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POTENTIAL EFFECT OF PLATELET RICH PLASMA AS A PROMOTER OF BONE HEALING IN OBESE RATS (HISTOLOGICAL AND ULTRASTRUCTURAL STUDY)

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ABSTRACT

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The study aimed to examine the impact of platelet rich plasma (PRP) on the healing of a mandibular bone defect in obese rats. In this research, forty-eight adult male albino rats were divided into two equal groups; Group 1: were fed on standard diet. Group 2: were fed on high fat diet for four weeks for obesity inducing. A bone defect on the right side of mandible was created in each rat then all rats were subdivided into two equal subgroups. In subgroups 1.1 and 2.1, the bone defects were left without treating whereas in subgroups 1.2 and 2.2 the bone defects were filled with PRP. Two and four weeks postoperatively, six rats per group were euthanized and the samples were processed histological examination, X-ray elemental microanalysis and scanning electron microscopic examination. The results showed that rats received high fat diet and their bone defects left untreated revealed delayed bone healing and depicted significant decrease in bone calcium level in comparison with other subgroups. Interestingly, rats received high fat diet and their bone defects filled with platelet rich plasma revealed marked progress in bone healing and exhibited significant increase in bone calcium level in comparison with rats received high fat diet and with untreated bone defect. Conclusion: Obesity impaired the healing of mandibular bone defect. However, PRP has the ability to offset the negative effect of obesity and promote bone healing in obese rats. Therefore, it is highly recommended in obese individuals to fill the surgical bone defects with PRP.

KEYWORDS: Bone healing, Obesity, Platelet rich plasma

INTRODUCTION

Obesity is a complicated illness characterized by fat excessive accumulation that may be detrimental to health. The body mass index (BMI) is a basic ratio of height to weight that is used to differentiate between those who are overweight and those who are obese. Overweight is described by the World Health Organization (WHO) as a BMI of 25 to 30 kg/m2 and obesity as \geq 30 kg/m2⁽¹⁾.

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Although the discrepancy between calorie consumption and expenditure is the most common cause of obesity, the major causes of obesity include genetic, social, environmental, psychological, physiologic, and economic factors that cooperate to the advance of obesity, as well as drug-induced weight gain as an adverse biological effect ⁽²⁾.

Obesity is seen as a severe threat to public health. Previous researches have proven that obesity is linked with a chronic inflammatory response, aberrant cytokine production, activation the inflammatory signaling pathways and elevated acute-phase reactants; these mechanisms contribute to the onset of obesity-related illnesses. Thus, obesity raises the risk of a number of chronic diseases, including dyslipidemia, fatty liver disease, gallstones, obstructive sleep apnea, osteoarthritis, type depression, hypertension, 2 diabetes mellitus stroke, coronary artery disease and some malignancies (3-5).

Long-standing dispute has surrounded the effect of obesity on bones. Tradition holds that obesity is advantageous to bone and may be regarded a preventative factor against osteoporosis and fracture risk due to the well-established positive effect of mechanical stress on bone development ⁽⁶⁾. Recent studies have revealed, however, that obesity-related fat accumulation does not protect individuals against osteoporosis and, in fact, that greater fat content is deleterious to bone mass. In addition, obesity impact bone metabolism by upregulating proinflammatory cytokine production, increasing adipocyte differentiation and fat accumulation, reducing osteoblast differentiation and bone formation and interfering with intestinal calcium absorption thereby reducing calcium availability for bone formation (7-10).

Platelet-rich plasma (PRP) is a blood-derived substance newly recognized for its potent healing qualities and expanding importance in regenerative medicine ⁽¹¹⁾. It is designed to achieve a high platelet concentration in a small amount of plasma. Both plasma and its preparation include a number of growth factors and physiologically active chemicals that stimulate tissue repair and bone regeneration effectively ⁽¹²⁾.

PRP has the capacity to bring greater concentrations of physiologically active growth factors and different proteins to wounded tissues, with the added benefits of simplicity, safety, efficacy, and continuous availability⁽¹³⁾. Since the plasma used in autologous transplantation treatment is the patient's own, there is no chance of sensitization, genetic manipulation or disease transmission⁽¹⁴⁾.

PRP therapy is a widely used method for tissue repair/regeneration in several medical fields such as sports medicine, dermatology, orthopedics, otolaryngology, cardiothoracic surgery, neurosurgery, obstetrics and gynecological surgery ⁽¹⁵⁾. Likewise, PRP has yielded promising results in regenerative dentistry, includes endodontics (pulpotomy, apexification, and apical surgery), periodontics (treatment of periodontal plastic surgery and infrabony periodontal abnormalities), and maxillofacial and oral surgery (soft tissue and bone surgery, tooth extractions, and implant surgery) ⁽¹⁶⁾.

Recently, there is a growing concern in the therapeutic capability of PRP in bone regeneration. Hence, this research was conducted to determine the proficiency of PRP as an advocate of healing of a mandibular bone defect in experimental obese rat model.

MATERIALS AND METHODS

Animals:

Forty eight adult male albino rats weighting an average of 150-180 grams were used in this study. According to the methods described by Charan and Biswas, a suitable sample size was determined ⁽¹⁷⁾.

Rats were housed in polycarbonate cages with wire lids (six rats per cage), numbered and housed in a well-ventilated animal house at Faculty of Medicine, Zagazig University. The room was kept at 23 °C with a humidity level of 60%, while maintaining a typical photoperiod. Dry rat pellets were fed to rats and they were allowed unrestricted access to water. After one week acclimatization period, the animals were randomly allocated into two equal groups:

Group 1: twenty four rats were fed on the standard diet (SD) for rodents throughout the experiment. A bone defect on the right side of mandible was created in each rat then the rats were subdivided into two equal subgroups, 12 rats each; *Subgroup 1.1 (SD + untreated):* the bone defects were left without any treatment. *Subgroup 1.2 (SD + PRP treated):* the bone defects were filled with 0.35 ml of PRP⁽¹⁸⁾.

Group 2: twenty-four rats were given an high fat diet (HFD) for four weeks consisting of 20 g fat/100 g diet by weight (the fat consisted of 19 g of butter oil and 1 g of soybean oil to supply necessary fatty acids)⁽¹⁹⁾. After induction of obesity, a bone defect on the right side of mandible was created in each rat similar to group 1 then the rats were divided into two equal subgroups, 12 rats each; *Subgroup 2.1 (HFD* + *untreated):* the bone defects were left without any treatment similar to subgroup 1.1. *Subgroup 2.2 (HFD* + *PRP treated):* the bone defects were filled with 0.35 ml of PRP similar to subgroup 1.2.

All animal studies were done in compliance with the ARRIVE standards and the National Institutes of Health protocol for the care and use of Laboratory animals (NIH Publications, revised 1985). Animal handling and experimental protocols were approved by the Institutional Animal Care and Use Committee at Zagazig University ZU-IACUC.

Preparation of Platelet Rich Plasma (PRP)

The rats were anesthetized by intramuscular injection of xylazine (6 mg/kg) (Sigma-Aldrich, USA) and ketamine (70 mg/kg) (Hospira, USA).

Then, 3.15 ml blood was taken from each animal through jugular vein cannulation using a 5 ml disposable syringe with 0.35 ml of 10% sodium citrate to avoid coagulation. Blood was stored in silicone vacuum tubes containing 5 ml. The infusion of sterile saline quickly replaced the same quantity of blood from each animal. After removing the cannula from the jugular vein and using a hemostatic agent locally, the tissues were realigned and sutured. The blood was centrifuged at 160g for 20 minutes at room temperature. Blood was separated into three components: erythrocytes, buffy coat, and plasma containing platelets. The plasma containing platelets was then aspirated, transferred to a fresh tube, and centrifuged for 15 minutes at 400g. While the platelet-poor plasma was discarded, the PRP was extracted and activated via adding 0.05 ml of 10%

Surgical Procedure of Mandibular Bone Defect

calcium chloride solution to 1 ml of PRP^(18,20).

Immediately after withdrawal of the blood samples, the anesthetized rats underwent aseptic preparation then the skin and the muscle of the mandible were incised, and the soft tissues were dissected. On the right side of the body of the mandible of each animal, a 5x5 mm complete thickness defect was made using a slow-speed dental drill under continual normal saline irrigation to minimize overheating. After the defect was formed, the region was thoroughly irrigated with normal saline to remove any leftover bone fragments. In subgroups 1.2 and 2.2, mandible defects were ultimately filled with PRP. The soft tissues above the defect were then relocated and sutured in layers with 4-0 vicryl sutures (Ethicon, Lenneke Marelaan, Belgium), and the rats were treated intramuscularly with penicillin (40,000 IU/ml, 1 ml/kg) (Sandoz, Austria) for three days after surgery ⁽²¹⁾.

Euthanasia and Sample Collections

Two and four weeks following mandibular bone defect surgery, six rats from each subgroup were sacrificed with an overdose of sodium thiopental (EIPICO, Egypt), confirmed with cervical dislocation and their mandibles were dissected and their right halves were collected and immediately fixed with 4% buffered formalin solution. At each experimental period, three samples per subgroup were prepared for histological examination and three other samples per subgroup were prepared for X-ray elemental microanalysis and scanning electron microscopic (SEM) examination.

Histological Examination

After fixation, 5% formic acid was used to decalcify the specimens. After full decalcification, specimens were rinsed in deionized water, dehydrated in escalating ethyl alcohol concentrations, cleared in xylene, and embedded in paraffin. Five micrometer-thick serial sections were cut and stained with hematoxylin and eosin (H&E). The slides were examined and photos were taken with a digital color CCD camera (Olympus, DP73, Tokyo, Japan) placed on a light microscope (Olympus BX53, Tokyo, Japan) at Faculty of Dentistry, Zagazig University.

X-ray Elemental Microanalysis

After fixation, the samples treated with 5% sodium hypochlorite (commercial bleach), for 1 hour, to remove the organic material. After washing in distilled water, specimens were dehydrated in ethanol then air-dried and examined by energy dispersive x-ray analysis (EDAX) attached with the SEM unit which is designed to analyze the inorganic constituents (mainly calcium level) of the specimens.

Scanning Electron Microscopic Examination

After determination of their elemental composition, the samples were prepared for SEM examination. They were vacuumed, coated with gold through Blazers' SCD-050 sputter that changed electrically non-conductive samples into conductive ones, so enabling SEM scanning of the sample surface with a tightly focused electron beam (JEOL JSM-636 OLA at an accelerating voltage of 15kv) in Electron Microscopy Unit, Mansoura University.

Statistical Analysis:

Statistical analyses of the calcium levels were performed using one-way analysis of variance (ANOVA) and Dunnett's post hoc test to determine whether or not there are statistically significant differences between subgroups. In this study, a value of P < 0.05 was regarded to indicate statistical significance and the calcium levels were expressed as the mean \pm standard error (SE). Statistical analyses were conducted using SPSS, version 11. (SPSS Inc, Chicago, IL, USA).

RESULTS

Histological Findings:

Two weeks after induction of bone defect, histological examination of subgroup 1.1 (SD + untreated) revealed the bone defect area contained granulation tissue and newly formed woven bone (Fig. 1-A). In subgroup 1.2 (SD + PRP treated), the bone defect area showed angiogenesis and filled with granulation tissue, irregular fibrous callus and newly formed woven bone (Fig. 1-B). On the other hand, subgroup 2.1(HFD + untreated) revealed an immature granulation tissue with inflammatory cells infiltration, hematoma and some empty spaces (Fig. 1-C). Similarly, subgroup 2.2 (HFD + PRP treated), revealed granulation tissue characterized by inflammatory cells infiltration with some empty spaces and hematoma along with minute bone trabeculae (Fig. 1-D). Four weeks after induction of bone defect, subgroup 1.1 (SD + untreated) revealed angiogenesis and the bone defect site was almost filled with new bone trabeculae (Fig. 2-A). In subgroup 1.2 (SD + PRP treated), the bone defect area showed well-formed bone trabeculae and medullary spaces (Fig. 2-B). On the other hand, subgroup 2.1(HFD + untreated) showed fibrous connective tissue characterized by few inflammatory infiltrate

and scanty bone trabeculae (Fig. 2-C). However, the bone defect area in subgroup 2.2 (HFD + PRP treated) was almost filled with well-formed bone trabeculae and medullary spaces except a minimal area containing granulation tissue (Fig. 2-D).

X-Ray Elemental Microanalysis:

The sites of mandibular bone defects showed variation in their elemental composition among the different subgroups at the two experimental intervals. Statistical analysis demonstrated that two and four weeks post bone defect induction there was significant decrease in bone calcium level of subgroup 2.1 (HFD + untreated) in comparison with all other subgroups including subgroup 1.1

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(SD + untreated), subgroup 1.2 (SD + PRP treated) and subgroup 2.2 (HFD + PRP treated). On the other hand, calcium level of subgroup 2.2 (HFD + PRP treated) simulated that of subgroup 1.1 (SD + untreated) with no significant difference between them. Moreover, there was significant increase in calcium level of subgroup 1.2 (SD + PRP treated) in comparison with subgroup 1.1 (SD + untreated) and subgroup 2.2 (HFD + PRP treated). Besides, comparison of bone calcium level between the two experimental periods of each subgroup revealed significant increase in calcium level of each subgroup at four weeks compared with the same subgroup at two weeks after induction of bony defect (Table 1 & Fig.3).



Fig. (1): Decalcified H&E stained sections in the defect site of rat mandibular bone two weeks after induction of bone defect. A) Subgroup 1.1 (SD + untreated) granulation tissues (G), new bone (arrow) and small area of hematoma (arrow head). B) Subgroup 1.2 (SD + PRP treated) granulation tissues (G) and new bone (arrow heads). C) Subgroup 2.1(HFD + untreated) granulation tissue with inflammatory cells infiltration (G), hematoma (arrow heads) and vacuoles (arrows). D) Subgroup 2.2 (HFD + PRP treated) granulation tissues (G), hematoma (arrow) and minute bone trabeculae (arrow heads). (Original Magnification A- D X 400)



- Fig. (2): Decalcified H&E stained sections in the defect site of rat mandibular bone four weeks after induction of bone defect. A) Subgroup 1.1 (SD + untreated). B) Subgroup 1.2 (SD + PRP treated). C) Subgroup 2.1(HFD + untreated). D) Subgroup 2.2 (HFD + PRP treated). Bone trabeculae (T). Medullary spaces (arrow) and granulation tissue (G). (Original Magnification A- D X 400)
- TABLE (1): Statistical analysis of the bone calcium level two and four weeks postoperatively in different groups

Groups		Time (week)	
		Two weeks	Four weeks
Subgroup 1.1	Range	10 – 16	15 – 21
	Mean ± SD	$12.4 \pm 2.30^{\text{ B,C}}$	$18.2 \pm 2.39^{B,C,E}$
Subgroup 1.2	Range	15 – 20	25 - 30
	Mean ± SD	$17.8 \pm 1.92^{A,C,D}$	$27.4 \pm 2.07^{A,C,D,E}$
Subgroup 2.1	Range	5 - 10	10 - 17
	Mean ± SD	$7.2 \pm 1.92^{\rm A,B,D}$	$12.6 \pm 2.70^{\text{A,B,D,E}}$
Subgroup 2.2	Range	8 – 12	17 – 22
	Mean ± SD	$10 \pm 1.58^{B,C}$	$20 \pm 2.35^{B.C.E}$

Notes: Each value represents mean \pm standard deviation; significance level p < 0.05. A: significance vs subgroup 1.1, B: significance vs subgroup 1.2, C: significance vs subgroup 2.1, D: significance vs subgroup 2.2 and E: significance vs two weeks value



Fig. (3): Bar chart shows the mean calcium level between the different study groups

Scanning Electron Microscopic Findings:

Two weeks after induction of bone defect, topographical examination of subgroup 1.1 (SD + untreated) showed the bone defect area was filled by new bone trabeculae (Fig. 4-A). However, subgroup 1.2 (SD + PRP treated) revealed the bone defect area was packed with thicker new bone trabeculae (Fig. 4-B). On the other hand, subgroup 2.1 (HFD + untreated) showed the defect area was almost devoid of bone (Fig. 4-C). In subgroup 2.2 (HFD + PRP treated), the defect area contained few bone trabeculae (Fig. 4-D). Four weeks after induction of bone defects, the bone defect areas of subgroup 1.1 (SD + untreated), subgroup 1.2 (SD + PRP treated) and subgroup 2.2 (HFD + PRP treated) showed complete closure of the defect sites with almost normal bone surface (Fig. 5 A, B& D). However, the bone defect area of subgroup 2.1(HFD + untreated) appeared filled with bone trabeculae but still incompletely closed (Fig. 5-C).



Fig. (4): Scanning electron micrographs of the defect site of rat mandibular bone two weeks after induction of bone defect. A) Subgroup 1.1 (SD + untreated). B) Subgroup 1.2 (SD + PRP treated). C) Subgroup 2.1(HFD + untreated) shows area devoid of bone (*). D) Subgroup 2.2 (HFD + PRP treated).



Fig. (5): Scanning electron micrographs of the defect site of rat mandibular bone four weeks after induction of bone defect. A) Subgroup 1.1 (SD + untreated). B) Subgroup 1.2 (SD + PRP treated). C) Subgroup 2.1(HFD + untreated) with incomplete closure of defect area (*). D) Subgroup 2.2 (HFD + PRP treated).

DISCUSSION

The present study involved two research points. First, the negative effect of obesity on the healing of mandibular bone defect. Second, the ability of PRP to offset the effect of obesity and promotes bone healing. In the current study, a mandibular bone defect was created in two groups (SD and HFD) then the defect area was filled with PRP in one subgroup per group while left empty in the other subgroup. Two and four weeks after the procedure, the progress of bone healing was studied by qualitative (histological and SEM) assessments and quantitative X-ray elemental analysis.

The adult male rats were the experimental model of choice in this study owing to similarities in pathophysiologic responses between rats and human skeletons, along with the financial and husbandry advantages of rats ⁽²²⁾. In addition, HFD feeding is used in the current study to induce obesity in rats because HFD-induced obesity rat model is the best to simulate the metabolic deterioration seen in human obesity ⁽²³⁾.

Qualitative histological and SEM results of rats received HFD and their mandibular bone defects left untreated (HFD + untreated subgroup) revealed obvious delay in osteogenesis and retarded healing of the bone defect area in comparison with other subgroups. This is in agreement with Damanaki et al ⁽²⁴⁾ who shown that obesity hinders the ability of rat periodontal abnormalities to mend on their own. They arrived at the conclusion that obesity may have an adverse effect on bone repair. Similarly, Gao et al ⁽²⁵⁾ verified showed bone repair in the obese mice was substantially slower than in the normal animals. They contributed this delay in fracture healing to the lower levels of fibroblast growth factor (FGF), transforming growth factor beta 1 (TGF- β 1), plasma calcitonin gene-related peptide (CGRP), and elevated concentrations of tumor necrosis factor- α (TNF- α).

In accordance with the depicted histological and SEM findings, quantitative X-ray elemental analysis of HFD + untreated subgroup revealed a significant decrease in bone calcium value below those of other subgroups through the two experimental periods. This finding was in agreement with Hsu et al. ⁽²⁶⁾ who showed a correlation between increased fat mass and decreased bone mineral content and bone mineral density. The significant decrease in bone calcium level indicated that obesity could induce changes in mineralization kinetics as proved by Lavet et al. ⁽²⁷⁾ who reported disruption of mineral metabolism and its main regulators in addition to progressive hyperphosphatemia and hypocalcemia in rats fed HFS diet.

Multiple lines of evidence have shown that obesity and bone metabolism are linked; first, since both osteoblasts and adipocytes are differentiated from the same multipotent mesenchymal stem cell, obesity suppresses bone production while enhancing adipogenesis⁽²⁸⁾. Second, obesity may enhance bone resorption through upregulating proinflammatory cytokines which are capable of stimulating bone resorption and osteoclast activity by altering the receptor activator of NF-xB (RANK)/RANK ligand/ osteoprotegerin pathway⁽²⁹⁾. Thirdly, persistent usage of steroid hormones such as glucocorticoids causes obesity and fast bone loss⁽³⁰⁾. Fourth, obesity is related to significant increase in serum leptin which is adipocyte-derived cytokines⁽³¹⁾. Overproduction of leptin from adipocytes was previously reported to have adverse impacts on bone metabolism⁽³²⁾. Also, obesity is associated with decrease in adiponectin which is another adipocyte-derived cytokines

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supposedly inhibits osteoclastogenesis, decreases bone resorption, and increases bone mass in addition to its anti-inflammatory effect ^(33,34).

Surprisingly in the present qualitative study, rats received HFD and their bone defects filled with PRP (HFD + PRP treated subgroup) revealed acceleration in bone healing and marked improvement in the histological and the topographical features of bone at the site of defects in comparison with HFD + untreated subgroup. These results could be related to beneficial effects of PRP on bone healing as reported by previous studies ^(35,36). As well, quantitative X-ray elemental analysis of bone calcium level in PRP treated rats either of SD or HFD subgroups revealed significant augmentation in comparison with their corresponding untreated subgroups. Moreover, HFD + PRP treated subgroup simulated that of SD + untreated subgroup but exhibited significant decrease than SD + PRP treated subgroup. These findings demonstrated that PRP could enhance bone mineralization in normal rats and alleviate obesityinduced changes in mineralization in obese rats.

These beneficial effects of PRP on bone formation and mineralization were confirmed by studies indicating that PRP contains key growth factors involved in bone regeneration, including FGF, TGF- β , vascular endothelial growth factor (VEGF), platelets derived growth factor (PDGF), insulin-like growth factor-1 (IGF-1), and epidermal growth factor (EGF) (37,38). In addition, PRP contains a variety of macrophage/monocyte mediators and interleukins capable of mediating inflammation ⁽³⁹⁾. In addition, the plasma component of PRP comprises albumin, fibrinogen, a number of immunoglobulins, and a number of bioactive compounds that play a crucial role in bone regeneration⁽⁴⁰⁾. Moreover, Bida et al.⁽⁴¹⁾ showed that PRP has the capacity to boost alkaline phosphatase activity, a crucial sign of mineralization and bone growth. In addition, Wei et al.⁽⁴²⁾ revealed that PRP unregulated the levels of osteogenesis-specific markers, such as runt-related transcription factor 2 (RUNX2), osterix (OSX), and osteopontin (OPN), which play crucial roles in the control of bone formation and mineralization. On the other hand, adipocyte-specific marker, peroxisome proliferator-activated receptor gamma 2 (PPAR γ 2), was inhibited by PRP, indicating that PRP mediates the balancing of osteogenesis and adipogenesis.

CONCLUSION

Obesity has adverse effects that could cause delay healing of mandibular bone defects in rats. However, this study proved the capability of PRP to alleviate the impaired bone healing in obese rats.

RECOMMENDATION

Further studies are recommended to prove the proficiency of PRP to treat obesity damaging effects on different body tissues and whether its valuable role will differ among different gender and ages.

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