AJDS LAZHAR *Al-Azhar Journal of Dental Science*
Vol. 22- No. 1- 87:93- January 2019
Record 1110-2624 | the ISSN Portal
portal.issn.org

EVALUATION OF THE EFFECT OF BETA-TRI-CALCIUM PHOSPHATE NANO PARTICLES ON BONE DEFECT HEALING IN DIABETIC RATS (HISTOLOGICAL STUDY)

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ABSTRACT

The aim of this study was to evaluate histologically the bone defect's regeneration after application of beta-tricalcium phosphate nanoparticles in diabetic rats. The experimental part was carried out on 16 adult male albino rats and their weight almost 250 gm., the animals were grouped into 2 equal groups as the following; group I (control diabetic) which had not receive the graft, group II (experimental diabetic) which had received the graft material (Nβ-TCP). Specimens were harvested on days 7 and 28 after surgical procedures, prepared and examined histologically by H&E stain, there were wide histological differences between the groups of this study along the different intervals of the study.

The histological results demonstrated that there was obvious retardation in granulation tissue formation, organization and bone formation in the control diabetic group I than the other group along the different intervals of this study. This retardation in the healing of diabetic control group was due to the effect of diabetes as it reduces cellular proliferation in early callus, reduces collagen synthesis content, reduces osteoblastic activity and reduces bone mineralization.

Moreover, there was great acceleration in granulation tissue formation, organization and bone formation in experimental diabetic group II which received the graft material. This enhancement in bone healing process was due to the stimulatory effect of Nβ-TCP on osteoblastic differentiation and enhanced functions of osteoblasts on nanophase ceramics.

INTRODUCTION

Diabetes mellitus (DM) defined as a group of metabolic disorders characterized by hyperglycemia resulting from defects in insulin secretion, insulin action, or both, it is caused by either autoimmune destruction of insulin-producing cells (Type I) or resistance of the body to insulin (Type II)[1]. Poorly controlled diabetes is associated with adverse systemic sequelae including increased susceptibility to infection, delayed wound healing and microvascular complications that lead to decreased immune response[2].Hyperglycemia produces deleterious effects on bone matrix and its components, and also affects adherence, growth and accumulation of extracellular matrix[3]. Many mechanisms have been reported by which diabetes may affect bone including inhibition of both osteoblast differentiation and expression of growth factors that promote bone formation and an increased osteoclast proliferation, resulting in lower bone mineral density and increased risk of fractures[4]. Therefore, there is need to develop new treatment plan for the bone healing in patients with DM.

The nanotechnology is fast approaching, which was unheard of two decades ago, the growing interest in the nanotechnology field has given emergence to the new field of "nanomedicine",the science and technology of diagnosing, treating, preventing

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diseases , preserving and improving human health[5]. In recent years, nanotechnology has allowed for the possibility of fabricating nanophase particles with functions suitable for targeting and treating various bone diseases [6]. These nanomaterials have shown superior properties compared to their conventional (or micron structured) counterparts due to their distinctive nanoscale features and novel physical properties [7,8].

Nano-sized β-tricalcium phosphate (Nβ-TCP) granules with higher phase purity (> 99 %), better mechanical compressive strength (> 2.22 MPa), higher porosity (> 75 %) including macropores, mesopores, and micropores, and larger specific surface area (> 2.50 m2/g) were successfully fabricated from wet chemically precipitated method [9]. Conventionally, the nano-sized particles in porous granules could play a significant role in increasing porosity and specific surface area of granules, the presence of macroporous (> 100 μm) and mesoporous structure (10 – 100 μm) favors cell ingrowth and newly bone formation while the microporosity (< 10 μm) allows the penetration of the body fluids into implant [10].

Typically, it had been reported that both the mesopores (10 – 100 μm) and the macropores (> 100 μm) play a significant part in stabilization of the initial blood clot and subsequent vascularization and integration of the material in the bony tissue [11]. In particular, the vascularization is important for successful bone regeneration, especially when using a biomaterial because of its role in the nutrition of the migrating cells [12]. The microporosity also can be a strategy to manipulate resorption and dissolution rate; greater microporosity means greater degradation rate [13,14]. In particular, it has also been demonstrated that the large specific surface area which can be achieved by increasing the number of micropores is essential for the osteoconductivity for bone regeneration [15-20].

In the present study, critical bone defects were made in rat tibial bone, and the effect of nano-sized particles of β-TCP on bone healing was evaluated using histological investigations.

MATERIALS AND METHODS

This study was carried out on sixteen adult male albino rats, their average weight was about 250 gm. The procedure management was carried out at house animals of Faculty of medicine, Cairo University.

Induction of diabetes

Diabetes was induced by a single intra-peritoneal injection of 120 mg/kg monohydrate alloxan dissolved in sterile 0.9% saline. Rats had been fast for 12 hours, followed by alloxan administration. After 12 hours 10% glucose solution was offered to the animals to prevent hypoglycemia. After 7 days, blood samples will be collected from the caudal vein of the animals for evaluation of plasma glucose levels. The animals with glycemic level higher than 250 mg/dl, were considered diabetic and used in this study.

Grouping and experimental planning

The animals were divided randomly into two main groups as follows:

Group (I), control diabetic group consisted of eight diabetic rats which had not receive the graft.

Group (II), experimental diabetic group consisted of eight diabetic rats which had received the graft material.

RESULTS

Seven days interval of control diabetic group (I) demonstrated blood clot filling the defect which infiltrated with inflammatory cells mainly macrophages engulfing the clot. No signs of granulation tissue formation were present and no bone resorption at the side of the defect. ((fig.1).

On day 28 of control group (I) at the surface of the defect there was thick band of typically organized granulation tissues which characterized by tightly packed collagen bundles with proliferating blood capillaries. The base of the defect was almost closed by thin trabeculae of woven bone connected to the old bone forming a bridge. Also, in some areas bone trabeculae were harboring marrow haemopoietic cavities in between them which filled with haemopoietic tissue. Moreover, there was recruitment of osteoblasts within the granulation tissue around the woven bone trabeculae, also there were numerous osteoblasts imprisoned within the bone trabeculae with narrow osteocytic spaces (fig.2).

Seven days interval of experimental diabetic group (II) demonstrated some remnants of the material (Nβ-TCP) surrounded by well-organized granulation tissue at the surface of the defect. The obvious feature was wide irregular spaces containing remnants of the material mixed with the blood surrounded by fibrovascular connective tissue which infiltrated the macropores of the material. Notable proliferating blood vessels were observed in the granulation tissue , also the newly formed granulation tissue was infiltrated by macrophages and multinucleated cells. Moreover, there was recruitment of osteoblasts and at the base and sides of the defect there was new woven bone formation demarcated from old bone by a reversal line, also the newly formed bone was highly cellular, fibrillar and characterized by imprisoning of multiple osteoblasts with wide osteocytic spaces (fig.3).

On day 28 of experimental group (II) demonstrated at the surface that the defect was closed by new woven bone formation with multiple reversal lines and small amount of imprisoning osteoblasts.

In as much as there were multiple dispersed bony trabeculae of different sizes and shapes filled the defect, harboring in between chemobiotic tissue with active mitotic index, also there was configuration of primary osteons and narrowed blood vessels. There was a reversal line demarcating the old bone from the newly formed bone at the periphery of the defect. (fig.4).

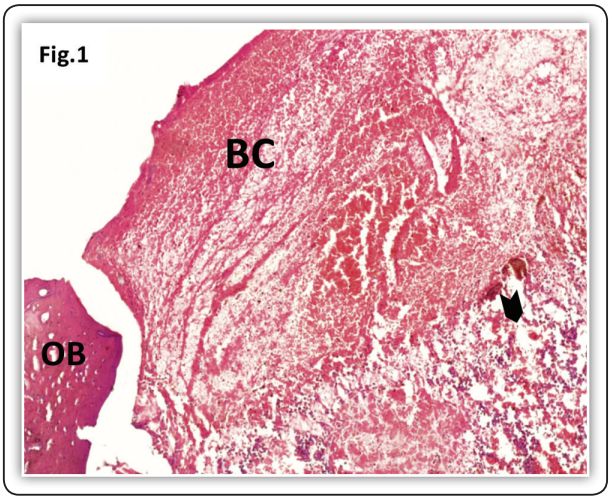

FIG (1) Histological section of control diabetic group (I) at 7 days interval showing: old cortical bone (OB), blood clot (BC), inflammatory cells infiltration (arrow head). (H&E. 100x)

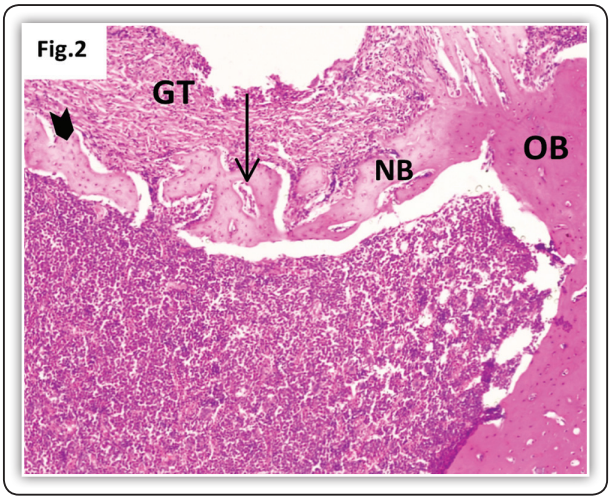

FIG (2) Histological section of control diabetic group (I) at 28 days interval showing: typically, organized granulation tissue (GT), Old bone (OB), thin bridge of new woven bone (NB), osteocytes with narrow osteocytic space (arrow head), marrow cavity with haemopoietic tissue (arrow) (H&E. 100x)

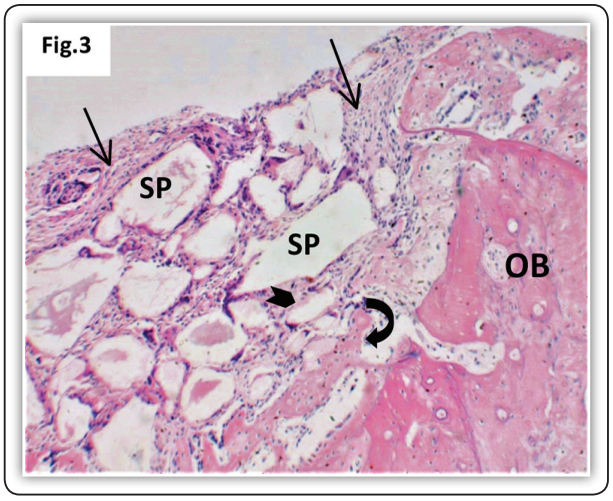

FIG (3) Histological section of experimental diabetic group (II) at 7 days interval showing: new bone formation (curved arrow), wide spaces (SP) surrounded by well-organized granulation tissue(arrows), which infiltrated the macropores of the material, blood vessel (arrow head) **(H&E. 100x)**

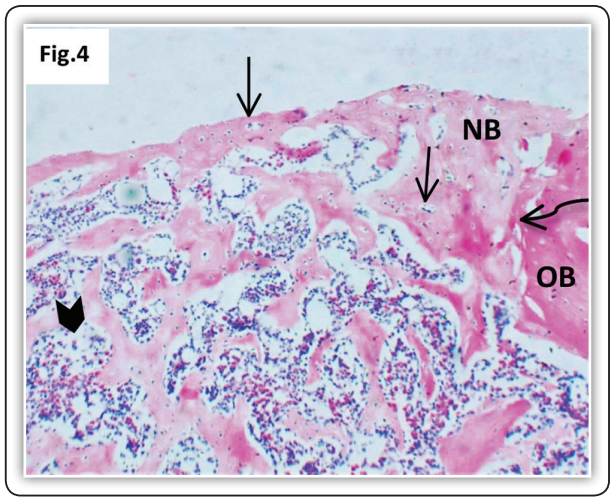

FIG (4) Histological section of experimental diabetic group (II) at 28 days interval showing: Old bone (OB), new woven bone (NB), marrow cavities with haemopoietic tissue (arrow head), primary osteons (arrows), reversal line (curved arrow). **(H&E. 100x)**

DISCUSSION

Diabetes is one of the most important sugar metabolic disorders which have a great effect on bone healing, many mechanisms have been reported by which diabetes may affect bone including inhibition of both osteoblast differentiation and expression of growth factors that promote bone formation and an increased osteoclast proliferation, resulting in lower bone mineral density and increased risk of fractures[4]. Therefore, there is need to develop new treatment plan for the bone healing in patients with diabetes mellitus.

Various calcium phosphate biomaterials had been developed for use as bone substitutes owing to their biocompatibility and osteoconductivity, one such material, beta-tricalcium phosphate (β-TCP), had been shown to have good biocompatibility and osteoconductivity in both animal experiments and clinical settings [21].Many biomaterials do not have Osteoinductive properties in the absence of additional osteoinductive agents, such as bone morphogenic proteins.[16] However, several reports have described osteogenesis by calcium phosphate alone after implantation in soft tissues

[22,19], and such osteoinductivity is a desirable characteristic of a bone-substitute material.

At seven days interval there were remarkable histological differences among the groups of this study, the control diabetic group (I) showed blood clot with high infiltration of inflammatory cells with no signs of granulation tissue formation suggesting retardation in healing process, these findings were parallel with Retzepi M, et al. [23], and Stolzing A, et al. [24], who stated that diabetes reduce cellular proliferation in early callus and reduce collagen synthesis content. Likewise, Andriankaja OM, et al. [25], Pacios S, et al. [26], referred the retardation in bone regeneration to the prolonged inflammation associated with diabetes mellitus and stated that diabetics have difficulty in down regulating inflammation once induced, moreover, increased levels of TNF may limit the capacity of diabetics to downregulate other inflammatory genes and increase apoptosis, which has been shown to reduce bone coupling in diabetic animals.

While at the experimental diabetic group (II) there were thin rim of well-organized granulation

tissue surrounding wide irregular spaces containing remnants of the material dissolved with the blood, moreover, there was new woven bone formation within the macroporosity created by the material, the new bone was highly cellular and fibrillar due to rapid formation .Our findings matched the results of Robert D.A. et al.[27] ,who observed several large spaces which represent β-TCP lost during decalcification and there was pink stained material inside these spaces suggesting that material "fibrin or proteins" had penetrated into the micropores of the implanted material. Also, notable proliferating blood vessels were observed in the granulation tissue, these finding were in agreement with Zerbo et al. [28], who postulated that there is invasion of fibro-vascular tissue in the large pores of the β-TCP at early time of implantation.

On the basis of the present study we observed from the different histological findings between these groups at seven days interval that beta tricalcium phosphate nanoparticles (Nβ-TCP) encouraged earlier neovascularization and enhanced bone induction in the experimental diabetic group (II) when compared with control group, and this enhancement was explained by Bignon A, et al.[29] , Ohtsubo S, et al.[30], Who stated that Increased microporosity may provide an increased surface area for the action of angiogenic and other proteins leading to the formation of blood vessels and promoting bone induction by osteoblasts at early time points. Furthermore, Chazono M, et al. [31], suggested that micropores of β-TCP play an important role as the storage space for extracellular matrix components, including collagen, as well as providing ideal conditions for osteoinductivity.

At twenty eight days interval there were remarkable histological differences among the groups of the study , the control diabetic group (I) demonstrated that at the surface of the defect there was thick band of typically organized granulation tissues and the base of the defect was almost closed by thin trabeculae of woven bone connected to the old bone forming a bridge ,the decreased thickness, size and number of bone trabeculae suggesting the retardation of the healing process in this group. These results were in agreement with that of Vashishth D, et al., Garnero P, et al., Kume S, et al. [32-34], who postulated that Hyperglycemia results in the accumulation of advanced glycation end products (AGEs), which affects the structure of the collagen resulting in a compromised organic bone matrix quality, these AGEs may also reduce osteoblast proliferation and function and increase osteoclast-related bone resorption leading to an overall deterioration in bone quality.

Moreover, at the experimental diabetic group (II) the defect was closed by new woven bone formation characterized by irregular bone trabeculae coalescent and anastomosed with each other in some areas forming trabecular network and harboring active bone marrow with multiple angiogenesis, this enhancement in bone healing process in compare with the control group was confirmed by the results of Webster TJ,et al.[8], who investigated the long-term (in the order of days to weeks) functions of osteoblasts on nanophase ceramics and provided the first evidence of enhanced osteoblast proliferation, alkaline phosphatase synthesis, and concentration of extracellular matrix calcium on nanophase ceramics.

On the basis of the current study we observed from the different histological findings between these groups at twenty-eight days interval that beta tricalcium phosphate nanoparticles (Nβ-TCP) supported bone regeneration, and such observation was confirmed by Knabe C, et al. [35], who studied the Effect of b-tricalcium phosphate particles with varying porosity on osteogenesis, and stated that these particles had a stimulatory effect on osteoblastic differentiation. Furthermore, it was shown that these particles attracted osteoprogenitor cells that migrated into the Interconnecting micropores of the bone substitute material and these cells differentiated into osteoblasts and thus brought about bone deposition.

CONCLUSION

1. The drastic reduction to the nano-sized level of the particle of β-TCP granules could contribute to inducing both higher porosity and larger specific surface area, and it is considered that these both factors played a significantly role in the remarkable bone regeneration effects.
2. Nβ-TCP induces blood vessel formation at an early stage after implantation and the blood vessels then appear to facilitate induction of osteoblasts and osteoclasts in macropores.
3. Nβ-TCP has been shown to have good biocompatibility and osteoconductivity and can be used to enhance bone healing specially in retarded conditions as in diabetes mellitus.

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