development of a Topical Insulin Polymeric Nanoformulation for Skin Burn Regeneration: An Experimental Approach. Int. J. Mol. Sci, 2021; 22, 4087.
29. Hrynyk, M. and Neufeld, R.J. Insulin and Wound Healing. Burns, 2014; 40 (8), 1433-1446.
30. Pastar, I., Stojadinovic, O., Yin, N.C., Ramirez, H., Nusbaum, A.G., Sawaya, A., Patel, S.B., Khalid, L., Isseroff, R.R. and Tomic-Canic, M. Epithelialization in Wound Healing: A Comprehensive Review. Adv Wound Care, 2014; 3 (7): 445-464.
31. Mirhoseini, M., Kianezhad, M.A., Rezaeipour, B., Ghasemi, M., Gatabi, Z.R., Nia, H.S. and Amiri, F.T. The Synergistic Effect of Topical Insulin and Clindamycin on Acute Dermal Wound Healing in Rat Model: A Histological Study. Journal of Histotechnology. 2021.
32. Martinez-Jimenez, M.A., Valadez-Castillo, F.J., and Aguilar-Garcia, J. Effects of Local Use of Insulin on Wound Healing in Non-Diabetic Patients. Plastic Surg, 2018; 26 (2): 75-79.
33. Ehterami, A., Salehi, M., and Farzamfar, S. In Vitro and in Vivo Study of Pcl/Coll Wound Dressing Loaded with Insulin-Chitosan Nanoparticles on Cutaneous Wound Healing in Rats ' Model. Int J Biol Macromol,2018; 117: 601-609.

34. Besson, J., Hernandes, L., Campos, J.M., Morikawa, K.A., Bersaniamado, C.A. and Matioli, G. Insulin Complexed with Cyclodextrins Stimulates Epithelialization and Neovascularization of Skin Wound Healing in Rats. Injury, 2017;48 (11): 2417-2425.
35. Abianeh, S.H., Bajestani, S.M., Rahmati, J., Shahrbaf, M.A. and Shirzad, N. The Effect of Local Insulin Injection on the Healing Process of Split Thickness Skin Graft Donor Site: A Randomized, Double-Blind, Placebo Control Clinical Trial. European Journal of Plastic Surgery, 2020; 3-3.

36. Cirmanova, V., Zofkova, I., Kasalicky, P., Lanska, V., Bayer, M., Starka, L. and Kanceva, R. Hormonal and Bone Parameters in Pubertal Girls. Physiol. Res, 2017; 66 (Suppl. 3), S419-S424.
37. Shah,V.N.,Carpenter R.D., Ferguson V.L., and Schwartz, A.V. Bone Health in Type 1 Diabetes. Curr. Opin. Endocrinol. Diabetes Obes, 2018; 25: 231-236.
38. Cao, W., Li, J., Yang, K. and Cao, D. (2021). An Overview of Autophagy: Mechanism, Regulation and Research Progress. Bull. Cancer, 108, 304-322.
39. Mu, W., Liang, G., Feng, Y., Jiang, Y. and Qu, F. The Potential Therapeutic Role of Metformin in Diabetic and Non-Diabetic Bone Impairment. Pharmaceuticals, 2022; 15, 1274.
40. Thomas, N., Chittoria, R.K., Gupta, S., Reddy, C.L., Mohan, P.B. and Shijina, K. Role of Topical Insulin in Venous Ulcer Management. Arch Med Case Rep,2020; 2 (1), 20-22.

تأثير الأنسولين الموضعى فى شفاء تجويف قلع الأرانب المصابة بداء السكرى (دراسة تجريبية)

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الملخص

الأهداف: تهدف هذه الدراسة إلى التحقيق في تأثير الأنسولين على شفاء تجويف الأسنان بعد القلع في الأرانب، فضلا عن تقييم شفاء الغشاء المخاطي الفموي وشفاء العظم السنخي خلال ثلاث فترات زمنية 3 و 10 و30 يومًا بعد قلع السن. المواد وطرائق العمل: تم استخدام اثني عشر أرنبًا أبيضًا ذكرًا في هذه الدراسة، وتم تقسيمها عشوائيًا إلى ثلاث مجموعات فرعية (أربعة ارانب لكل مجموعة) لعمل التقييم النسيجي المجهري والتقييم النسيجي المناعي-الكيميائي خلال 3 و 10 و30 يومًا بعد القلع ومقارنة النتائج مع المجموعة الضابطة. تم قتل الأرانب لكل مجموعة وحسب الفترات الزمنية المذكورة انفا، حيث مثل كل أرنب عينتين (العينة الضابطة والعينة المعالجة). تمت إزالة الضرس الأول من الفك العلوي لكل ارنب من الجهتين اليمين واليسار (الجانب المعالج والجانب الضابط)، تلاها حقن الأنسولين في تجويف قلع الضرس. تم وضع رغوة هلام اسطوانية بأبعاد (3*5 مم) في كلا الفتحتين. بعدها تم حقن جرعة مكونة من 10 وحدة دولية بحجم 0.1 مل من محلول بتركيز 100 وحدة دولية/مل من الأنسولين لكل المجموعات المعالجة. حيث تم حقنها في رغوة الهلام. بينما بالنسبة للجانب الضابط لم يتم حقن اية مادة معالجة. تم تحضير جرعة قياسية من الانسولين وتم حقنها بشكل فموي وحنكي حول حواف اللثة للفجوة المدروسة في اليوم الثاني والرابع والسادس بعد القلع حيث تم الحقن فقط للجانب المعالج. بعد إتمام العملية تم جمع العينات من جميع المجموعات وحسب الفترات الزمنية لكل مجموعة. حيث اخذت العينات من الغشاء المخاطي الفموي ومن تجويف قلع الضرس من عظم الفك العلوي وتم ترتيبها على الفور وتجهيزها للمختبر. تم تقييم الاستجابة الالتهابية، وتكوين النسيج الحبيبي، وإعادة تبطين النسيج الظاهري، وتقييم شفاء العظام، ومقياس تأثير معاملات CD34 وKi67. تم إجراء تحليل إحصائي غير معلمي، وتم تسجيل الدلالة عند p≤0.05. **النتائج**: أظهرت نتائج التقييم النسيجي استجابة التهابية مخفضة بشكل كبير، وتكوين نسيج حبيبي أقل، وإعادة تبطين النسيج الظاهري بشكل أسرع، فضلا عن تكوين العظام المعزز للمجموعة المعالجة مقارنةً بالمجموعة الضابطة في فترات زمنية معينة من الفترة الإجمالية للدراسة. كما أظهرت نتائج التقييم النسيجي المناعي-الكيميائي زيادة ملحوظة في كميات معاملات CD34 وKi67 في المجموعة المعالجة مقارنة بالمجموعات الضابطة في فترات دراسية معينة. **الاستنتاجات**: في حدود الدراسة الحالية، أظهرت النتائج أن التطبيق الموضعي للأنسولين له تأثيرًا إيجابيًا على عملية شفاء الغشاء المخاطي الفموي وكذلك تجديد العظام في تجويف الأسنان. مما يرشح مادة الانسولين لتكون مناسبة للاستخدام الموضعي لتعجيل الشفاء بعد قلع الاسنان.