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

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Abstract

Aims: The present study aims to investigate the effect of insulin on the healing of dental sockets in diabetic 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:** Twelve male albino diabetic rabbits were used in this study, these were randomly sub-divided into three sub-groups (N=4) for the histological and immunohistochemistry evaluation at 3, 10, 30 days post-extraction and compared to the controls. The rabbits were euthanized at the corresponding time interval, each rabbit represented a control and 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 10 IU in 0.1 ml from a solution of 100 IU/ml concentration of Insulin; for each corresponding group was injected into the gel foam whilst 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 palataly 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, 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, and 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 treated group when compared to 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 of diabetic rabbits. The local injection of Insulin would be suitable candidate to be used in the enhancement of post-extraction healing.

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

الملخص

الأهداف: تهدف هذه الدراسة إلى التحقيق في تأثير الأنسولين على شفاء العظم السنخي للأرانب المصابة بداء السكري بعد قلع السن، فضلا عن تقييم شفاء الغشاء المخاطي الفموي وشفاء العظم السنخي خلال ثلاث فترات زمنية 3 و 10 و30 يومًا بعد القلع. **المواد وطرائق العمل:** تم استخدام اثني عشر أرنبًا محليا أبيضًا ذكرًا في هذه في هذه الدراسة من الأرانب المصابة بداء السكري 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 $p \leq 0.05$. **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.

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INTRODUCTION

Wound is basically known as disruption of the cellular and anatomic continuity of tissues, injuries can extend further to other tissues and structures such as skin, oral mucosa, subcutaneous tissue, muscles, tendons, nerves, vessels as well as bone.[1] Tooth extraction is one of the most common surgical procedures in oral and maxillofacial surgery, tooth loss is one of the most critical oral health indicators.[2] The healing of extraction sockets has similar stages of fracture healing, when diagnosed with hopeless tooth condition due to periodontitis, dental caries, trauma, etc., tooth extraction is performed.[3] Alveolar socket wound healing process involves soft tissue and bone remodelling, and it starts right after tooth extraction.[4] The healing process is affected by various factors such as systemic metabolite imbalances, local factors, diet supplementations, radiation, or others [5] .

Diabetes mellitus (DM) can disrupt various wound-healing processes. These includes haemostasis, inflammation, proliferation, and remodelling phases. This problem impacts long-term adverse effects on quality of life, morbidity and mortality characterized by chronic wounds that cause impaired healing due to delayed or uncoordinated healing.[6] DM shows a persistent inflammatory phase due to obstacles in forming granulation tissue and reduced wound tensile strength caused by damage to blood vessels that cause ischemia.[7] Endothelial dysfunction is strongly associated with the main reason of impaired wound healing ability, higher rate of complications associated with DM. The inflammatory phase is lengthened, the concentration of growth factors and proliferative phase cells is reduced and neovascularization is impaired [8].

Insulin (INS) is a peptide hormone and growth factor which can stimulate wound healing through the cell migration [9]. This hormone improves the infiltration of macrophages and chemo-like monocytes, which stimulates phagocytosis and reduces necrotic tissue at the wound site, INS and its derivatives like IGF-1 are effective in keratinocyte proliferation and migration to the wound; in addition, it can increase the TGF- β production which accelerates the migration of the epithelial cells to the wound [10]. It has been suggested that local application of INS improves wound healing in diabetes. It also has been found to reduce inflammation, accelerate wound healing and causes rapid re-epithelialization of the wound by promoting protein synthesis [11]. Clinical studies have also shown that topical application of INS can promote the healing of diabetic foot and skin ulcers and improve wound tissue morphology. [12] The positive effects of INS on bone strength are direct or indirect (i.e., via glucose control, anti-inflammatory, and antioxidant effects).

Therefore, INS do not affect bone micro architecture/BMD in humans [13] or animals. [14] Indirect evidence suggests that intensive diabetes management with INS therapy increases fall risk. It is an anabolic agent for bone, a lack of INS leads to an impaired osteoblast function giving rise to a low bone-turnover state, which results in a decreased peak bone mass during puberty,[15] and a reduced bone mineral density during adult life.[16] The physiological concentration of INS can stimulate the proliferation and differentiation of osteoblasts in the high-glucose environment, and its application to the local tooth extraction nests of diabetic rats can promote bone wound healing. In addition, topical application of INS in bone defects induced new bone formation and healing of the defect.[17] Induction of insulin-dependent diabetes mellitus by chemical treatment of experimental animals is relatively well understood several compounds can be used to mimic human diabetes, Including alloxan, streptozotocin, vacor, dithizone, gold-thioglucose monosodium glutamate and 8-hydroxyquinolone.[18] Compared to other diabetogenic compounds it appears to be the most used compound for the induction of diabetes in animal experimental studies, probably because of its affordability and availability, it is the most common chemical compound used to induce experimental diabetes due to its selective destruction of beta cells in the pancreatic islets through its ability to generate superoxide radicals, hydrogen peroxide and hydroxyl radicals with fatty degeneration signs of mitochondrial damage leading ultimately to apoptosis.[19]

The present study aims to investigate the effect of Insulin on the healing of dental socket of diabetic 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 control *in vivo* study design was used. The total sample was consisted of 12 of alloxan-induced diabetes 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 as 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 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%. [20] 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 into three experimental groups according to the time intervals (rabbits were euthanized at 3, 10, 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 control side (left) in order to minimize any bias. Each group was identified by coding colours on the animal's tail.

Material of the study:

Insulin solution of 100 IU/ml concentration, total volume of 10 ml that contains 1000 IU of (Actrapid®, Novo nordisk, Denmark), as seen in Figure (1). While the extraction socket was filled with a cylindrical of 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).

Induction of diabetes mellitus:

Diabetes mellitus was induced in overnight fasted male rabbit by a standard dose of intra-peritoneal injection of freshly prepared alloxan monohydrate (Waterloo Ltd, UK), the concentration was determined by the body weight of each rabbit at 400 mg/kg. The material was dissolved in normal saline (Gibco, UK), (0.9% NaCl) at a rate of 1 ml of normal saline for each 1kg of rabbit's body weight, [21] using a 5 ml plastic disposable syringe (Carmine Ltd, China). Prior to injection the abdominal skin was cleaned with 4% Povidine Iodine solution (India) with the help of cotton to avoid any kind of infection. Animals were treated with 5% glucose solution orally to combat the early phase of alloxan drug-induced hypo-glycemia, then after one hour of alloxan monohydrate injection, the rabbits were served with standard diet and fresh water.

The fasting blood glucose level was determined by using a single-touch glucometer (Accu-Chek sensor, Roche Diagnostics, Germany) using blood collected from the rabbit's ear by vein tipping of all experimental rabbits every two day for the first seven days post alloxan injection. [21] Two blood glucose readings were taken using two similar devices and the average value was considered to decrease any bias. The experimental rabbits with fasting blood glucose level above 200 mg/dl were considered as diabetic and chosen for the study [22]. In addition, signs of diabetic were observed on the selected animals as rabbits were suffered from extreme tiredness and frequent urination [21].

Surgical Procedure:

1. General Anaesthesia:

One to four hours prior to the initiation of the study (pre-operative), rabbits were food deprived, while they were water fasted 1 hour before the administration of anaesthesia. 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 [23].

2. Surgical procedure:

Surgical procedures were performed under standardized protocol and approved by the ethical committee of 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 anaesthesia, each rabbit was laid on its lateral side on the surgical board, covered with sterile towel exposing the head only. The rabbit's mouth was opened using rabbit mouth gag (Starlabs, UK). The surgery was performed through bilateral extraction of upper first premolars using rabbit extraction forceps, (Starlabs, UK). Resulting in two sockets per rabbit providing a treated side and 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 bucally and palataly around the gingival margins of the studied socket on the 2nd, 4th, and 6th day post-extraction for the treated side only. The surgical procedure is detailed in Figure (2).

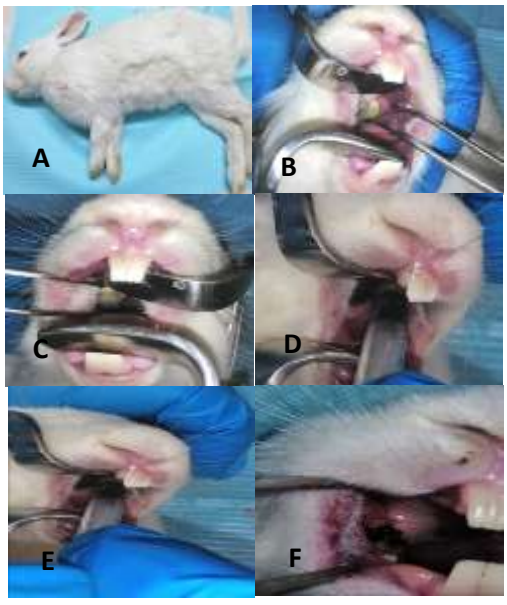

Figure (2): Photographs of the steps of the surgical procedure: [(A) anesthetized rabbit, (B-C) tooth extraction, (D-E) gel foam insertion and local insulin injection, (F) final socket]**.**

Postoperative Procedures:

1. 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 veterinarian, monitoring their feeding and physical activity.

2. Specimen collection and preparation:

After euthanization 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 required 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) [24]. After bone decalcification, the specimens were removed from the solution and rinsed thoroughly with distilled water and stored ready for histological analysis.

3. Histological preparation and staining:

The specimens (soft tissue and bone) were prepared prior to 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 labelled in paraffin wax blocks. The specimens were then frozen for 24 hours then sliced into 4 microns 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 labelled glass slide (Citoplus, China). The obtained slides were then, stained with hematoxylin and eosin stains (Thermo scientific, UK), mounted with DPX (Iso-com Ltd., UK) for further microscopical examination [25].

4. 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 then let 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 contains 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 slides were let 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 moisture chamber for 60–90 min followed by two times gentle rinsing with PBS. Once completed the slides were incubated for 10 min with appropriate secondary antibody according to manufacturer's recommended protocol at room temperature then washed with PBS three times and prepared to receive chromogenic solution. The slides were then incubated with DAB tetra hydrochloride solution, which is peroxide-compatible chromogen for 5 minutes at room temperature as a substrate chromogen solution to produce brown color, followed by twice rinse with distilled water for 5 min 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 media (permount) and covered with cover slip for microscopical analysis [(26)].

Histometric analysis and evaluation of oral mucosa and alveolar bone healing:

1. Histometric analysis and evaluation of Haematoxylin and Eosin-stained sections:

The extracted tooth socket healing was evaluated histologically via means of semi quantitative scale, measuring the intensity of inflammatory response, amount of granulation tissue formation within the socket, wound re-epithelialization whether partial or complete and regular mature or not, bone healing. Three examiners were recruited to examine the slides, the researcher and other two experienced histopathologists whom both recorded their own scores 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 explained in Tables

(1, 2, 3 and 4). The histometric evaluation was done using light microscope (Optica, Italy) at power magnification of X10 and X40.

Table (1): The criteria for inflammatory response intensity scoring [27].

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 [28,29].

Score	Observed histopathological response
0- Nil	Absensce of granulation tissu formation in the field of operation at (X10).
1- Mild/Scanty	Scanty amount of granulatio tissue formation, less than hal 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 [30].

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 [31].

Score	Observed histopathological response
0	No newly formed vessels. None to 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.

2. Histometric analysis and evaluation of immunoreactive socket tissue sections against CD34, Ki67 epitopes:

Angiogenesis process of socket (wound) healing was evaluated on a semi-quantitative scale measuring the intensity of CD34 immunoreaction expression in the operation field under light microscope (Optica, Italy) at X10 magnification. The specific protein molecules of CD34 presented mainly in endothelial cells. Positively marked cells (immunoreactive cells against CD34 antigen) in the wound area were counted and expressed, counting cells was performed according to the documented procedure, cells with any degree of staining were scored positively [32].

Ki67 is well-established marker for cellular proliferation during wound repair [33]. The immunostaining of Ki67 appeared as brown/yellow nuclear stain, the expression of Ki67 is considered as positive when there is a brown/yellow granule in the nuclei of cell or in the cytomembrane beside the cell nuclei. All stained nuclei were counted as positive, irrespective of the staining intensity [34]. All slides for the immune-staining were evaluated using 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 other two experienced histopathologists using special criteria for scoring as 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 number in more than 3/4 of the field of operation at (X10) magnification.

Statistical analysis:

The Statistical analysis was done by using Sigma Plot software program, data were expressed as mean ± standard error. The scores are descriptive expression of the inflammation response granulation tissue, re-epithelialization, bone formation and CD34, Ki67 expression for each of the study group. The collected scores were analysed statistically by Mann-Whitney U test for all histological and IHC variables of groups comparisons, whereas the comparisons of the time intervals were statically analysed using the Kruskal-Walli's test. The statically 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).

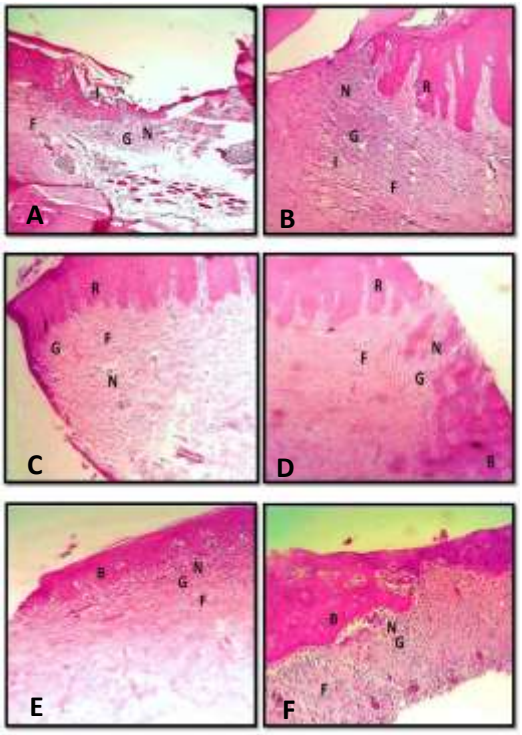

Figure (3): Photomicrographs of the oral mucosa and maxillary alveolar bone (left-control, right- treated): [At the 3 (A-B), 10 (C-D), and 30 (E-F) days post-extraction. (G) = granulation tissue, (N) = new vasculature, (F) = fibrous connective tissue, (I) = inflammatory response, (R) = re-epithelialization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

1. Post-operative histological findings at three days period:

Sections of oral mucosa and maxillary alveolar bone of control group that obtained at the third day post-extraction showed marked infiltration by inflammatory cells and presence of profound amount of granulation tissue with re-epithelialization were seen at the wound edges only. The bone sections exhibited none to very minimal number of fibroblasts with no newly formed vessels, osteoid or bone. The oral mucosa sections of the treated group exhibited moderate infiltration by inflammatory cells, moderate amount of granulation tissue with re-epithelialization were seen covering less than half of the area at the wound surface. The bone sections demonstrated none to very minimal number of fibroblasts with no newly formed vessels, osteoid or bone. A statistically significant reduction in inflammatory response and granulation tissue formation as well as a significant increase in re-epithelialization scoring in the treated group specimens as compared with control. In contrast, there was no statistically significant difference between the two group specimens in 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).

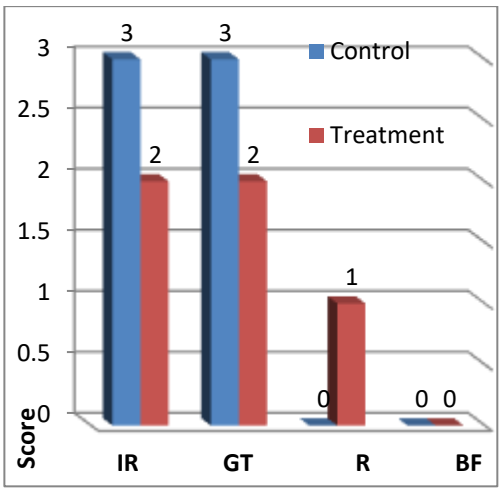

Figure (4): Means of histological evaluation scores: For inflammatory response (IR), granulation tissue (GT), re-epithelialization (R), bone formation (BF); at 3 days post-extraction period, for control and treatment groups.

2. Post-operative histological findings at ten days period:

Sections of oral mucosa and maxillary alveolar bone of control group that obtained at the 10th day post-extraction showed mild infiltration by inflammatory cells, presence of profound amount of granulation tissue with re-epithelialization were seen covering less than half of the area at the wound surface. The bone sections exhibited none to very minimal number of fibroblasts with no newly formed vessels, osteoid or bone. The oral mucosa sections of the treated group exhibited no inflammatory cells in the field of operation, with moderate amount of granulation tissue

and re-epithelialization were seen covering more than half of the area at the wound surface.

The bone sections demonstrated predominant number of fibroblasts with moderate newly formed vessels, osteoid and bone cells. A statistically significant reduction in inflammatory response and granulation tissue formation. As well as a significant increase in re-epithelialization and bone formation scoring was observed in the treated group specimen as compared with 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).

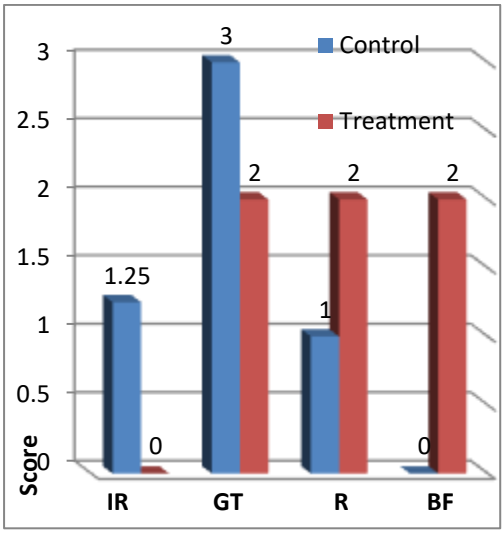

Figure (5): Means of histological evaluation scores: For inflammatory response (IR), granulation tissue (GT), re-epithelialization (R), bone formation (BF); at 10 days post-extraction period, for control and treatment groups.

3. Post-operative histological findings at thirty days period:

Sections of oral mucosa and maxillary alveolar bone of control group that obtained at this period showed mild infiltration by inflammatory cells, moderate amount of granulation tissue with re-epithelialization were seen covering more than half of the area at the wound surface.

The bone sections exhibited predominant number of fibroblasts with moderate newly formed vessels, osteoid or bone cells. The oral mucosa sections of the treated group exhibited no inflammatory cells were seen in the study field, as well as moderate amount of granulation tissue with re-epithelialization covering the entire wound area. The bone sections demonstrated fewer number of fibroblasts, highly organized osteoid and extensive newly formed vessels with bone cells. A statistically significant reduction in inflammatory response and significant increase in re-epithelialization and bone formation scoring in the treated group specimens as compared with control group, while there was no statistically significant difference between the two groups specimens in inflammatory response and granulation tissue formation, as seen 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).

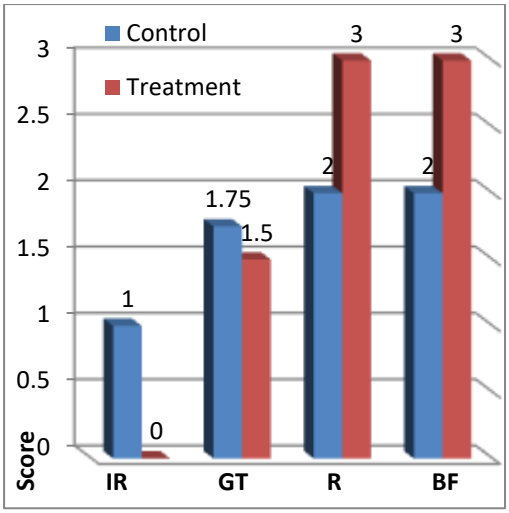

Figure (6): **Means of histological evaluation scores**: For inflammatory response (IR), granulation tissue (GT), re-epithelialization (R), bone formation (BF); at 30 days post-extraction period, for control and treatment groups.

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	1.25 ± 0.00 Ab	0.00 ± 0.00 Bb	0.029
	30 days	1.00 ± 0.00 Ab	0.00 ± 0.00 Bb	0.029
	***p*-value (N=4)**	<0.001	0.015	
G. T	**3 days**	3.00 ± 0.00 Aa	2.00 ± 0.00 Ba	0.029
	10 days	3.00 ± 0.00 Aa	2.00 ± 0.00 Ba	0.029
	30 days	1.75 ± 2.25 Ab	1.50 ± 0.28 Aa	0.686
	***p*-value (N=4)**	0.015	0.415	
R.	**3 days**	0.00 ± 0.00 Bc	1.00 ± 0.00 Ac	0.029
	10 days	1.00± 0.00 Bb	2.00 ± 0.00 Ab	0.029
	30 days	2.00 ± 0.00 Ba	3.00 ± 0.00 Aa	0.029
	***p*-value (N=4)**	0.001	<0.001	
B.F.	**3 days**	0.00 ± 0.00 Ab	0.00 ± 0.00 Ac	1.00
	10 days	0.00 ± 0.00 Bb	2.00 ± 0.00 Ab	0.029
	30 days	2.00 ± 0.00 Ba	3.00 ± 0.00 Aa	0.029
	***p*-value (N=4)**	<0.001	<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).

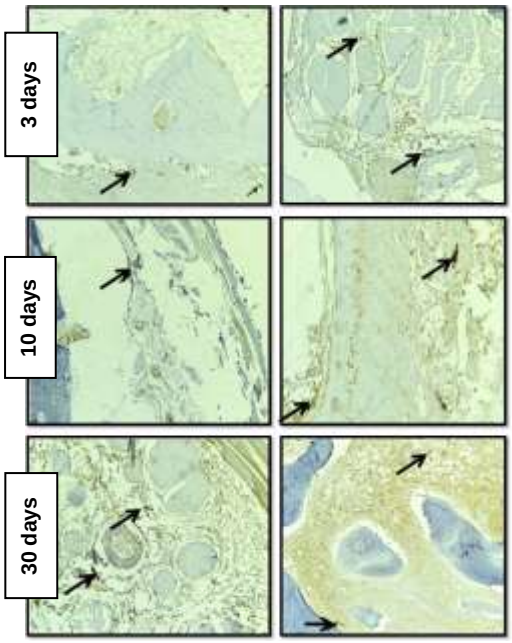

Figure (7): Photomicrographs of the oral mucosa and maxillary alveolar bone (left: control, right: treated); at the 3 (A-B), 10 (C-D), and 30 (E-F) days post-extraction stained with immunohistochemistry technique for CD34 biomarker. Black arrows mark the positively stained cells (immunoreactive against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

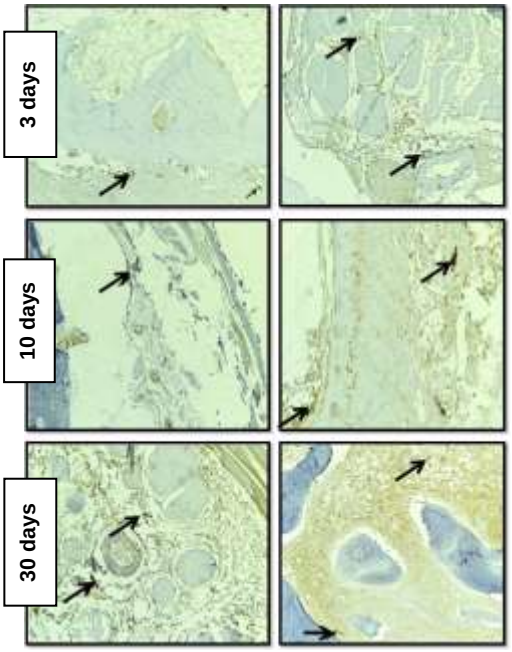

Figure (8): Photomicrographs of the oral mucosa and maxillary alveolar bone (left: control, right: treated); at the 3 (A-B), 10 (C-D), and 30 (E-F) days post-extraction stained with immunohistochemistry technique for Ki67 biomarker. Black arrows mark the positively stained cells (immunoreactive against 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 showed mild expression of positively stained cells in the study field, also histological sections of the treated group specimens demonstrated moderate positively stained cells that were present in few numbers for CD34 antibody. There was no expression of positively stained cells observed in the study field, with mild positively stained cells that were seen in the study field of histological sections of the treated group specimens for Ki67 antibody. Statistically, a significant increase in the expression scoring of both antibodies was observed in treated group as compared to control group, 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 showed mild expression of positively stained cells, and histological sections of the treated group the specimens showed moderate positively stained cells present in few numbers for both (CD34 and Ki67) antibodies. Statistically, a significant increase in expression scoring of CD34 antibody and a non-significant increase in the expression scoring of Ki67 antibody was observed in the treated group as compared to control group, 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 and marked positively stained cells that were seen in the study field of histological sections of the treated group specimens for both (CD34 and Ki67) antibodies. Statistically, a significant increase in the expression scoring of both antibodies was observed in the treated group as compared to control group, as seen in Figure (9) and Table (7) for CD34, Figure (10) and Table (8) for Ki67.

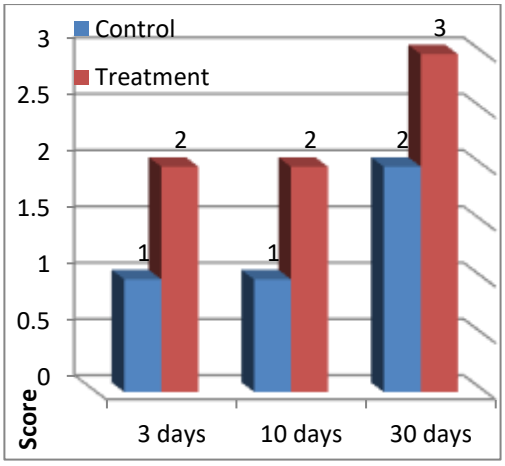

Figure (9): IHC analysis of the CD34 shows the mean scores for both control and INS treated groups; at 3-, 10-, and 30-days post-extraction period.

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	1.00 ± 0.00 Bb	2.00 ± 0.00 Ab	0.029
10 days	1.00 ± 0.00 Bb	2.00 ± 0.00 Ab	0.029
30 days	2.00 ± 0.00 Ba	3.00 ± 0.00 Aa	0.029
***p*-value (N=4)**	0.015	0.015	

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 time intervals at $p \leq 0.05$.

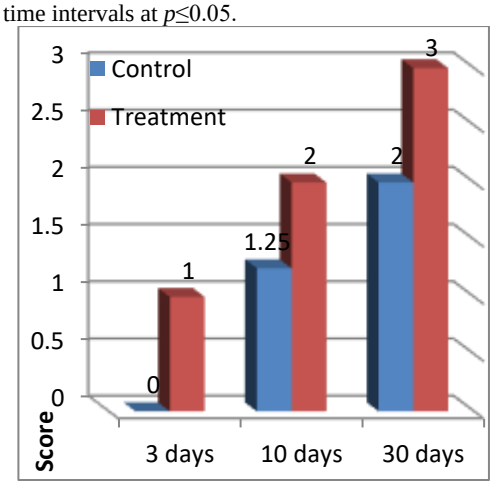

Figure (10): IHC analysis of the Ki67 shows the mean scores for both control and INS treated groups; at 3-, 10-, and 30-days post-extraction period.

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 Ac	0.029
10 days	1.25± 0.25 Aa	2.00 ± 0.00 Ab	0.114
30 days	2.00 ± 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 $p \leq 0.05$. The small letters refer to differences between post-extraction time intervals at $p \leq 0.05$.

DISCUSSION

Insulin has positive effects on wound healing when used in diabetic patients both T1D and T2D therapy either systemically [36] or even locally [37]. In present study, the INS was locally administrated into the wound site i.e. the extraction socket of the diabetic rabbit. The local administration of INS would require fewer doses as compared to systemic administration, which reduces potential side effect; these are the main advantages for management and wound healing using local INS administration [38]. In contrast, the systemic administration of INS has limited applicability because of its significant side effects, including hypoglycaemia, and hypokalaemia [39]. The outcome of our study suggested that the INS solution when administered locally has had a positive effect on the healing process of oral mucosa and alveolar bone including haemostasis,

inflammatory response, granulation tissue proliferation, and bone remodelling phases.

Inflammatory response

The persistent presence of pro-inflammatory macrophages and chronic inflammatory response on the wound area are characteristic pathological challenges of diabetic impaired healing. High glucose induces macrophage polarization towards pro-inflammatory M1 phenotype; INS promotes macrophage phenotype transition from M1 to M2 and attenuates high glucose-induced inflammatory mediator secretion *in vitro*. Low dose of INS with topical application on diabetic wounds validly stimulates macrophage phenotype to switch from pro-inflammatory to anti-inflammatory healing phenotypes, this summary the anti-inflammatory effect of insulin [(40)]. All oral mucosa and alveolar bone specimens of INS treated diabetic group showed significantly lesser inflammatory cells infiltrate means than that recorded in the control specimens at all study intervals. These findings are consistent with Abdelkader *et al*. who reported that diabetic wounds treated with topical INS loaded nanoparticles demonstrated a reduction in the inflammatory process in cutaneous wound healing in rats.[(41)] Similar findings were reported by Mieczkowski *et al*. who found that INS treated group showed small infiltration of inflammatory cells as compared with control group at 3 and 9 days post-extraction intervals.[(42)] In addition to the above articles the results also are in agreement with Mirhoseini *et al*., who investigated the inflammation response after 3 days of INS administration that showed higher inflammatory response which in turn decreased in the INS group from 3 days to 14 days as compared to a control, their results showed that wounds treated with INS exhibited mild inflammation in the wound bed [(43)] .

On the other hand, the present study contradicts Azevedo *et al*., study who showed that topical insulin cream increased the inflammatory cell infiltration in the diabetic group after 7- and 14-days post-burn [(44)] . However, there are many differences between this study and our study regarding; study unit, study design, and type of tissue healing, material dose.

Granulation tissue and re-epithelialization:

Insulin acts on human growth hormone receptors and enhances the collagen formation, granulation tissue, and the stimulation of fibroblasts to produce insulin-like growth factor (IGF). Insulin also stimulates migration and proliferation of human keratinocytes, which enhances cell growth and accelerates wound healing [(45)].

The present study results showed a favourable effect on neovascularization process in the topical INS treated diabetic group as compared with control group, it associated with significant decrease of new connective tissue generation during wound

healing than in control specimens at 3 and 10 days of the study intervals. This comes in agreement with Zhang and Lv study, who reported the positive effect of topical INS on the growth of granulation tissue and the enhancement of new blood vessels formation [46]. These also agree with Zhu *et al*., who concluded an excellent wound healing performance when treated with INS through promotion of fibroblast proliferation and deposition of collagen [47].

The re-epithelialization results revealed a significant increase and quicker re-epithelialization that was observed in INS treated specimens as compared to control specimens at all study intervals of diabetic rabbits. This comes in agreement with Azevedo *et al*, who found that re-epithelialization and granulation-tissue formation in the diabetic INS group was similar to that of the healthy skin from animals without diabetes and significantly differs from that of the diabetic control group after 7 and 14 days post burn.[44] The study results also match with Mirhoseini *et al*., who indicated that the rate of epithelial growth from the wound edge to the center of the wound in the INS treated group.[43] In our study all wounds in treated groups showed epithelial growth at the edges of the wound, while the control untreated wounds had less epithelial growth.

Bone formation:

Our results showed that the local use of INS resulted in significant increase in bone formation as it promotes new bone formation in the treated specimens as compared to control specimens at 10- and 30-days post-extraction. Wang *et al*., reported similar findings as they reported that the physiological concentration of INS can stimulate the proliferation and differentiation of osteoblasts in high glucose environment, and its application to the local tooth extraction nests of diabetic rats can promote bone wound healing through promoting the phagocytosis of macrophages.[48] In addition, macrophages play an important role in all phases of adult wound healing including proliferation, inflammation, and remodelling, which suggests that macrophages take a distinct role to ensure proper wound healing [49] .

CD34 and Ki67 bio-markers expression

All specimens from the oral mucosa and alveolar bone that were treated with INS demonstrated significant increase in CD34 expression as compared to the control group at all study intervals. This finding is in agreement with Zhang and Lv., who showed that the expression of CD34 increases at 5 days after local INS injection as compared with control group [46].

Our results showed that Ki67 marker demonstrated an increased expression in the INS treated group when compared to the control group at all study intervals, this increase was significant at 3- and 30-days post-extraction intervals. Azevedo *et al*., analyzed Ki67 expression as a marker for cell proliferation status. They observed a significantly more

expression of Ki67 in the diabetic INS group compared with the diabetic control group, suggesting that they were modulated by topical insulin to improve both the inflammatory and proliferative phases via recruitment of macrophages and increased cell proliferation.[50] Similarly, Yang *et al*., reported that local application of INS promotes cell proliferation, as they observed increased expression of Ki67 of the corneal epithelium wound healing of diabetic mice[51] . Mieczkowski *et al*., also concluded similar finding in that INS accelerates wound healing in diabetic rat models under chronic hyperglycaemia, causing increased expression of Ki67 [42].

CONCLUSIONS

Within the limits of the present study: Local application of Insulin demonstrated a positive effect on the healing process of oral mucosa of diabetic rabbits.

Local application of Insulin demonstrated a positive effect on the healing process of the dental socket and bone regeneration of diabetic rabbits. Also, insulin would be a suitable candidate to be used to enhance the post-extraction healing.

Conflict of Interest

The authors declare that there are no conflicts of interest regarding the publication and/or funding of this manuscript.

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