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The Effect of Systemic Delivery of Aminoguanidine versus Doxycycline on the Resorptive Phase of Alveolar Bone Following modified Widman Flap in Diabetic Rats: A Histopathological and Scanning Electron Microscope (SEM) study

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Abstract:

Objectives: Aminoguanidine (guanylhydrazinehydrochloride) is a drug that prevents many of the classical systemic complications of diabetes including diabetic osteopenia through its inhibitory activity on the accumulation of advanced glycation end –products (AGEs). The aim of the present study was to evaluate the effectiveness of aminoguanidine versus doxycycline in reducing alveolar bone resorption following mucoperiosteal flap in diabetic rats, using the conventional histopathology and scanning electron microscope (SEM).

Methods: Twenty-seven male albino rats were used in this study. Periodontal defects were induced experimentally on lower anterior teeth. All rats were subjected to induction of diabetes, by IV injection of the pancreatic B-cells toxin alloxan monohydrate. After eight weeks following the establishment of periodontal defects in all rats, the ligation was removed and 3 rats were scarified as negative control (group1). The remaining animals were divided into three group based on treatment applied following mucoperiosteal flap surgery. Group 2receivedsaline treatment only, group 3 received doxycycline periostat (1.5 mg/kg/day) for 3 weeks, and group 4 received aminoguanidine (7.3 mmol/kg) for 3 weeks. The fasting glucose level was measured weekly post operatively. After 21 days all rats were sacrificed. Three anterior parts of the mandible of each group was prepared for histopathologicalexamination and two parts were prepared for SEM.

Results: Aminoguanidine treated group (group4) showed statistically significant increased new bone formation, higher number of osteoblasts and decrease osteoclasts number, resorptive lacunae and existing inflammatory cell infiltration as compared to positive control group (group 2) ($P<0.05$). Doxycycline was also effective in reducing bone loss as documental by histopathological study.

Conclusion: The present study showed that aminoguanidine was significantly effective in reducing alveolar bone loss and can modify the detrimental effects of diabetes in alveolar bone resorption.

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Introduction:

Diabetes mellitus is a heterogeneous group of disorders affecting the metabolism of carbohydrates, lipids and proteins. The characteristic feature of diabetes is an abnormal elevation in blood glucose level (hyperglycemia) that is due to deficiency of insulin secretion by pancreatic B-cells and /or insulin resistance in liver and muscles (American Diabetic Association, 2005). Diabetic complications are related to chronic long-term elevation of blood glucose concentration (chronic hyperglycemia) which results in the formation of advanced – glycation end products (AGEs). [1]

Advanced glycation end-products (AGEs) alter the function of numerous extracellular matrix components and modify matrix-matrix and cell-matrix interactions. These alterations have an adverse effect on target tissues. For example, collagen stability and vascular integrity is reduced as AGEs formation leads to increased cross-linking between collagen molecules. This cross-linking of collagen significantly contributes to reduced solubility and decreases turnover rate. Consistent with these results, diabetic gingival collagen exhibited decreased solubility properties.[2, 3]

The formation of AGEs results in collagen accumulation in the periodontal capillary basement membranes, causing membrane thickening. [4] AGE-stimulated smooth-muscle proliferation increases the thickness of vessel walls, which leads to decrease tissue perfusion and oxygenation.[5] AGE-modified collagen in gingival blood vessel walls binds circulating low-density lipoproteins (LDL), which is frequently elevated in diabetes, resulting in atheromaformation and further narrowing of the vessel lumen. These changes in the periodontioum dramatically alter the tissue response to periodontal pathogens, resulting in increased tissue destruction and diminished repair potential.[6]

Periodontal disease, which is characterized by excessive extracellular matrix (ECM) degradation, has been recognized as one of the complications of diabetes and was found to be more prevalent and markedly severe in diabetic populations compared to non-diabetic. It has been reported as the sixth complication of diabetes along with neuropathy, nephropathy, retinopathy, microvascular and macrovascular diseases.[7]The incidence of periodontal disease is increased by nearly three folds in diabetic patients compared to healthy subjects [8] and patients with diabetes have been reported to be susceptible to more severe periodontitis.[9]

Mucoperiosteal flap is used routinely in many surgical procedures to access bone and root surfaces, for debridement, and during regenerative procedures and implant surgery. The resorptive effects following mucoperiosteal surgical procedures were well documented.[10] In addition, histological studies in human and experimental models have found a significant change in diabetic bone metabolism as represented by increased alveolar bone loss, decreased bone formation, hypercalciuria, and hypomagnesaemia that have also been found to be associated consistently with human diabetes.[8, 11, 12]

Altered collagen synthesis, maturation, homeostasis metabolism, in addition to increased collagenase enzyme production and the development of diabetic osteopenia in diabetic patient demands searches for pharmacologic agents that might prevent the detrimental effects of diabetes on wound healing.[13-15] Efforts have been made to pharmacologically inhibit this increased collagenase activity by using tetracycline or chemically modified tetracyclines (CMTs). CMTs may have potential in lowering collagenase levels in animal models, which may affect bone formation and remodeling process.[16, 17]

Doxycycline, like tetracycline and minocyline, is a broad-spectrum antimicrobial agent. Doxycycline offers advantages over the two other drugs as its absorption from the gastro-intestinal tract is not altered by calcium, metal ions or anti-acids, it has more potent compliance and it is associated with less photo and renal toxicity.[18]

It was found that 20 mg bid doxycycline yields serum concentration of 0.6 to 0.8 mg/ml. this level is considerably below the minimal inhibitory concentration determined for the majority of the bacteria isolated from the sub-gingival flora. Therefore, this dose termed sub-antimicrobial dose doxycycline (SDD) is marketed as Periostat.[17, 18]

Several short-term clinical studies and a recent long-term multi-centered clinical study indicated that the adjunctive use of sub-anti

microbialdose doxycycline (Periostat) provided a significant benefit to scaling and root planning due to its anticollagenase and anti-inflammatory activities rather than to its antimicrobial activity.[19, 20] Long term multi-centered study of have proved the efficiency of using Periostat in decreasing collagenase activity which was accompanied by a beneficial and significant improvement in attachment level, probing depth, and bone level.[20]

Currently researchers have shown interest in a new drug that prevents many of the classical systemic complications of diabetes mellitus, including cardiovascular and renal pathology. Aminoguanidine (AG) is a neutrophilic hydrazine, a prototype a,b-dicarbony1 scavenging agent that has been studied as one of the most promising compounds for the treatment of diabetic complications, because the compound has both advanced-glycation inhibitory activity and antioxidant activity.[21]

Aminoguanidine (AG) has been shown to be effective in preventing hyperglycemia induced tissue damages in experimental animal models and in diabetic patients.[22, 23] Because of its chemical reactivity, aminoguanidine blocks the vicious cycle of reactions initiated by glucose condensation with amino groups of proteins that results in the formation of advanced glycation end products whose levels not only play a pathogenic role in the development of most diabetes complication but can also predict the extent of the renal, ocular and cardio vascular damages occurring in diabetic patients.[24, 25]

Therefore, the present study was designed to evaluate the effectiveness of aminoguanidine versus doxycycline in reducing alveolar bone resorption following mucoperiosteal flaps in diabetic rats, using the conventional histopathology and SEM.

Materials and Methods

The study was carried out on 27 adult, male Albino rats with an average weight 200-250 grams and housed in the same environmental condition.

Periodontal defects were induced experimentally on the lower anterior teeth using an orthodontic elastic ligature. The ligature was changed twice weekly for eight weeks. All animals were subjected to induction of diabetes immediately following the placement of elastic ligature around the teeth by IV injection of the pancreatic B-cells toxin alloxanmonohydrate (Johnson & Johnson, California, USA) in a dose of 50 mg/kg dissolved in saline.[26] Twenty-four hours after alloxan administration, the induction of diabetes was confirmed by measuring the blood sugar level >200mg/dl, and this value were further checked once a week throughout the study period. After eight weeks following, the establishments of periodontal bony defects the ligation was removed. Three rats were scarified and were considered as negative controls **(Group I).** The remaining 24 animals received modified Widman flap surgery and then divided into three groups according to the type of treatment applied. Each group consists of eight animals.

Group 2: injectedvia intraperitonealwithsaline twice a week for 3 weeks.
Group 3: given doxycycline (Periostat)[R*] (1.5 mg/kg/day Periostat suspended in carboxymethylcellulose orally) for 3 weeks [27]
Group 4: treated with modified hydrochloride (guanylhydrazine hydrochloride 7.3 mmol/kg)[**] via intraperitoneal injectiontwice a week for 3 weeks.[28]

Surgical Procedure:

The rats were anaesthetized prior to surgery by using a mixture of 25 mg/kg body weight of Ketalarand 42mg/kg by body weight of xylocaine.

A reverse bevel incision was made 0.5 m from the gingival margin crivecular incision was carried out then a full thickness flap raised and granulation tissue and marginal tissue collar were removed.

The flap was elevated using a small periosteal elevator on both sites of anterior teeth. A notch was made on sites on the root surface at the bottom of the defect. The topography of the intrabony defect was assessed by a graduated periodontal probe after flap reflection. The root were scaled and root planed, The inner surface of flap was trimmed to remove the granulation tissue and

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*** Ketamin HCL, Park-Davis, S.A., Barcelona, Spain.

[**]Sigma- Aldrich corp., st Louis, MO.

remaining dentogingival Junction and the flap was closed using interrupted sutures. Rats were randomly assigned to three different treatment modalities.

All animals of each group were sacrificed 21 days post surgically.

Histopathological evaluation:

After the scarification of animals, three anterior parts of the mandible were removed in each group. One part fixed in 10% neutral phosphate buffered formalin for at least 24 hours, decalcified in 10% ethylene diaminetetraacetic acid (EDTA) solution containing 5% sodium sulfide. The decalcified specimens were then embedded in paraffin following the routine processing; then 4.0 u sections were cut in a mesiodistal plane.Deparaffinized sections were subjected to the conventional technique for staining with hematoxyline and eosin stain.

For the histopathological determination of inflammatory cell infiltration (ICL), scoring was determined as 1 or 0 according to existence or non-respectively and the values obtained for the saline and treated groups were recorded.

Histopathological examination was focused upon evaluation of the interdental alveolar bone and periodontal tissue with the semi-quantitative objective method as follows: Existing inflammatory cell infiltration of the periodontal tissue, fibrous tissue formation, number and morphology of osteoclasts of the alveolar bone and existing resporptive lacunae.[29]

Osteoclast morphology observed normally with their ruffled borders was scored as 1, and osteoclasts that lacked ruffled borders and exhibited more regular cell margins were scored as 2.

Existing resorptive lacunae was defined as regions of bone beneath vacuolated multinucleated cells flattened against trabecular bone. If the surfaces were visible, they were scored as 1; if they were not visible and they were scored as 0.

Scanning electron microscopic study (SEM):

The remaining two anterior parts of each group were fixed in 2.5% glutaraldehyde in phosphate buffer (PH 7.3) for 48 hours and washed twice in the same buffer. The specimens were then dehydrated in the graded series of aqueous ethanol solution 50%, 70%, 90% and 100% ethanol for one hour for each specimen. Then they were air-dried, mounted on aluminum SEM stubs with silver paint and sputter coated with gold using a coater. The specimens were examined by (SEM, JXA-840A, JEOL, Japan) and photograph of in the same magnification.[30]

Laboratory analysis:

The fasting glucose levels were carried for each animal weekly during the experimental period after the surgical procedure.

Data analysis:

The results were recorded, tabulated, and statistically analyzed using statistical package for Social Science (SPSS version 10).

Comparison between the studied groups was performed with student t-test and ANOVA test.

Results

Histopathological evaluation:

In the control non-operated animal (group 1) (negative control) the periodontal tissue showed vasodilatation, marked cellular infiltration (12 ± 3 / cm^2) and irregular bone surface with many resorptive lacunae containing osteoclasts (10 ± 2 / cm^2) with ruffled border formation (Fig. 1, Table 1).

In saline-treated group (group 2) (positive control), there was marked alveolar bone resorption with the presence of a large number of osteoclasts (9 ± 5 / cm^2), many resorptive lacunae with ruffled border. An intense inflammatory cell infiltration was also observed within periodontal tissue as well as the marrow space of alveolar bone (Fig. 1, Table 1).

In the doxycycline treated group (group3), there was a significant increase of the number of osteoblasts (7 ± 2 / cm^2) and decreased number of osteoclasts (4 ± 2 / cm^2) and inflammatory cell infiltration (5 ± 3 / cm^2) compared to positive control group (Fig. 1, Table 1). In addition, there was a layer of newly formed bone with clearly visible line of demarcation between new and old bone (Fig. 1).

In the aminoguanidine treated group (group4), there was a statistically significant

reduction in inflammatory cell number (4±2 / cm^2), osteoclasts (3±2 / cm^2),resorptive lacunae (3±1 / cm^2) compared to control group. There was also significant increased the number of osteoblasts (10±1 / cm^2) compared to control group (Fig. 1, Table 1).

Marked newly formed bone with smooth surface, numerous osteoblasts and lines of demarcation between the new and old bone were noted (Fig. 1). The periodontal ligament showing more arranged collagen bundles and cementablasts.

	Groups	Inflammatory cells M±SD	Osteoblast M±SD	Osteoclast M±SD	Resorptive lacunae M±SD
1	Negative control	12±3/cm^2	3±2/cm^2	10±2/cm^2	8±4/cm^2
2	Positive control	11±4/cm^2	4±1/cm^2	9±1/cm^2	7±5/cm^2
3	Doxycycline	5±3/cm^{2*}	7±2/cm^{2*}	4±2/cm^{2*}	4±2/cm^{2*}
4	Aminoguinidine	4±2/cm^{2*}	10±1/cm^{2*}	3±2/cm^{2*}	3±1/cm^{2*}

* Statistically significant (P < 0.05)

Table 1: Mean (M) and standard deviation (SD) of the number of osteoblasts, osteoclasts, inflammatory cell and resorptive lacunae in test groups versus control groups.

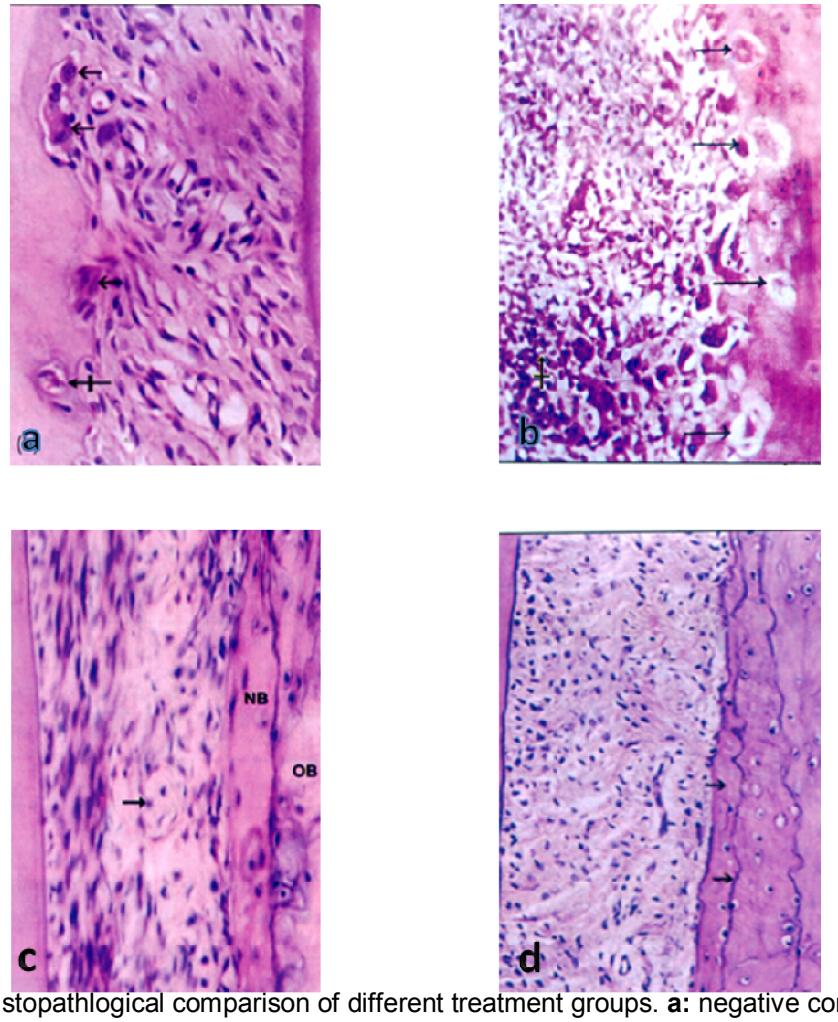

Fig. (1): Histopathlogical comparison of different treatment groups. **a:** negative control **(group 1)** showing irregular bone surface and resorptive lacunae (arrows), rounded osteoclasts with ruffled

border (cross arrow) and vasodilation. **b**: positive control **(group 2)** showing alveolar bone surface is markedly irregular and many resorptive lacunae housing osteoclasts (arrows) and intense inflammatory cell infiltration. **c**: doxycycline group **(group 3)** showing nearly healthy periodontal ligament and mild inflammatory cell infiltrate (arrow). Note the line demarcating newly formed bone (NB) and old bone (OB). **d**: aminoguanidine group**(group 4)**showing healthy periodontal ligament and smooth surface of newly formed bone, Note the line demarcating newly formed bone and old bone(arrow). (H&E×400)

Blood sugar analysis:

Table 2 showed the blood sugar level in the different treatment modalities at baseline 1, 2 and 3 weeks post operatively. The negative and positive control groups (group 1 & 2) showed the highest results as blood sugar level increase by time.

At base line there was no significant difference between all groups. At the end of the treatments there was a significant difference when comparing each group to its baseline. On the other hand, there was a statistically significant difference between aminoguanidine group (group 4) comparing to doxycycline (group 3) versus control groups (group 1&2) ($P<0.05$).

	Groups	Blood sugar level			
		M±SD	M±SD	M±SD	M±SD
		Baseline	1 week	2 week	3 week
1	Negative control	319±17	387±19	410±15	430±25
2	Positive control	320±15	385±25	405±22	420±15
3	Doxycycline	319±18	291±20	285±23	280±19
4	Aminoguanidine	318±20	277±19	266±15	254±15*

*Statistically significant ($P < 0.05$)

Table 2: Mean (M) and standard deviation (SD) of the blood sugar level at baseline first second and third week for different treatment modalities.

Results of SEM

The negative control (group1) specimens exhibited numerous pores and multiple areas of bone destruction (Fig. 2 a.). The phenomenon of these pores documented the type of osteoporosis related to diabetic specimens. The positive control specimens (group2) revealed narrowing of some holes while others were still patent (Fig. 2 b.).

The specimens treated with doxycycline group (group3) represented granulated surface that cover the defects but without closing some of the bony defects (Fig. 2 c.).

Consequently, the best results were indicated with specimens treated with aminoguanidine (group 4) which illustrated smooth surface, it was similar to that of the normal bone (Fig. 2 d.).

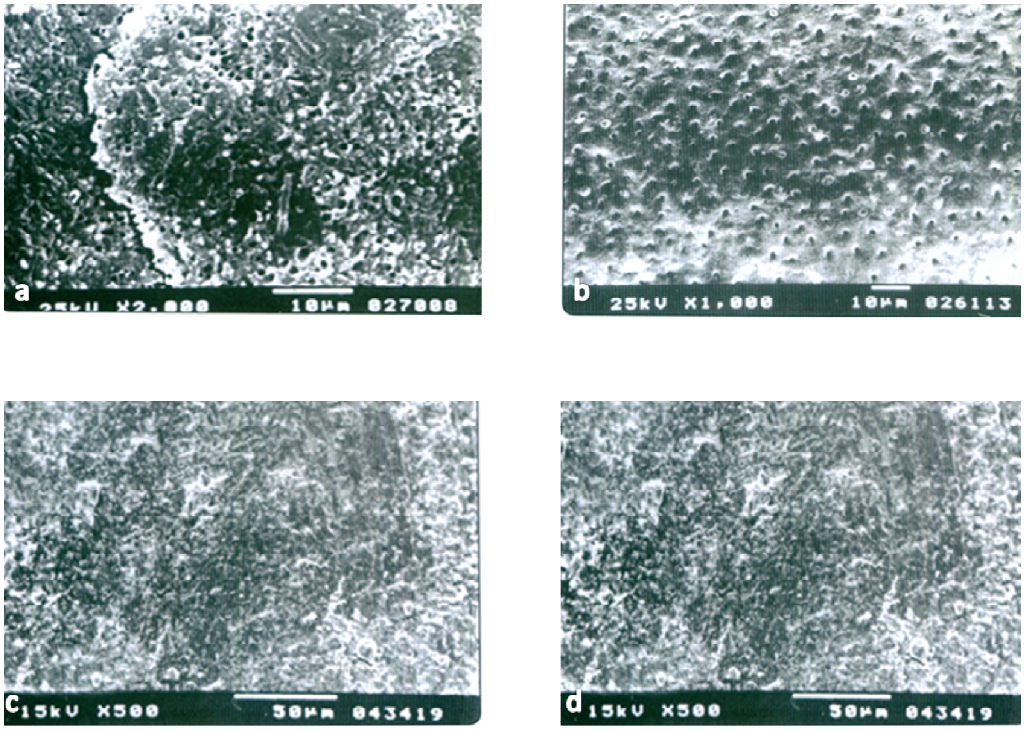

Fig. (2): SEM. **a:** negative control (group 1) showing destruction of bone surface as different diameter pores and fractured parts of the surface (Mic Mag× 2.000). **b**: positive control (group 2) showing narrowing of some pores while others still patent(Mic Mag× 1.000). **c:** doxycycline group (group 3) showing incomplete closure of some pores and granulated bone surface(Mic Mag× 500). **d**: aminoguanidine group (group 4) showing closure of all bony defects and fine granulated surface that resembles normal bone surface (Mic Mag× 500)

Discussion

Evidence supports the bidirectional relationship between diabetes and a periodontal disease, that diabetes is associated with increased occurrence and progression of periodontitis and periodontal infection is associated with poorer glycemic control in people with diabetes.[8] While treating periodontal infection in people with diabetes is important in maintaining oral health, it may also have an important role in establishing and maintaining glycemic control.[31]

One of the complications of diabetes is the development of either osteopenia or osteoporosis. These phenomena due to suppression of osteoblastic activity and induction of osteoclastic differentiation leading to increase bone resorpation and inhibition of bone formation. [11, 32] Diabetic osteoporosis impaired the production of mineral density which leads to rapid resorption of alveolar bone.[33, 34]

Surgical procedure is a mode of treatment of chronic periodontitis in diabetic or non diabetic patients to stop the subgingival infection and compensate for the bone destruction. However, increased tooth mobility immediately following periodontal surgery is considered one of the side effects to the surgical therapy.[10] This was attributed to increased osteoclastic activity and alveolar bone resorpation.[35]

Animal models using either streptozotocin or alloxan to induce diabetes have been used extensively to evaluate the wound healing process after mucoperiosteal flaps.[11, 12, 36] Bone formation, mineralization and resorpation was measured over a l0-day period using double-tetracyline labeling of bone in control, untreated diabetic and insuline – treated diabetic rats. Results showed reduction of bone formation and osteoid volume early in the course of diabetes.[37] Studies that evaluated bone morphology and function in diabetic rats after 3 to 4 weeks of diabetes found that number and function of osteoblasts areseverely suppressed in diabetic rats, resulting in decreased osteoid surfaces, mineral apposition rate, and plasma osteocalcin levels.[38]

In the present study, the histopathological evaluation of positive control group revealed intense inflammatory cell infiltration, irregularity of bone surface with many resorptive lacunae containing osteoclasts and minimum amount of new bone formation. The histopathological findings documented the presence of still bony holes on the surface of SEM observation. This was in agreement with the results of other studies were increased alveolar bone loss; decreased bone formation in non-treated diabetic rats has been found.[11, 12, 37]

Doxycycline was used in the present study not for its antimicrobial properties, but for its unique ability to reduce degeneration of collagenase matrix by inhibiting the activity of metalloproteinase (MMPs) and its potent inhibitor of osteoclastic function.[20] Doxycycline appeared to prevent the development of the bone deficiency disease without affecting the severity of hyperglycemia and suggests that the tetracyclines might inhibit diabetic osteopenia via mechanisms unrelated to their antimicrobial properties.[16] Yu et al. [39] continued the investigation of tetracyclines, hypothesizing that the diabetic osteopenia may be a result of excess matrix metalloproteinase activity in the connective tissue of diabetic rats.

In the doxycycline treated group (group 3), bone surface showed a few resorptive lacunae, a few osteoclasts and the surface appeared smoother and regular when compared tocontrol group, indicating decreased bone resorping activity. This results was agreement with the results of other studies which showed that tetracycline used in the treatment of periodontal disease have therapeutic effect unrelated to their antimicrobial activity.[16, 20, 39, 40] Studies proposed that tetracycline, even in low dose inhibit MMPs activity and therefore, it was recommended to be used where degradation of connective tissue matrix was marked.

Studies on the classic complication of diabetes are linked to the accumulation of AGEs. Li et al. [41] studied the effect of aminoguanidine on aging related renal and vascular changes and they suggested that early interference with AGE accumulation by aminoguanidine treatment might impart significant protection against the progressive cardiovascular and renal decline afflicting the last decades of life.

Fong et al. [42] investigated the inhibition of matrix induced bone differentiation by AGEs in vitro and found that the addition of aminoguanidine reduced AGE fluorescence to normal and restored bone differentiation. Their results suggest that formation of AGEs on bone matrix inhibits its ability to induce bone formation.

Aminoguanidine treated group (group 4) showed statistically increased new bone formation, more osteoblastcell numbers and decrease osteoclastsnumber, lessresorptivelacunae and existing inflammatory cell infiltration compared to saline treated group (group 2). This result was in agreement with previous results.[28, 42]

In the present study, the histopathological findings appeared to correlate with SEM observation. The histopathological evaluation of positive control group (group 2) revealed minimum amount of new bone formation documented by the presence of still bony holes on the surface of SEM observation. These results were documented by SEM results in which the illustrated in gradual closure of bony holes by calcified granules on bone surfaces until reached as similar as normal appearance.

Comparing the changes of the recorded glucose level assay along the experimental period and the histopathological feature of the different treatment modalities, it was found that the aminoguanidine treatment of diabetic rats results in least resorptive changes following periodontal flap surgery. This might be due to the role of aminoguanidine in preventing AGEs accumulation and subsequently reducing diabetic osteopenia. The results of this study

are in accordance with those reported form Guimaraesetal [(43)] which evaluated the effect of aminoguanidine on preimplant healing in mice with induced diabetes.

Further investigations are required to enhance the understanding of aminoguanidine mechanisms of action and or probable its side effects.

Conclusion:

The periodontal healing process was negatively affected within the model of diabetes induced by alloxan. Aminoguanidine was significantly effective in reducing alveolar bone loss following periodontal flab surgery in comparison to control.

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