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Effect of the parotid salivary gland on calcium and amylase enzyme levels in blood and its influence on bone healing in albino rats (Histological and radiographic study)

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ABSTRACT

The healing potential of bone is influenced by a variety of biochemical, cellular, hormonal and pathological mechanisms. As previous studies stated that parotid salivary glands may have endocrinal role, the aim of this study was to evaluate the effect of parotidectomy on bone healing and on calcium and amylase enzyme levels in blood. The rats were divided into two groups; control and experimental group. The control group was subjected to unilateral surgical mandibular defects, while the experimental group was subjected to the same procedure in addition to bilateral surgical removal of the parotid glands. Each of the control and experimental groups was further subdivided into 3 subgroups, A, B and C according to the time of termination corresponding to 4, 8 and 12 weeks respectively. Blood samples were obtained in order to determine calcium and amylase levels in blood. The surgically defected mandibles of each subgroup were analyzed postoperative to determine the radiographic bone density of the surgical defect throughout the healing process, then processed and examined histologically. Examination of the H & E stained sections of the mandibles at 4 weeks showed minimal bone formation from the defect margin in comparison with the control group. At 8 weeks, the experimental group showed increased bone formation from the defect margin. At 12 weeks, the center of the defect was filled by a considerable amount of spongy bone and a definite reversal line between new and old bone. The Masson trichrome stained sections of the experimental group at 12 weeks presented a considerable amount of green collagen fibers. The average (mean) percentage of radiographic bone densities of the surgical defect of the experimental group slightly raised to 82.06 at 12 weeks. The serum amylase level at 4 weeks was less than the normal value then was slightly increased at 8 weeks and finally at 12 weeks increased more than the normal value. However, the serum calcium level was within the normal value in all experimental and control subgroups. It was concluded that bilateral parotidectomy in albino rats resulted in delayed bone healing and was associated with an initial drop in serum amylase level at 4 weeks, however serum amylase level was self-compensated at 8 and 12 weeks postoperatively, while it didn't significantly influence serum calcium level.

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1. Introduction

Bone is a specialized mineralized connective tissue which makes up the skeletal system together with cartilage. Bone serves three major functions, a mechanical function serving as a support and site for muscle attachment for locomotion, a protective function for vital organs and bone marrow and finally a metabolic function where it acts as a reserve for calcium and phosphate used for maintenance of serum homeostasis and electrolyte balance [20].

Healing of bone restores the tissue to its original physical and mechanical properties. Healing occurs in three distinct but overlapping stages; the inflammatory stage, the repair stage and the late remodeling stage [12].
Bone continuously remodels by coordinated cellular mechanisms to adapt its strength to the changing needs of growth and physical exercise. Old, damaged and unneeded bone is removed by resorption, and new bone is subsequently deposited by formation. Diseases affecting either or both of these processes lead to disturbed calcium homeostasis [19].

Calcium balance refers to the state of the calcium body stores, which are primarily in bone and which are largely a function of dietary intake, intestinal absorption, renal excretion and bone remodeling. Bone calcium balance can be positive, neutral and even negative, depending on a number of factors which include growth, aging, acquired and inherited bone disorders [17].

The salivary glands are generally considered as exocrine glands, which secrete their proteins and fluids externally into a lumen or a duct. However many previous studies stated that parotid salivary glands were found capable of endocrine secretion, dispensing their proteins as amylase directly into the blood stream [10,18].

The glands were found capable of endocrine secretion, dispensing their proteins and calcium, also whether the parotid gland has an impact on bone healing or not.

2. Materials and methods

The experimental procedures were conducted in compliance with ethical principles for animal research as reviewed and approved by institution guidelines of Ain Shams University ethical committee.

Forty two adult male albino rats (weighing about 200–250 gm each) were used in this study. They were recorded in The Research Center, Faculty of pharmacy, Future University. The animals were housed in wire mesh dated cages (three rats each) and were fed certified pelleted diet and tap water ad-libitum. Temperature and humidity conditions were controlled as possible on housing the animals during the experimental period.

2.1. The rats were equally divided into two main groups (21 rats each)

2.1.1. Group I (control)

Twenty one rats were subjected to unilateral surgical mandibular defects on the right side using 3 mm trephine bur under copious amount of saline irrigation.

2.1.2. Group II (experimental)

Twenty one rats were subjected to unilateral mandibular defect as in Group I in addition to bilateral surgical removal of the parotid glands.

- Bilateral removal of the parotid glands was chosen over unilateral removal to avoid any unreliability of the results as the non-removed gland might compensate for the enzyme deficiency.

Each of the control and experimental groups was further subdivided into 3 subgroups, A, B and C according to the time of termination corresponding to 4, 8 and 12 weeks respectively [16].

2.2. Surgical procedure

2.2.1. Animals anesthetization

- Surgical intervention was performed under general anesthesia using intraperitoneal injection of Xylaine 5–10 mg/kg and Ketamine 20–40 mg/kg [21].

- Post operative care: Prophylactic antibiotic coverage and analgesics were given for 3 days post-operative [4].

2.3. Blood samples collection

Before each scarifying time of both groups blood samples were obtained in order to determine: Calcium blood level and Amylase blood level.

Blood samples were also taken from not operated rats to act as a base line (normal) value for obtained data comparison. Samples were centrifuged to extract the serum.

2.4. Specimens collection

At the end of the experimental period of each sub group, the rats were terminated by an overdose anesthesia. The surgically defected mandibles were excised free and were fixed in 10% buffered formaldehyde for 24 h.

2.5. Radiographic evaluation

Radiographs of the rats' mandibles were taken using a standard x-ray machine. A digital radiograph of the surgical defected mandibles was taken using vista scan digital radiographic system of both the control and experimental groups at 4, 8 and 12 weeks post-operative.

2.5.1. Radiodensitometric analysis

The mandibles of each subgroup of the control and experimental groups were analyzed post-operative by the Digora software to determine the radiographic bone density of the surgical defect throughout the healing process. Also a radiographic density of normal bone has been taken to compare each result with the normal bone density of each rat mandible to avoid individual variations.

2.6. Histological examination

After complete decalcification, specimens were processed, infiltrated in paraffin wax and embedded in the center of wax blocks. The embedded specimens were cut into 5 microns thick sections. Specimens were sectioned bucco-lingually to show a wider view of the bony defect and were stained by:

2.6.1. Hematoxyline and Eosin (H&E) stain

After fixation, specimens were washed properly under running water, dehydrated by transferring through ascending concentrations of alcohol. The sections were stained by Hematoxyline and Eosin (H&E) stain to be examined by light microscope.

2.6.2. Masson goldner trichrome special stain

It was used to detect areas of new bone formation as green color while old bone areas without new collagen formation appear redish in color when examined by light microscope [5].

Paraffin sections were fastened to slides with Masson's gelatin.

2.7. Statistical analysis

The resultant data from radiographic examination and blood samples were analyzed statistically. All gathered records were statistically evaluated by Microsoft Office Excel 2007 Statistical Functions to determine the mean (AVERAGE), the standard deviation (STDEV) and p value (T TEST).
3. Histological results

Examination of the H & E stained sections of the mandible of sub group IA (control) showed the experimental cavity partially filled with newly formed woven bone budding from the periphery of the surgical defect. An apparent line of demarcation between new and old bone was obviously detected. Resting lines were also observed, straight and gently undulated between successive layers of bone.

The newly formed woven bone exhibited irregular bone trabeculae containing numerous irregularly arranged entrapped large sized osteocytes with intervening wide marrow cavities. Few inflammatory cells and numerous eosinophilic giant cells were detected. Numerous active basophilic stained fibroblasts were obviously seen in both marrow cavities as well as in the connective tissue of the defect cavity. On the other hand, sub group IIA showed minimal bone formation from the defect margin. The newly deposited bone trabeculae were apparently reduced in both thickness and in comparison with subgroup IA. Numerous dilated blood vessels congested with red blood cells (RBCs) and inflammatory cells infiltrations, were encountered in marrow spaces as well as the connective tissue (Figs. 1 and 2).

In sub group IIB, the H & E stained sections of the experimental defects showed increase of the bone formation from the defect margin. The newly formed woven bone exhibited irregular bone trabeculae with wide marrow cavities. The blood vessels appeared normal with no congestion and few inflammatory cells were sometimes encountered. Scattered Osteoblasts lining the bone trabeculae and the marrow cavities were detected. Osteocytes appeared relatively large in size with darkly stained nuclei and surrounded by lacunae of variable size and shape (Fig. 3).

In sub group IIC, the H & E stained sections presented a considerable amount of spongy bone with relatively small marrow spaces filling a considerable part of the bony defect. A definite reversal line between new and old bone was obvious. The most peripheral part of the bony defect presented an almost uniform definite band of lamellar bone with frequent resting lines. The marrow cavities appeared narrow and lined by osteoblasts (Fig. 4).

The Masson trichrome stained sections subgroup IIA showed few newly formed collagen fibers bundles lining the defect wall, while the rest of the defect was apparently filled with numerous collagen fiber bundles. In subgroup IIB, the amount of newly formed collagen fibers increased, moreover, most of the marrow spaces showed new collagen fibers. Finally in subgroup IIC, the masson trichrome stained sections presented a considerable amount of green collagen fibers while the old collagen and the new mineralized bone appeared red (Figs. 5–8).

4. Radiographic statistical results

The average (mean) percentage of radiographic bone densities of the surgical defect of the control subgroups was found to be 56.32% of the normal bone at 4 weeks, which increased to 81.79% at 8 weeks and continued to increase in density till reaching 93.10% of that of the normal bone at 12 weeks. The experimental subgroups average (mean) percentage of radiographic bone densities of the surgical defect at 4 weeks was found to be 48.38% of that of the normal bone, increased to 80.27% at 8 weeks and then slightly raised to 82.06% at 12 weeks. By comparing the results of the surgical sites bone densities of the control and experimental groups statistically; the p value was equal to 0.054 at 4 weeks, at 8 weeks was equal to 0.62 and at 12 weeks was equal to 0.05 (Significance level p ≤ 0.05) (Chart 1).

5. Hematology statistical results

The results of serum amylase laboratory tests of the control group showed a mean serum amylase level of 640 ± 13.7 U/L at 4 weeks, where at 8 weeks it was 698 ± 24.2 U/L, while at 12 weeks post-operative the mean serum amylase level was 765 ± 28 U/L. While for the serum amylase level for the experimental group the 4 week interval showed a mean of 571.5 ± 30.5 U/L, a mean of 666 ± 20.1 U/L for the 8 weeks interval and a 12 weeks mean of serum amylase of 804.5 ± 13.4 U/L. Statistical evaluation of the results was performed comparing the results of the control and experimental groups at each interval by the p value (with significance mark of p value ≤ 0.05). The calculated p value comparing the results of the control and experimental groups at 4 weeks was 0.173, 8 weeks p value of 0.151 and 12 weeks p value of 0.13.

The results of serum calcium laboratory tests for the control
group showed a mean serum calcium level of 10.35 ± 0.21 mg/dl at 4 weeks, where at 8 weeks it was 11.2 ± 0.2 mg/dl, while at 12 weeks post-operative the mean serum calcium level of the control group was 11.6 ± 0.21 mg/dl. While for the serum calcium level for the experimental group the 4 week interval showed a mean of 9.9 ± 0.13 mg/dl, a mean of 11.8 ± 0.2 mg/dl for the 8 weeks interval and a 12 weeks mean of serum calcium of 11.9 ± 0.12 mg/dl. The calculated p value comparing the results of the control and experimental groups at 4 weeks was 0.061, 8 weeks p value of 0.014 and 12 weeks p value of 0.142 (Chart 2 and 3).

6. Discussion

Histological results of the present study showed that at 4 weeks postoperative the experimental group (sub group IIA), presented few relatively thin bone trabeculae and wide marrow cavities when compared to its control counterpart (sub group IA). Such features reflect a slight delay in bone healing of the parotidectomized rats in this duration. This was emphasized by the absence of a continuous...
layer of osteoblasts in sub group II A in contrast to its presence in sub group IA specimens.

On the other hand sub group II A presented apparently numerous fibroblasts, such finding might reflect altered osteoblastic differentiation. This was explained by a previous study which stated that fibroblasts and osteoblasts have the same origin and can change into one another depending on stimulation or on the surrounding micro environment [3]. The irregular bone trabeculae with numerous entrapped osteocytes with large lacunae were evident in both groups regardless to the size and the extension of the newly deposited bone. It was stated that the number of osteocytes depends on the rapidity of bone formation, so they increase in woven bone and decrease in lamellar bone. Osteocytes present in large lacunae during the early stages of their transformation from active osteoblasts to osteocytes as they have a well developed golgi apparatus for collagen storage [14].

A previous study reported mild inflammatory reaction at 4 weeks postoperatively and there was a decrease in the inflammatory cells existence [2]. This comes in accordance with the control group IA of the present study where few inflammatory cells and gaint cells were sometimes detected. The presence of multi nucleated giant cells or macrophages were noticed in the injury site, as they are responsible for phagocytosis of debris and dead cell remnants [1]. On the contrary sub group II A presented numerous inflammatory cells infiltration and extravasated RBCs, these findings might reflect prolongation of the inflammatory phase of bone healing.

In the present study the amount of formed bone in the experimental group at 8 weeks (subgroup II B) was relatively greater than their counterpart at 4 weeks (subgroup II A) which indicated progression of bone healing process, however it is still relatively less than those of the control group (sub group IB) at the same duration. The defect of sub group IB showed continuation of bone healing process by further bone deposition towards the center of the defect and remodeling of the recently formed bone at the peripheries. It was stated that the remodeling phase is the final phase in bone healing during which the rapidly and randomly formed bone matrices become replaced by a more organized bone structure and resorption of excess callus tissue [6].

At 12 weeks the experimental group (sub group IIC) of the current study showed continuation of bone healing process, which was showed by the presence of lamellar bone at the peripheries of the bony defect. This indicated the transition between the repair phase and the remodeling phase. A picture was more or less similar to those of the control group at 8 weeks (sub group IB).

The effects of parotid gland ablation were studied on experimental fracture in albino rats, stating that there were no significant difference in the healing of the fracture sites of both the control and parotid removed groups despite there were differences in the cellularity of the healing callus of both groups [15], which in turn

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**Chart 1.** Showing radiographic bone densities of the control and experimental groups at 4, 8 and 12 weeks post operative.

**Chart 2.** Showing the changes in the mean values of the serum amylase of the control and experimental groups at the pre-operative, 4 weeks, 8 weeks and 12 weeks post-operative intervals.

**Chart 3.** Showing the mean values of the serum calcium levels of the control and experimental groups at 4 weeks, 8 weeks and 12 weeks postoperative and comparing them to each other.

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Fig. 8. Photomicrograph of subgroup IIC showing intermingling green and red areas of the bone trabeculae and green color in the marrow cavities indicates presence of collagen fibers. (Masson Trichrome X 100).
It was mentioned that serum amylase level depressed initially then rose again to normal values over time despite absence of the glands. They suggested that other sources of the enzyme compensated for their loss [18]. Also it was stated that the serum amylase level of rats may recovered to normal values in 4 weeks postoperative and that serum amylase is mainly derived from the parotid gland, but pancreas and liver may contribute to serum amylase in compensation for the parotid glands [11].

The serum calcium results of the control and experimental subgroups were statistically evaluated to compare it using the p value. At 4 weeks, the p value was 0.061 indicating a non significant difference, while at 8 weeks the p value was 0.014 which claims that the 8 weeks results shows a statistically significant difference in the collected data between the two groups. Finally at 12 weeks the p value was 0.142 which again returns to state that the results were statistically non significant.

Analyzing the calcium level results of the current study showed that the level of serum calcium of both the experimental and control subgroups were within the normal range at 4 weeks post operative period, while at 8 weeks and 12 weeks the serum calcium levels slightly rose but still close to the normal level, this was thought to be due to the increased absorption from intestine to meet the mineralization demand in these stages. This comes in accordance with a study which stated that calcium reaches the skeleton by being absorbed from the diet in the gastrointestinal tract, where the absorbed dietary calcium then enters the extracellular fluid space and becomes incorporated into the skeleton through the process of mineralization of the organic matrix of bone (osteoid tissue) [13].

7. Conclusions

From the present study, it could be concluded that bilateral parotidectomy in albino rats resulted in delayed bone healing. It was associated with an initial drop in serum amylase level at 4 weeks, however serum amylase level was self-compensated at 8 and 12 weeks of postoperatively. It didn't significantly influence serum calcium level. The role of parotid salivary gland in osteogenesis is mostly mediated through its endocrine function. Further investigations targeting the endocrical role of the parotid gland seems essential.

References


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