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HISTOLOGICAL EVALUATION OF BONE REGENERATION AFTER TOPICAL APPLICATION OF STRONTIUM RANELATE GEL IN CRITICAL SIZE BONE DEFECTS IN THE TIBIA OF INDUCED DIABETIC RATS

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ABSTRACT

Objective: The aim of this study was to evaluate bone regeneration histologically after topical application of Strontium Ranelate gel in critical size bone defects in the tibia of induced diabetic rats. **Subjects and methods**: sixty adult male albino rats were used in this study and divided into four groups equally, fifteen rats for each group. These groups were Normal control group (N), Diabetic control group (D), Normal experimental group treated with SrR gel (NSR), and Diabetic experimental group treated with SrR gel (DSR). Five animals from each group were sacrificed at 5, 10 and 30 days alternatively postoperatively. After termination of the experiment, the specimens were prepared, and bone formation was evaluated histologically by H&E stain and Masson's trichrome stain. **Results:** In experimental groups treated with SrR, there was acceleration in the formation and mineralization of granulation tissue and woven bone, also there was acceleration in the conversion of woven bone to spongy bone. While in Diabetic control group there was obvious retardation in formation and mineralization of granulation tissue and bone than all other groups. **Conclusion:** The study showed that the topical application of SrR gel can accelerate formation and maturation of granulation tissue. Also, it accelerates bone formation and mineralization in diabetic rats treated with SrR gel.

KEYWORDS: Strontium ranelate, Bone regeneration, Diabetes mellitus, Topical application, Critical size defect.

INTRODUCTION

Bone undergoes different types of diseases and different forms of surgeries lead to problems in the continuity, normal architecture, and function of bone. In some clinical conditions, significant bone loss, the existence of a broad variety of illnesses, or the removal of a tumor might result in delayed healing or even nonunion [1, 2].

Diabetes mellitus (DM) is a chronic form of diabetes characterized by high blood glucose levels. Uncontrolled diabetes is linked with unfavorable systemic sequelae, such as delayed wound healing, greater susceptibility to infection, and micro vascular problems, which contribute to a decline in the immune system [3, 4] and an elevation in bone fragility [5] Diabetes mellitus diminishes osteoblastic activity and bone mineralization [6]. Furthermore, it

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diminishes the fracture's biomechanical qualities, cellular proliferation in the earliest callus, and collagen production and composition[7,8]. Numerous clinical and experimental research have demonstrated that diabetes inhibits bone production, reduces its mechanical strength, and promotes bone architectural degeneration [9,10,11]. In addition, hyperglycemia has negative consequences on the bone matrix and its constituents, as well as on the adhesion, proliferation, and accumulation of extracellular matrix [12].

A viable alternative to growth factors might be the local injection of a systemic medication that modulates bone and is routinely used to treat bone disease. Local medication delivery has the benefit of releasing the antibiotic locally at the infection location for a sufficient time without systemic exposure, hence minimizing bacterial resistance and drug-related systemic adverse effects [13]. There are several medicines that may be administered systematically to stimulate bone metabolism. In recent years, researchers have paid particular attention to strontium ranelate (SrR), one of these medications[14, 15].

Current osteoporosis treatment SrR looks to be a potential option because of its ability to stimulate osteoblast activity and suppress osteoclast activation, hence promoting bone production [16,17]. Several in vitro investigations have indicated that SrR promotes osteoblast proliferation, differentiation, survival, and function, while also triggering apoptosis and decreasing osteoclast formation and activities [18, 19]. In vivo, the systemic administration of SrR promotes the repair of bone deficiencies [20, 21].

In past few years, the incorporation of inorganic ions (inorganic angiogenic agents) into biomaterials to stimulate the production of neovascularization for bone regeneration has gained popularity. In comparison to growth factors, these inorganic angiogenic factors have garnered more interest due to their greater stability, lower cost, and superior clinical safety [22, 23, 24].

Among such inorganic angiogenic agents, strontium ions (Sr2+) can stimulate angiogenesis. In earlier studies on Sr-based bone healing materials, the release of Sr2+ ions were shown to increase the production of proangiogenic components, such as basic fibroblast growth factor (bFGF), vascular endothelial growth factor (VEGF), and matrix metalloproteinase-2 (MMP-2) in tissue-engineered bone [22]. Additionally, it was shown that Sr2+ ions released from biomaterials might stimulate the proliferation, migration, and development of tubular structures in vitro, suggesting that Sr2+ ions may be advantageous for angiogenesis [25].

In both cell lines, SrR administration induced a considerable rise in alkaline phosphatase (ALP) activity, confirming its impact on osteoblastic development. ALP is a characteristic sign of osteoblastic maturation[26]. Especially in mesenchymal stem cells, SrR has been discovered to have a positive influence on Runx2 expression. It has been demonstrated that SrR can direct aged osteopenic mesenchymal stem cells to the osteoblastogenesis route by upregulating Runx2 expression and matrix mineralization. Inhibiting adipogenic development via decreasing the expression of peroxisome proliferator-activated receptor gamma 2 (PPAR-γ2) in these cells [27].

SUBJECTS AND METHODS

Animal models:

This research used sixty adult male albino rats. The average age of these rats was around 2.5 months old, their weight approximately 250 gm. The procedures of management were carried out at animal house of Cairo University and approved by the local ethical committee.

Experimental design:

The animals in this study were divided randomly into three main groups as the following:

(I) Normal control group (N), 15 animals, which was not received SrR gel.

(II) Diabetic control group (D), 15 animals, which was not received SrR gel.

(III) Normal experimental group (NSR), 15 animals which treated with SrR gel.

(IV) Diabetic experimental group (DSR), 15 animals which treated with SrR gel.

Induction of diabetes:

For groups (D) and (DSR), Diabetes was induced by a single intraperitoneal injection of 120mg/kg monohydrated alloxan (Sigma, St. Louis, MO) dissolved in sterile 0.9% saline. Before alloxan administration, rats were made to fast. The animals were given a 10% glucose solution after 12 hours to avoid hypoglycemia. After seven days, blood samples were drawn from the caudal vein of the animals to determine plasma glucose. Animals with hyperglycemia levels above 250 mg/dL were designated diabetic and utilized in this investigation[28].

Preparation of SrR gel:

In the pharmaceutical and industrial pharmacy department, Faculty of pharmacy, Boys, Cairo, Al-Azhar University, SrR gel was made as follows: - A 4.0% (w/v) methylcellulose (4,000 cps) gel (Sigma chemicals Co., St. Louis, MO) was initially made as the vehicle for SrR by adding the needed quantity of polymer to warm distilled water and allowing it to cool to gel at room temperature. Then 2.5 mg of SrR, (Sigma chemicals Co., St. Louis, MO), was dissolved in 1 ml of methylcellulose. The SrR gel was loaded into plastic insulin syringe until we can load it into the defects of the experimental groups[29].

Surgical procedures:

The animals were weighted, premedicated with atropine (IM 0.04mg/kg) and anesthetized intramuscularly with a mixture of 2% xylazine in a dosage of 5mg/kg (ADWIA, Egypt) and 50mg/kg ketamine (ROTEXMEDICA, Germany). The tibia's covering skin was scraped and cleaned with iodate alcohol. On the medial aspect of the tibia, a 2cm incision was created, and the epidermis, subcutaneous tissue, and muscle layer were dissected to expose the tibia bone. A circular 5-mm diameter flaw was generated using a carbide rose head surgical bur installed on a dental hand piece coupled to a micro-motor with a speed of 2,000 rpm. A metal template with a 5-mm diameter circular cavity was utilized to regulate the defect's location and size. To prevent bone scorching and preserve the viability of bone cells surrounding the defect, the procedure of creating the deficiency was performed under abundant saline irrigation. In group (N) and group (D) the defect was not received SrR, while in groups (NSR) and (DSR) SrR gel was placed in the defect, after repositioning the flap, the muscle layer was sutured with resorbable (#4.0) catgut and the skin was stitched back using interrupted (#4.0) silk sutures.

Postoperative care:

It was necessary to administer postoperative drugs and conduct frequent examinations for evidence of inflammation. Rats having a blood glucose level of less than 250 mg/dl were omitted from the study by periodic monitoring of blood glucose levels. Moreover, any excluded rat was replaced by another one to maintain the total number of the diabetic rats until the day of scarification which determined for each rat.

Scarification of Animals:

Five animals out of each group were slaughtered 5, 10, and 30 days, respectively, following surgery. The specimens were prepared for histological examination to study the bone regeneration in each group histologically by Hematoxylin and eosin stain (H&E) and by Masson's trichrome stain for collagen evaluation.

RESULTS

The following results were obtained after histological examinations of the groups at the several intervals.

Five days interval:

1- **Normal control group (N5):** was shown that the core of the defect was filled with fragments of blood clot that had been invaded by inflammatory cells, fibroblasts, and BV. At the defect's sides and base there were organized granulation tissues underneath the blood clot which characterized by more condensed and tightly packed collagen bundles. Figure (1- A)

   The collagen evaluation at 5 days interval of this group revealed an evidence of tightly packed collagen bundles formation indicated by the green colour at the defect's sides and base. Figure (1- E)

2- **Diabetic control group (D5):** indicated that the center of the defect was filled with blood clot remnants that had been invaded by inflammatory cells, fibroblasts, and BV. At the defect's sides and base there was early formation of granulation tissue which were seen as few collagen bundles arranged and tightly packed together. Figure (1- B)

   The collagen evaluation at 5 days interval of this group revealed early formation of dispersed collagen fibers indicated by light green colour at the defect's sides and base. Figure (1- F)

3- **Normal experimental group treated with SR gel (NSR5):** demonstrated that the center of the defect was filled with remnants of blood clot which stabilized by a fibrin network. Inflammatory cells, fibroblasts, and BV were found within the blood clot. Underneath the blood clot there were well organized granulation tissues which characterized by more condensed, tightly packed collagen bundles, which is interlaced with fibroblasts and blood capillaries that are growing. There was recruitment of osteoblasts were observed within the granulation tissue on the top of the newly formed bone to creep on the preformed mature collagen bundles. At the defect's sides and base there were new woven bone trabeculae formations with irregular diameter. The bone trabeculae were lined by osteoblasts and osteoprogenitor cells. There were osteoblasts imprisoned within the bone matrix of the newly formed bone trabeculae forming osteocytes with wide osteocytic spaces. There were wide sinusoidal spaces filled with haemopiotic tissue and vascular buds in between the newly formed bone trabeculae. Figure (1- C)

   The collagen evaluation at 5 days interval of this group showed tightly packed collagen bundles indicated by the green colour and newly formed bone matrix indicated by deep green colour with evidence of few areas of mineralization indicated by red colour. Figure (1- G)

4- **Diabetic experimental group treated with SR gel (DSR5):** demonstrated that the center of the defect was filled with remnant blood clot which still replaced by formation of granulation tissue. The clot was stabilized by a fibrin network and infiltrated by inflammatory cells, fibroblasts, and BV. At the defect's sides and base there were well organized granulation tissues which characterized by more condensed, tightly packed collagen bundles which interspersed by fibroblasts and notable proliferating blood capillaries. Fig. (D)

   The collagen evaluation at 5 days interval of this group revealed heavy formation of mature tightly packed collagen bundles indicated by the green colour at the sides and base of the defect. Fig. (H)

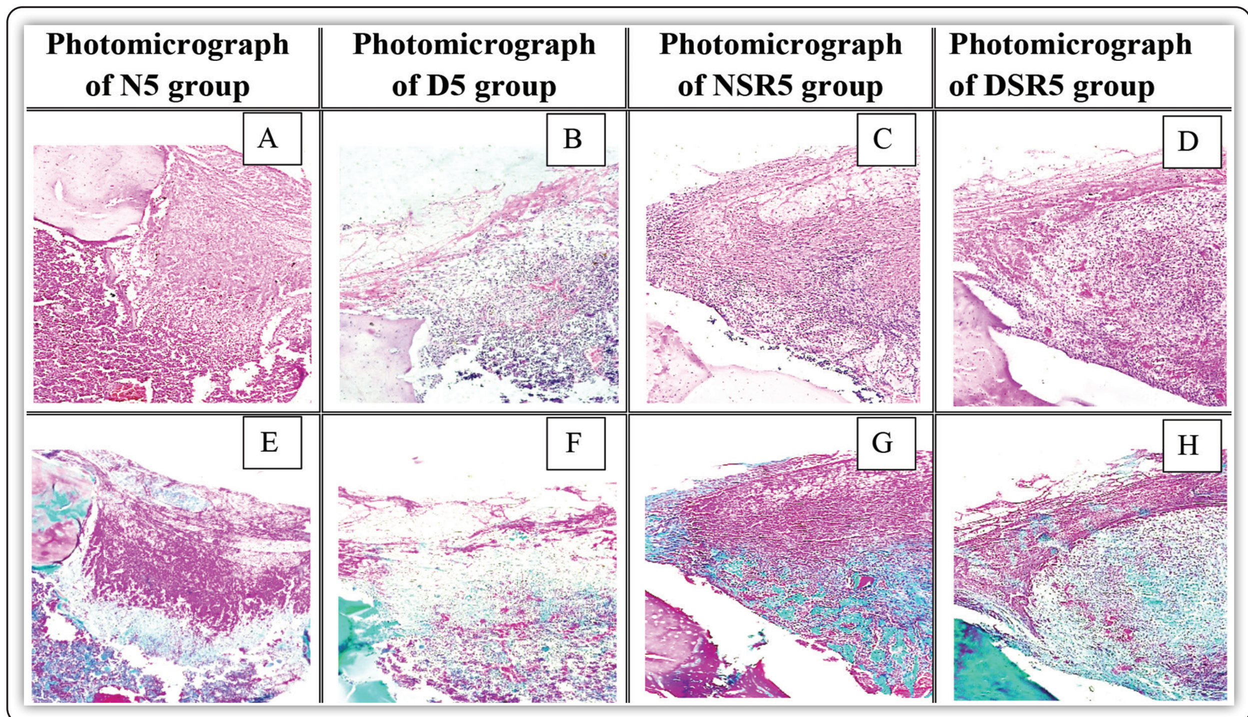

FIG (1) Showing comparison between groups at 5 days interval. **A, B, C** and **D** (H&E stain X100), while **E, F, G and H** (Masson's trichrome stain X100)

Ten days interval:

1- **Normal control group (N10):** demonstrated remnants of the blood clot in the center of the defect which still replaced by granulation tissue. Also, the granulation tissues demonstrate recruitment of osteoblasts along the surface of the newly formed bone. At the defect's sides and base there was new woven bone formation as bone trabeculae of variable sizes and shapes. The bone trabeculae were dispersed away from each other and lined by osteoprogenitor cells and osteoblasts. There were osteocytes with wide osteocytic spaces were imprisoned within the newly formed bone trabeculae. In some areas the bone trabeculae showed slight features of coalescence and harboring wide marrow cavities in between them which filled with haemopiotic tissue. Figure (2- A)

   The collagen evaluation at 10 days interval of this group showed tightly packed collagen bundles characterized by the green colour and newly formed irregular bone trabeculae in different degree of formation and mineralization ranging from green to red colour. Figure (2- E)

2- **Diabetic control group (D10):** demonstrated remnant of the blood clot in the center of the defect which still replaced by granulation tissue. At the defect's sides and base there was early formation of new woven bone which formed of thin irregular bone trabeculae which lined by osteoblasts and osteoprogenitor cells. These trabeculae were dispersed away from each other and contain large osteocytes with wide osteocytic space. There was early formation of very wide irregular marrow cavities filled with heamopiotc tissue in between the newly formed bone. Figure (2- B)

   The collagen evaluation at 10 days interval of this group showed tightly packed collagen bundles indicated by the green colour and

newly formed bone matrix indicated by deep green colour with evidence of few areas of mineralization indicated by red colour. Figure (2- F)

3- **Normal experimental group treated with SR gel (NSR10):** demonstrated at the center of the defect thin rim of typically organized granulation tissues. Moreover, the granulation tissues were over hanging the edge of the defect and demonstrated recruitment of osteoblasts along the surface of the newly formed bone. At the defect's sides and base there was formation of new bone trabeculae of variable thickness which were lined by osteoblasts and osteoprogenitor cells. Also, there were osteocytes imprisoned within the trabeculae with wide osteocytic space. The bone trabeculae were coalescent and anastomosed with each other in most of the defect forming trabecular network as initial stage of spongy bone formation. There were multiple irregular marrow cavities with different diameters in between the newly formed bone trabeculae which filled with hemopoietic tissue and lined by osteogenic cells and osteoblasts. Figure (2- C)

The collagen evaluation at 10 days interval of this group showed tightly packed collagen bundles characterized by the green colour and newly formed trabecular network of bone in different degree of formation and mineralization ranging from green to red colour. Figure (2- G)

4- **Diabetic experimental group treated with SR gel (DSR10):** showed at the center of the defect there was thin rim of typically organized granulation tissues. Also, the granulation tissues

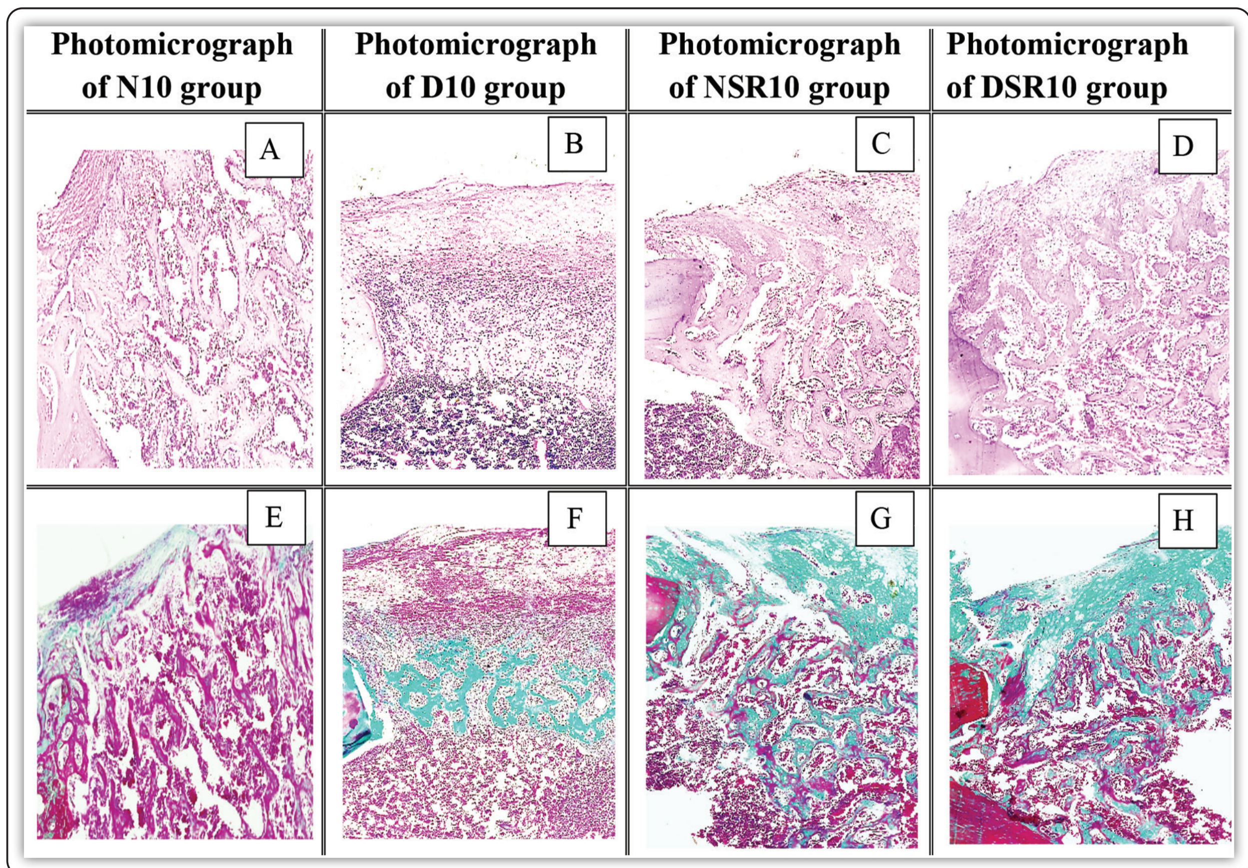

FIG (2) Showing comparison between groups at 10 days interval . **A, B, C** and **D** (H&E stain X100), while **E, F, G and H** (Masson's trichrome stain X100)

were over hanging the edge of the defect and there was recruitment of osteoblasts. At the base and sides of the defect there was new woven bone formation as bone trabeculae of variable sizes and shapes. The bone trabeculae were dispersed away from each other and lined by osteoprogenitor cells and osteoblasts. There were osteocytes with wide osteocytic spaces were imprisoned within the newly formed bone trabeculae. In some areas the bone trabeculae showed slight features of coalescence and harboring wide marrow cavities in between them which filled with haemopoietic tissue. Figure (2- D)

The collagen evaluation at 10 days interval of this group showed tightly packed collagen bundles characterized by the green colour and newly formed bone trabeculae in different degree of formation and mineralization ranging from green to red colour. Figure (2- H)

Thirty days interval:

1- **Normal control group (N30):** demonstrated that at the surface of the defect thin rim of typical organized granulation tissue. Also, there was recruitment of osteoblasts on the top of the newly formed bone to creep on the preformed mature collagen bundles. At the center of the defect there was new bone which forms primary closure of the defect. The newly formed bone appeared as thick bone trabeculae which increased in number and size. These trabeculae were coalescent and anastomosed with each other forming bone islands and trabecular network and were connected to the old bone. The bone trabeculae were harboring marrow cavities in between them with various size and shapes, however many of them become narrower. These marrow cavities filled with haemopoietic tissue and lined by osteogenic cells and osteoblasts. There were numerous osteocytes with wide osteocytic spaces imprisoned in an irregular way within these bone trabeculae. Figure (3- A)

The collagen evaluation at 30 days interval of this group showed the granulation tissue at the surface of the defect which revealed maturation through tightly packed collagen bundles and collagen matrix indicated by the green color. There were numerous bony spicules appeared in the preformed collagen indicated by red colour. The trabecular network of newly formed bone at the base and sides of the defect showed mineralization which indicated by red colour. Despite the center of each trabeculae have the red colour, the periphery of the same trabeculae has the green colour indicating continual increase in thickness of the trabeculae. Figure (3-E)

2- **Diabetic control group (D30):** demonstrated that at surface of the defect there was new bone formation which characterized by thin irregular trabeculae and were connected to the old bone. Moreover, there was recruitment of osteoblasts on the top of the newly formed bone trabeculae. The bone trabeculae were anastomosed with each other and harboring wide irregular marrow haemopoietic cavities in between them which filled with hemopoietic tissue. At the center of the defect there were thin irregular trabeculae of new bone which were dispersed away from each other. The freshly produced bone trabeculae were bordered by osteogenic cells and osteoblasts. There were numerous osteocytes with wide osteocytic spaces imprisoned in an irregular way within these bone trabeculae. Figure (3- B)

The collagen evaluation at 30 days interval of this group showed the newly formed bone trabeculae at the surface of the defect indicated by deep green colour with areas of mineralization indicated by red colour. At the center of the defect there were some bone trabeculae were scattered while those at the defect sides were fused with the old bone, however both showed green and red colours. Figure (3- F)

3- **Normal experimental group treated with SR gel (NSR30):** showed at the surface of the defect a thin rim of typical organized granulation tissue. Also, there were numerous osteoblasts aligned on the top of the newly formed bone trabeculae crowded to creep on the preformed mature collagen bundles. The defect was closed at its center by new bone which characterized by thick trabeculae. These trabeculae were coalescent and anastomosed with each other forming bone islands and trabecular network and were connected to the old bone. The bone trabeculae were harboring multiple marrow cavities in between them which filled with hemopoietic tissue and lined by osteogenic cells and osteoblasts. Also, there were numerous osteocytes dispersed in an irregular way with narrow osteocytic spaces. These marrow cavities become narrower in most of the defect as initial stage of osteon formation. Figure (3- C)

The collagen evaluation at 30 days interval of this group showed tightly packed collagen bundles on the surface of the defect indicated by the green colour. There were numerous bony spicules appeared in the preformed collagen indicated by red colour as evidence of rapid rate of mineralization. At the defect's sides and base the newly formed bone trabeculae were fully mineralized which indicated by the red color. The newly formed bone trabeculae showed a uniform colour pattern throughout the defect with exception of the central part at the side of the defect which showed a bias to the green colour which attributed to mature collagen not yet mineralized indicating continual increase in thickness of the trabeculae. Figure (3- G)

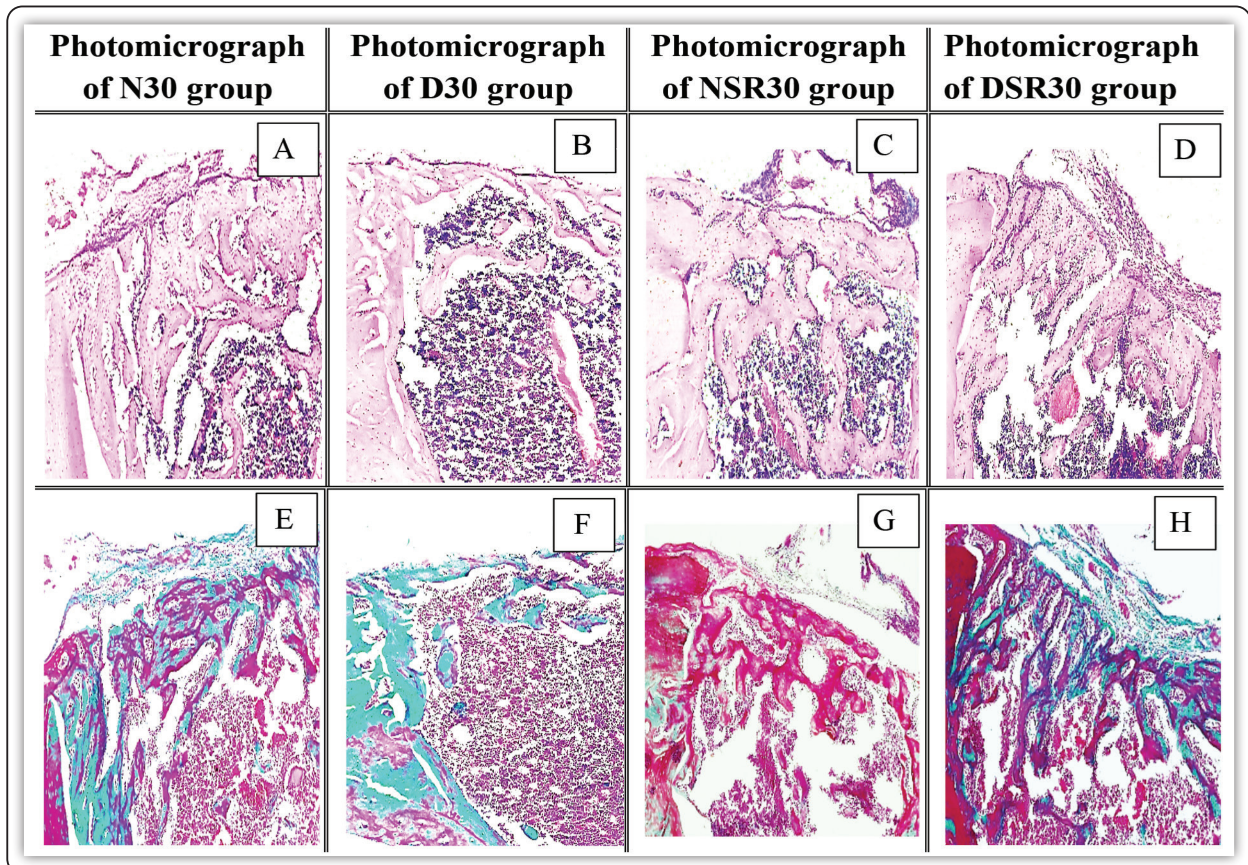

FIG (3) Showing comparison between groups at 30 days interval. **A, B, C** and **D** (H&E stain X100), while **E, F, G and H** (Masson's trichrome stain X100)

4- **Diabetic experimental group treated with SR gel (DSR30):** showed at the surface of the defect there were thin rim of typical organized granulation tissues with recruitment of osteoblasts on the top of the newly formed. At the center of the defect there was new bone formation which appeared as irregular bone trabeculae of different thickness which coalescent and anastomosed with each other forming trabecular network and were connected to the old bone. The bone trabeculae were harboring marrow cavities in between them with various size and shapes, however many of them become narrower. These marrow cavities filled with hemopoietic tissue and lined by osteogenic cells and osteoblasts. There were numerous large osteocytes with wide osteocytic spaces imprisoned in an irregular way within the new bone trabeculae. Figure (3- D)

The collagen evaluation at 30 days interval showed tightly packed collagen bundles on the surface of the defect indicated by the green colour. There were bony spicules appeared in the preformed collagen indicated by red colour as evidence of mineralization. At the base and side of the defect there were trabecular network of the newly formed bone in different degree of formation and mineralization ranging from green to red colour indicating continual increase in the thickness of the trabeculae. Figure (3- H)

DISCUSSION

Diabetes is among the most significant illnesses of sugar metabolism that has a significant impact on bone repair. Diabetes is linked to bone resorption, inadequate osseous repair, and decreased bone regeneration[(30,31)]. Diabetes inhibits bone production, decreases its mechanical strength, and promotes bone architectural degeneration [(9, 10, 11)]. In addition, hyperglycemia has negative effects on bone matrix and its constituents, as well as on the adhesion, proliferation, and accumulation of extracellular matrix [(12)]. Accordingly, it is necessary to establish a new treatment strategy for bone repair in diabetic patients.

A viable alternative to growth factors might be the local injection of a systemic medication that modulates bone and is routinely used to treat bone disease. Local medication delivery has the benefit of releasing the antibiotic locally at the point of infection for a sufficient time without systemic exposure, hence minimizing bacterial resistance and drug-related systemic adverse effects [(13)]. There are several medicines that may be administered systematically to stimulate bone metabolism. In recent years, researchers have paid particular attention to strontium ranelate (SrR), one of these medications [(14, 15)].

Current osteoporosis treatment SrR looks to be a potential option because of its ability to stimulate osteoblast activity and suppress osteoclast activation, hence promoting bone production [(16,17)]. Several in vitro investigations have indicated that SrR promotes osteoblast proliferation, differentiation, survival, and function, while also triggering apoptosis and decreasing osteoclast formation and activities [(18, 19)].

In the present study Alloxan was used for induction of diabetes and this was in agreement with De Amorim FP et al. 2008 [(32)] who revealed that alloxan increases the selective death of pancreatic β-cells, resulting in reduced insulin production and an increase in blood glucose levels in mice.

In our study rats were used as animal models and this was coincidental with Gomes PS and Fernandes MH 2011 [(33)] who stated that small and simple to manipulate, rats are one of the most often used animal models, considered effective in preclinical research for evaluating biomaterials as bone replacements and one of the model types of choice for in vivo testing of bone tissue regeneration.

In this work, the CSD model was utilized since it was a highly practical model for examining bone

regeneration effects. These was parallel to Choi et al. 2010 [34] who demonstrated that the CSD model is reasonably accessible, simple, and repeatable since errors do not spontaneously cure. In addition, it has been reported to be efficient for assessing the capacity for bone regeneration. Finally, the compressive force of this model is comparable to intraoral circumstances.

SrR was used topically not systemically in this study to obtain its advantages and to avoid its side effects which results from its systemic use. These was parallel to Puri K and Puri N 2013 **[13]** who stated that a viable alternative to growth factors might be the local injection of a systemic medication that modulates bone and is routinely used to treat bone disease. Local medication delivery has the benefit of releasing the antibiotic locally at the infection location for a sufficient time without systemic exposure, hence minimizing bacterial resistance and drug-related systemic adverse effects.

There were significant histochemical and histological differences between the four research groups at various study intervals. The histology and histochemical findings demonstrated that granulation tissue production, organization, new bone creation, and mineralization were significantly slowed down in the diabetic control group was very weak along the different intervals of the study, while in diabetic group treated with SrR gel, throughout the study's intervals, bone regeneration grew significantly. However, the bone regeneration was better in normal group treated with SrR gel than all other groups.

This delay in the recovery of diabetic groups was owing to the negative impact of diabetes. These results were parallel to findings of other studies which postulated that diabetes affects bone mineralization and osteoblastic activity. Additionally, micro vascular problems and diminished blood flow enhance bone fragility [5, 6]. Additionally, these finding were in agreement with others who reported that diabetes reduces early callus cell proliferation and collagen production and content [7, 8]. Moreover, our findings were coincidental with the results of other study which proposed that several mechanisms, including diminished angiogenic responses, growth factor synthesis, collagen buildup, and alterations in mineral metabolism, contribute to wound healing deficits in diabetic patients and affect bone healing due to diabetes [35].

On the other hand, granulation tissue growth, organization, and bone formation were significantly accelerated in groups treated with SrR gel due to the beneficial effects of SrR on bone regeneration. These findings were coordinated by **Bonnelye E., et al. 2008[18]** and **Caudrillier A., et al. 2010[19]** who stated that SrR stimulated osteoblast proliferation, differentiation, survivability, and function, while causing apoptosis and inhibiting osteoclast development and activity. Moreover, our findings agreed with **Zhang Y. et al. 2006 [36]** who demonstrated that SrR stimulate pre-osteoblast proliferation, osteoblast development, collagen type I production, and bone matrix mineralization.

In as much as, our results were parallel to **Ammann P, et al. 2004[37]** who observed that in rats treated with SrR, there was an increase in trabecular thickness and quantity, as well as a decrease in trabecular separation in the axial and appendicular skeleton. Furthermore, in a previous study [22] on Sr-based bone repair materials, the release of Sr2+ ions were shown to stimulate the utterance of proangiogenic factors including basic fibroblast growth factor (bFGF), vascular endothelial growth factor (VEGF), and matrix metalloproteinase-2 (MMP-2) in tissue-engineered bone.

CONCLUSIONS

1. The topical application of SrR gel accelerates bone formation and mineralization in diabetic rats.

2. The local application of SrR is a safe and cost-effective method to stimulate bone formation in case of diabetes.

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