Histopathological evaluation of bone healing using Nanobone in experimentally induced frontal bone defects of rabbits

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ABSTRACT

Background: The bone substitute biomaterials are used in reconstruction of bone defects and one of them is Nanobone. It consists of nanocrystalline hydroxyapatite embedded in a highly porous matrix of silica gel. It promotes the healing of bone defects and increases the amount of new bone formed. It is degraded and resorbed by osteoclasts after a period of time. The aim of study was to evaluate the effects of Nanobone on bone healing in experimentally frontal bony defects of rabbits.

Materials & Methods: Thirty five indigenous rabbits were used in this study. Granules of Nanobone were implanted in surgically induced defects of 5mm size in one side in the frontal bone of rabbits, while the other side defects were left free to heal spontaneously as controls. Samples were taken after 2, 6, 9, 12 and 24 weeks and demineralized. The amount of new bone formed around implanted granules and the inflammation present were analyzed by means of light microscopy in both Nanobone granules-filled defects and controls.

Results: At 2 weeks, there was no new bone formed in both defects with few amount of inflammation which considers normal at this time. At 6, 9 and 12 weeks, the amount of new bone formed in Nanobone filled defects was much more than that formed in controls (statistically significant correlation, P<0.05), no inflammation seen in boths. At 24 weeks, the Nanobone granules were degraded and completely replaced by new bone while in controls there was few spaces not filled with bone (statistically highly significant correlation, P<0.01), no inflammation seen also.

Conclusions: Results of present study revealed that Nanobone material promotes healing of bony defects in comparison to controls and is degraded and replaced by new bone after a period of time.

Key words: Nanobone, bone healing, frontal bone, rabbits.

INTRODUCTION

The life expectancy of the current population has increased considerably over recent decades due to improvements in the quality of life of individuals. One of the consequences of this phenomenon has been the increase in injuries and bone disease in older people who have a diminished bone regeneration capacity. Defects in oral and craniofacial tissues, resulting from trauma, tumors, infections, abnormal skeletal development or progressive deforming diseases, present a formidable challenge and restoration of these tissues is a subject of clinical, basic science and engineering concern. (1)

Bone is a highly vascularized and innervated connective tissue. It is composed of cells and mineralized organic matrix. Bone is the only body tissue capable of regeneration and remodeling process (it is in constant formation and resorption, this allows the renovation of 5-15% of the total bone mass per year under normal conditions). It is regulated by genetic, mechanical, vascular, hormonal, nutritional, and local factors. (2)

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(interconnecting nanopores) has been created by a newly developed production process, which produces a very large surface area of this bone augmentation material.\(^{(6)}\)

**MATERIALS AND METHODS**

The experimental animal study was carried out in the Animal House of Veterinary Medicine College of Al-Anbar University. Thirty five (35) indigenous healthy rabbits with average age of six months were used for the present experimental study. The weight range of the animals was (2.5-3kg), these animals were housed in wire mesh cages under the supervision of the veterinarian staff. Five vials of Nanobone granules (Artoss, Rostock, Germany; DentaurumImplants, Ispringen, Germany) were used in current study. The size of each vial was 0.6 ml. The size of granule was 0.6 mm x 2.0 mm. The rabbits were fastened 8 hours before surgery, the sites of the operations were shaved from the frontal bone. Prophylactic antibiotic ampiclox 500 mg was injected I.M. 30 min. before the operation. I.M. injection of 0.5 mg atropine was also given 15 min. before the induction of anesthesia, 5mg/kg xylazine was also given as an I.M. injection, this is followed after about 10 min. by an I.M. injection of 50 mg/kg Ketamine hydrochloride and this was repeated every occasion when there was reflex movement.

Ligocaine hydrochloride 2% with 1:80,000 adrenaline was infiltrated subcutaneously along the planned incisions. The operative fields, after achieving anesthesia were properly dropped by sterilized towels and aseptic application of betadine solution.

General anesthesia was given with I.M. injection of Ketamine 30 mg/kg and xylazine 6mg/kg atropine was used (one ml), application of eye ointment to prevent dryness of the cornea of the animal. The surgical fields were done in the bridge of the nose and cranium. The surgical fields were shaved carefully to avoid injury to the surgical field then sterilized by betadine (Povidine, iodine). A blade no.1 (BP) was used to make a vertical incision of 5cm length in frontal area, soft tissue and muscle retracted. The bone was exposed and defect created by using bur in low-speed micrometer hand piece (NSK, JAPAN) with irrigation by normal saline during procedure.

The bone defected areas were created in both sides of the frontal bone. The size of defect was 5 mm, induced area has been prepared in the right and the left frontal region. The created defect was dried from blood by pressure of gauze for five minutes, then Nanobone granules applied in the defects at right side while the left side was left free to heal spontaneously to consider as control group. Following the insertion of Nanobone granules, condensing it, filling it very well and the flaps were sutured by plan or chromic 3/0 catgut and 3/0 black silk. The animals were fed a green diet and received water added libitum. Procaine penicillin 2mg/day was injected I.M. after the operation for 3 days. Sutures were removed at the seventh day. The experimental animals were divided into five groups:-

I. Scarified after 2 weeks.
II. Scarified after 6 weeks.
III. Scarified after 9 weeks.
IV. Scarified after 12 weeks.
V. Scarified after 24 weeks.

The rabbits were scarified at 2, 6, 9, 12 and 24 weeks intervals after implantation, their biopsies were taken and fixed in 10% neutral buffered formalin (10%N.B.F.) for 5 days and refixed by sodium chloride formal for one day. The bone trim then decalcified in Sodium Citrate Formic acid. The decalcified solution was changed every 48 hrs till the bone becomes soft. Tissue placed in decalcified solution must be washed in water for a minimum of 24 hrs before processing neutral buffered formalin.

The decalcifying fluids were Solution A (90% formic acid 125mg, D.W. 25ml), Solution B (Sodium Citrate 50mg, D.W. 250ml, 5%m Formic acid). The decalcification will take approximately 2-4 weeks depending upon the thickness of the block and degree of calcification. After that the decalcified tissue were dehydrated through ascending grades of alcohol (70,80,90,95,100) clearing in xylene. Processed tissues were impregnated in paraffin wax (M.P.58 co). Paraffin embedded section of 6-7 micron were cut with rotary microtome and mounted on glass slides then stained with standard haematoxylin and eosin. Slides were examined on a light microscope equipped with photomosaic unit.

On an ordinal scale, sections were scored for bone healing in control & experimental groups in two parameters: (i) the amount of new bone according to Qiu grading system\(^{(7)}\) table (1); (ii) the degree of inflammation according to Mankin grading system\(^{(8)}\) table (2).

**Table 1: Grading system for new bone formation\(^{(7)}\)**

<table>
<thead>
<tr>
<th>New Bone: amount</th>
<th>Scoring</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>Score 0</td>
</tr>
<tr>
<td>Scattered islands</td>
<td>Score 1</td>
</tr>
<tr>
<td>Thin sheet</td>
<td>Score 2</td>
</tr>
<tr>
<td>Bone trabeculae</td>
<td>Score 3</td>
</tr>
</tbody>
</table>

\(^{(6)}\) Mankin grading system

\(^{(7)}\) Qiu grading system

\(^{(8)}\) Table (2)
Table 2: Mankin grading system for degree of inflammation\(^8\)

<table>
<thead>
<tr>
<th>Degree of Inflammation</th>
<th>Inflammation</th>
<th>Scoring</th>
</tr>
</thead>
<tbody>
<tr>
<td>Severe</td>
<td>Abundant macrophage, giant cells and PMN leucocytes</td>
<td>Score 0</td>
</tr>
<tr>
<td>Moderate</td>
<td>Many macrophages/giant cells with few PMN leucocytes</td>
<td>Score 1</td>
</tr>
<tr>
<td>Some</td>
<td>Few macrophages/giant cells</td>
<td>Score 2</td>
</tr>
<tr>
<td>None</td>
<td>No inflammation</td>
<td>Score 3</td>
</tr>
</tbody>
</table>

Differences in new bone & degree of inflammation between experimental and control groups of each period were statistically evaluated by using Spearman's rho and Pearson Chi-Square tests. \(P\)-value were calculated, and values of less than 0.05 were considered to be significant.

RESULTS

1 Microscopical Examination

1.1 Two weeks group

Control group

At this interval, there was no new bone formation within frontal bone defects. The defect filled with collagen fibers, large number of fibroblast and new blood vessel with moderate degree of inflammation. No osteoblasts were seen in figure 1.

Experimental group with Nanobone

The inorganic components of Nanobone was removed by decalcification process so that (NB) appear empty spaces except of that, there is a few amount of loose fibrovascular tissue found at the periphery and within the defect which contains a number of blood vessels, fibroblast cell with moderate degree of inflammation less than that seen at control group (figure 2).

Figure 1: H&E 20X Microscopical section for 2 weeks control group showed collagen fibers (CF) & fibroblasts (F).

Figure 2: H&E 10X Microscopical section at 2 weeks experimental group showed collagen fibers (CF) and fibroblasts (F).

1.2 Six Weeks Group

Control Group

The sections at this time show formation of new bone islands. The spaces between bone islands were filled with the cartilaginous callus, collagen fibers, large number of fibroblast and new blood vessel with mild degree of inflammation, osteoblasts present at the periphery (figure 3).

Experimental Group

Initial scattered islands of immature bone proliferation were seen extending from the peripheral bone cavities occupied the space under implanted (NB) granules along the surface of the cortical bone with few collagen fibers and new blood vessels (figure 4). There were no evidence of foreign body reaction, no inflammation and no bone resorption. The osteoblasts activity at the peripheral area of the bone were observed.

Figure 3: H&E 10X microscopical section at 6 weeks control group showed collagen fibers(CF) with new bone formation(NB) & old bone (OB)
1.3 Nine Weeks Group

Control Group
Microscopical observations of control showed increasing in bone formation with many spaces between them, the spaces filled with little amount of collagen fibers and few fibroblast with scant inflammatory cells, osteoblasts present at periphery of bone trabeculae with few number of osteoclasts, the inflammation is very mild (figure 5).

Experimental Group
Microscopical observations of granule-filled defects at the 9 weeks showed gradually increasing bone formation on the surface of the basal and central implanted granules. The connective tissue was moderately dense well vascularized and generally in immediate contact with the Nanobone granule (figure 6).

1.4 Twelve Weeks Group

Control Group
Microscopical observations of controls showed increasing in bone formation with few spaces between them, the spaces filled with little amount of collagen fibers and few fibroblast, osteoblasts present at periphery of bone trabeculae with few number of osteoclasts and no inflammation was seen (figure 8).

Experimental Group
Histological evaluation revealed that there were bone formation in direct contact with surface of (NB) granules, also there was obviously thick fibrous connective tissue that embedded in the calcified matrix as in figure 9. Mature connective tissue form the inter granule matrix.
Histopathological evaluation

1.5 Twenty Four Weeks Group

Control Group

Histopathological evaluations showed large amount of bone trabeculae filled the defect with few spaces between them, very little amount of collagen fibers and fibroblasts were seen. Osteoblasts present at periphery of bone trabeculae, with few amount of osteoclasts, no evidence of inflammation (figure 10).

Experimental Group

Microscopical observations revealed complete healing with new bone formation. The defect filled with mature bone, osteocyte located with lacunae, osteoblasts at periphery, osteoclasts within bone trabeculae. No inflammation seen, no foreign body reaction. No or very mild fibrous connective tissue was seen.

Biodegradation to (NB) particles during this interval were seen and completely replacement by new bone (figure 11).

Correlation of new bone formation and degree of inflammation in control and experimental groups regarding time intervals.

Results of present study revealed statistically significant correlation (P<0.05) in control and experimental groups concerning amount of new bone formation in the intervals of 6, 9 and 12 weeks, while statistically highly significant correlation (P<0.01) in the interval 24 weeks (figure 12).

Regarding degree of inflammation results showed statistically non significant correlation (P<0.127) between control and experimental groups in all time intervals (figure 13).

DISCUSSION

This study showed that the NB particles were encapsulated by fibrous tissue. There was evidence of new bone formed at (6, 9, 12 and 24) of NB implanted in frontal bone. The area under the grafted NB gradually filled with newly bone formation adjacent to peripheral defective area with bundles of connective tissue fibers extending to surround the NB implant were observed. The bone formation became apparent with increasing time of implantation at (6, 9, 12 and 24) intervals.

The present study indicates that early bone formation within and around NB particle found in 6 weeks that proves osteoinductive, osteoconductive and osteointegrative properties of NB material and this is in favour for facial defect reconstruction.

The proven high biocompatibility and angiogenic response of the NB may play a part in contributing to the fact that clinical no signs or symptoms of inflammation were found after augmentation.
Figure 12: Statistical relation of new bone amount between control & Nanobone with increasing the time (significant) (Pearson Chi-Square)

Figure 13: Statistical relation of degree of inflammation between control & Nanobone with increasing the time (non significant) (Pearson Chi-Square)

REFERENCES