Comparing the effect of human wisdom teeth pulverized in micron and nano particle dimensions as grafting material in healing of tibial bone defect

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ABSTRACT

Background: In this article, we decided to introduce an available, affordable and biocompatible material from human teeth using nanotechnology to repair bone defects. Totally impacted wisdom teeth of human, which had been removed by surgery, were prepared as powder in two particle sizes of 500 microns and nano (up to 100 nm) after sterilization.

Method: Test cases were eight white rabbits of New Zealand species that were divided into 2 groups. Pores with 6 × 6 mm dimensions were created at hamstring area of tibia bone. In left leg tibia’s pore, nanoparticles powder and in the right leg tibia’s pore, micro particles powders were placed. The groups of two were sacrificed after 4, 8, 12 and 16 weeks. Samples underwent histomorphometric analysis and radiological analysis. The results showed the superiority of nano-groups in the percentage of new bone formation (26.62±10.88) over micro-groups (14.36±8.4) to (P-value = 0.015). Obtained Hounsfield number for micro-particle group was 2477±480 and for nanoparticle group was 1387±429 (p-value = 0.001). The differences in value soft bone vitality, inflammation, and foreign body reaction were not significant between the two groups of micro and nano. In micro particle group, despite suitable biocompatibility and Osseo integration, due to higher density and degree of crystalline, absorption and replacement rates by new bone and overall percentage of new bone formed were lower than nano group.

Keywords: Tooth powder, Bone repair, Rabbits, Graft material


INTRODUCTION

Every year, about 2.2 million bone transplants are performed worldwide in orthopedic, neurosurgery, and oral and maxillofacial surgeries whose annual cost is around 2.5 billion dollars.¹ An ideal material for bone substitution should have four main characteristics:

1. Osseointegration means the ability to create chemical bond with the bone surface without an intermediate fibrous tissue layer. The term osseointegration in an article by Albrektsson et al. defined as the direct contact between living bone and implanted material observed by light microscopy.³ The material that is so toxic to tissues that do not allow osteoconduction will not be osseointegrated.

2. Osteoconduction means the ability to conduct and support the growth of bone by penetration of capillaries and bone-building cells into the graft material. Osteoconduction is defined as the ability of bone growth on a surface. A surface with osteoconductive properties will allow bone growth on that surface or within its pores.³

3. Osteoinduction means the induction of differentiation of polyvalent stem cells of the surrounding tissues to osteoblastic phenotypes to produce bone. Several papers have been published on heterotopic bone formation in various host locations (such as the anterior chamber of the eye or muscle gap).⁴ The surest way to show whether or not a matter has osteoinductive property is to create bag in a soft tissue such as muscle (where bone formation does not happen in natural conditions) and study bone formation by putting the matter there.

4. Osteogenesis is the ability of the graft material to produce new bone. New bone formation requires the presence of osteoblast cells in connective tissue.⁵ Osteogenic grafting materials have living cells capable to produce bone (osteogenic cells) or with the ability to differentiate into bone-forming cells (progenitor cells) that participate in the early stages of recovery. Osteogenesis, a property that exists only in fresh autogenous bone and bone marrow cells. Bone replacement materials are
classified into four general categories: autogenous, allogeneic, alloplastic (fabricated) and xenogeneic graft materials.

**Autogenous graft materials**

Known as the best choice in this area, due to their osteogenic, osteoinduction, and osteoconduction properties and compatibility with the immune system. However, a sufficient number of progenitor cells survive and osteogenic properties of this type of transplant are obtained from them. Small amounts of autogenous bone graft can be extracted from intraoral places like outer surface of ramous of mandible and chin. In cases where there is need for greater volume of autogenous bone, it can be elevated by obtaining it from iliac crest bone. Among the complications of using autogenous bone, donor site morbidity can be noted. In addition; autogenous bone grafts are prone to resorption. Among other limitation of this kind, the high cost of surgery for bone extraction can be noted.

**Allogenic graft materials**

Obtained by extracting bone from dead or the living resources during hip arthroplasty and have both osteoinduction and osteoconduction properties. These materials lack osteogenic properties that is due to lack of living cells. The use of allogenic bone graft has the risk of transmitting infectious agents such as HIV, HBV, HCV or toxins.

**Xenogeneic graft materials**

The removal of bones from a living organism and transferring it to another living being of another species. BioOss can be noted that includes non-organic and mineral bovine bone. All organic components have been removed during special processes (heating to 300 °C and processing by sodium hydroxide) leading to the production of apatite porous hydroxyapatite bone chips. This material is deproteinized has osteoconduction properties, and has good long-term stability.

**Fabricated graft materials (alloplast)**

Other fabricated or ceramics materials (non-mineral and non-metallic materials). Ceramics used in the field of biomedicine can be classified into two categories, bio-neutralized and bioactive ones. Among bio-neutralized materials alumina and zirconium can be cited that are identified as muscle and bone implants for oral and maxilla facial uses. The other group is bioactive group consisting of calcium phosphates, bio-glass, and glass ceramics. In the area of calcium phosphate bio-materials, a great deal of research is concentrated on hydroxyapatite (HA) compound. According to the studies conducted, it is reported that the process of HA absorption from bone mineral is different. Bone mineral crystals are nano sized and have a large surface area. These crystals are in an organic matrix and crystals connection to each other is very loose. Thus, absorption by osteoclast occurs quite evenly. Unlike that, HA has micro particle size with less surface area and stronger crystal bonds. In addition, bone mineral materials have demonstrated more biological activity compared to crystalline HA.

Additionally, in vitro and in vivo studies on the release of calcium ions from HA powder with nanoparticle size have shown that the results are similar to bone apatite and are considerably faster than those related to HA powder with micro particle sizes. Thus, nano-sized HA engineering results in improved functional properties because of its particle size compared to high volume and the degree of crystallinity similar to biological apatite, and this in turn has a positive effect on transplanted cell-material interaction in vivo. In numerous articles, the positive impact of nano-sized HA in improving bone regeneration with increase in alkaline phosphatase and mineral matrix synthesis has been proven.

In this paper, using nanotechnology, enamel and dentin is reduced to nano-scale particle size and thus crystalline degree and the properties of HA content of enamel and dentine are made close to the natural source of HA in bone. In the end, in explaining the importance of conducting this study, it should be noted that chemical production of HA has high cost and non-compliance with exact stoichiometry will result in products with high impurities. One of the ways to reduce costs in this field is the extraction of HA from natural resources. Thus, much attention is paid to the human teeth by researchers as one of the donors of intraoral sites due to its chemical similarity to the bone.

**METHODS**

Methodology of this study was Laboratory Experimental Trial. Sampling was conducted randomly on eight samples of male rabbits (according to a similar study). Data is collected manually and entered into SPSS software (version 16) after collection and analyzed using Mann-Whitney test and Fisher-Exact tests. It should be noted that we thank Kish Tissue Regeneration Corporation for sponsoring this study.

**Animal sample and anesthesia**

In this study, eight male rabbits of New Zealand White Rabbit species weighing 2.5 ± 0.5 kg were used that had been prepared by the Faculty of Medical Sciences Research Center of Fars. Rabb
were examined by a veterinarian for health and being eligible to enter the study. Inclusion criteria were age 1 year, no systemic disease, and lack of pathological lesions in the mouth. After receiving permission from Ethics Committee (ID: IR.SSU.REC.1394.117) about studying animal, rabbits were placed in four groups of two. Thirty minutes before surgery, rabbits were sedated with intramuscular injection of Xylazine for 5 mg per kg body weight. Then with IV injection (in marginal auricular vein) of Ketamine HCL 35 mg per kg body weight, samples were anesthetized.

During the study, the rabbits were kept in separate cages and were fed with vegetables water and protein supplements. It should be noted that considering the type of study that was animal study, painless and sterile surgical principles were adhered to and in postoperative antibiotics phase, analgesics and antibiotics were prescribed with appropriate dose to consider ethical considerations of animal studies. Controlling nutrition, light, air, and temperature of the animals’ shelter was done to provide suitable living conditions for them.

Preparation of dental particles for grafting
Completely impacted wisdom teeth removed surgically were washed and disinfected with 70% ethyl alcohol and any soft tissues attached to the teeth were removed. Teeth were kept in sterile physiological serum and were sent for the production of tooth powder. After all organic matters were extracted, they were milled for 5 hours with fast mill (Maybod Ceramic) along with solvent that was ethanol. Then the solution was filtered by the filter paper and then dried at 100 °C for 24 hours in the dryer, and finally by means of shakers (AZARPARS), particles to the size of 500µm were achieved.

Second stage was converting the particles into nanometer-size. First, solutions in ethanol were prepared from tooth powder, milled for extra 5 hours by the mill, then added to the solution of dispersant and rotated for 48 hours by a magnetic stirrer. After smoothing solution, powders were dried for 5 hours at 100 °C by AE54 dryer. Finally, the chemical compositions, identification of phases, the crystallite size were studied using X (XRD) radiotherapy with the help of X-ray diffraction device. One way to determine the grain size is using the peak in X-ray diffraction pattern known as Scherrer method. Scherrer did not consider the role of network strain in widening of the peak and considered all inherent widening related to fragmentation of seeds and proposed the following equation:

**Relation 1:** \( t = 0.89 \frac{\lambda}{\beta \cos \theta} \)

In this relation, \( t \) is grain size, \( \lambda \) is the wavelength used (for coils of copper equal to 1.5418 nm), \( \beta \) is the peak width selected at half height in radians, and \( \theta \) is peak angle in degrees (27, 28). The surface structure of tooth powder was identified by the SEM (Scanning electron microscopy) (S-4800, Hitachi, Japan) and the basic components and their values and Ca/P ratio (also determination of kinds of phases) were determined by EDS (Energy Dispersive X-ray Spectroscopy) (X’PertPRO, Netherlands) connected to the electron microscope. To study the size and morphology of nanoparticles and obtained powders agglomeration (confirmed characteristics of nanoparticles) in this part of the work, the observation of TEM (Philips EM208) microscopes were used that confirmed both their shape and nano sizes (Figure 2).

Finally, about 20 grams of tooth powder with micro-size (500 microns) and 20 grams of nano-sized tooth powder was obtained that was packaged and sealed in 2-gram vials and were sent to the atomic energy center of Taft for sterilization by gamma irradiation (13-15 kGy).

**RESULTS**

**Surgical procedure**
Skin of the surgery area that was tibia bone in this study was shaved and disinfected by 10% povidone-iodine antiseptic solution. For homeostasis
and vasoconstriction, 1.8 cc (a number of dental anesthetic cartridges) of lidocaine hydrochloride solution 1% with epinephrine 1/100000 was injected in the surgery area. A cut at a length of 2.5 cm was created below the joint area over the skin to the bone. Flap with full thickness was set aside (the periosteum was removed without traumatizing and ripping it) to expose the bone. In hamstring area of tibia bone of each leg, a bone defects were created through the standard method. This was done using surgery micro-motor and direct surgical hand pieces 1:1 (NSK, Japan) and by 3 mm round carbide hand piece bur with a rotation speed of 2000 rpm, buccal cortical bone and cancellous bone beneath were removed until reaching lingual cortical bone. Defects were prepared as 6 × 6 mm rectangle. The bone was continuously washed during preparing the cavity by sterile saline to prevent heat damage. Then the defects were completely filled by tooth powder prepared.

On one side, the nano-scale particles, and on the other particles in micro-size were placed in bone defects. Dental powder was turned into dough before placement in the defect with rabbit blood. The tooth powder in place was covered with resorbable collagen membrane (Kish Tissue Regeneration Corporation, Iran) at the size of 1 × 1.5 and the membrane was stabilized on both sides by two 3 mm pins (Figure 3 and 4). Periosteum was sutured by absorbable sutures 4.0 (Ethicon, Somerville, NJ, USA). Cutting of the skin was sutured with non-absorbable nylon 3.0 (Ethicon, Somerville, NJ, USA) was closed.

After surgery, to control pain and swelling, for three days, daily 0.1 ml ketoprofen under the skin. Moreover, to 0.6 ml of enrofloxacin (Baytril, Bayer Corp, KS, USA) was prescribed under the skin. During the perioperative period, rabbits were under constant surveillance. After 4, 8, 12, 16 week and each time two rabbits were sacrificed using ketamine overdose.

**Radiological process**

After sacrificing the samples, separating the tibia was done by surgery and was placed into a 50-cc...

![Figure 3](image-url)  
**Figure 3** The bone defect created in antero medial area of tibia

![Figure 4](image-url)  
**Figure 4** Fixation of membrane by two membrane tags

![Figure 5](image-url)  
**Figure 5** CBCT image of micro powder graft (A (4 weeks), (B (16 weeks))

![Figure 6](image-url)  
**Figure 6** CBCT image of graft powder: (A (4 weeks), (B (16 weeks))
syringe body and Digital chips were created by Cone Beam CT-Scan. Then by the special software density was spotted and healthy bone around was determined (Figures 5 and 6).

**Histopathological process and microscopic observation**

In order to prepare histological sections in defects areas, 5 mm behind the defect and 5 mm ahead were cut and the samples were extracted. Then the samples were immersed and fixed in 10% neutral and buffer formalin in the refrigerator. Then the samples were placed in formic acid 10% decalcified (as the hard tissue is required to be decalcified to prepare block sections) and dehydrated in alcohol (as the presence of water will prevent paraffin penetration for preparation) and the samples were placed in xylol (as an substitute to water).

Finally, the samples were embedded in liquid paraffin at 50 °C. After the penetration of paraffin to the samples, the serial of the sections was cut with a thickness of 3 to 4 micrometers by microtome, placed on a microscope slide, and stained with hematoxylin eosin methods, and examined by an oral and maxillofacial pathologist colleague using a light microscope (Olympus Optical co., Ltd, Japan) at 40x magnification. Bone quantity and quality were examined histomorphometrically.

Counting was done in five random fields at 40X magnification and then using the formula below, average of counting was obtained as Labeling index in each field:

**Relation 2:**

\[
100 \times \frac{\text{The total number or percentage of variable investigated in 5 randomized field}}{5}
\]

In this study, the following variables were evaluated: 1) the severity of inflammation: as the average number of lymphocytes in each field of microscope with a magnification of 40 times: Grade 0: absence of inflammatory cells, Grade 1: mild inflammation: the scattered presence of inflammatory cells, Grade 2: presence of 5 to 10 inflammatory cells, Grade 3: presence of 11 to 50 inflammatory cells, Grade 4: presence of more than 50 inflammatory cells, 2) presence or absence of foreign body reaction (grade 1 and 0, respectively) that due to the presence of giant cells, foreign body was found in one granulomatous reaction. 3) The amount of bone made that is on average, the percentage of bone formation in the grafted area regardless of its density with 40 times magnification. 4) The amount of residual graft material, on average, is the percentage of remaining graft material in the grafting area with 40 times magnification. 5) Bone vitality: the presence of osteocytes in trabecular bone Lacuna was a sign of vitality in bone and in the absence of osteocytes in trabecular bone Lacuna was considered as a non-viable bone. 6) Radiological density: in CBCT imaging to assess bone quantity and quality, bone relative density was calculated according to calibrating gray-value scale: air (-1000), water (0) and bones (from 350 to +1000) (Figures 7-10).

**Related studies**

Numerous articles are presented on the production of HA from natural resources such as marine crustacean shells\(^27\) and eggshell,\(^28\) one of the methods suitable for manufacturing ceramic material of HA can be extracting from human dental tissue. Another study conducted in this regard is by X Qin et al.\(^28\), which was conducted in 2014. They extracted lower left central teeth of 18 rabbits, shaped them as pieces of 8mm, placed them in the same size bone defects they had created in mandible and fixed them to the bone bed by a titanium screw of 5×1.5 mm. They sacrificed test cases at 3 and 6 months intervals and examined them histologically and radiographic.

The results showed that the gap between graft and host beds will gradually be filled by new bone and lead to ankylosis of tooth graft to the bone bed. The border between the tooth graft and the bone bed that was first radiolucent was gradually added to radiopacity. In a study by Pilloni et al.\(^29\) in 2014 on the effect of nano-HA on osteoblasts, it was revealed that HA becoming nano increases proliferation, differentiation, adhesion strength, osteoblast scattering, increased expression of bone morphogenetic protein (BMP), and increased bone conduction properties of bone and therefore improving the regeneration of bone.

Rohit Jain et al. (2014) conducted a study regarding comparing the effect of nano-HA powder with particle size of 20 nm and beta-tricalcium phosphate powder with particle size of 500µm to

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**Figure 7** Microscopic images of foreign body reaction (A- magnification of 400 ×, B- 100 ×) (Micro Group -4 weeks)

**Figure 8** Lamellar bone with new osteoid border (A- magnification of 400 ×, B- 100 ×) (Nano Group -4 weeks)
1000µm in the treatment of periodontal inside bone defects. They studied inside-bone defect filling at 3 and 6 months examined. The results showed that after three months in nano-group more bone defect was filled compared to micro group, but after six months, the same bone defect was filled in both groups.

DISCUSSION

According to Table 1, Mann-Whitney test, and P-values offered, values of variables of new bone, remaining bone graft material, and Hounsfield of reconstructed bones between the two groups have significant differences. According to Table 2, there is no significant difference between the two micro-size and nano-size groups in the rate of bone vitality.

Bone substitute material is a suitable alternative to autogenuously bone graft in the regeneration and repair of bone defects including jaw and face. The challenge that exists in the use of bone substitute materials is their biodegradability and succession by new bone. One of the materials used for repairing bone defects that has received much attention is hydroxyapatite. HA has chemical similarities to bone and tooth tissue. In this study, teeth were used as a natural source of HA and prepared at micro (500µm) and nano (up to 100 nm) particle size. The reason to use nano size is that mineral crystals of bone are nano-sized, have large surface, and their absorption process is different from larger crystals.

Histomorphometric analysis of the samples in this study showed that in nano powder group, HA obtained from the teeth significantly makes higher rate of new bone formation (26.62%) compared to micro-size powder (14.36%). These results are consistent with the results of Schnettler et al. (2004). According to the study it was found that nano-HA attaches to the bone and by stimulating osteoblast activity stimulates bone repair. Early absorption of HA nanoparticles after 12 weeks was reported by Thorwarth et al. in 2005, which can explain the reality of why in nano group after 1-month intervals, more new bone is formed compared to the same period in micro group. In a study conducted in 2007, Strietzel et al. found that nano-crystalline of HA are different from HA microcrystalline in terms of dissolution and degradation, where the particle size of nano-crystals leads to faster dissolving and replacing by host bone. This is consistent with lack of decomposition of micro-crystals of HA and their stability until the end of the fourth month. However, new bone formation was observed around HA microcrystalline whose amount was significantly less than nano-hydroxyapatite (P-value <0.05).

Nanoparticles provide more levels to connect osteoblasts compared to micro particles and lead to increased connectivity, proliferation, and differentiation of osteoblasts. The reason behind the effect of these HA nano-particles is that these nanoparticles due to the smaller size enter osteoblast cells more easily (by endocytosis or other mechanisms) and with lysosomal degradation in the cells, they produce calcium ion and by increase in the amount of calcium ions in the cytoplasm, cell moves towards proliferation and differentiation. This confirms the results of our research.

<table>
<thead>
<tr>
<th>Particles</th>
<th>Inflammation (Grade 0-6)</th>
<th>Foreign body reaction (grade 0 and 1)</th>
<th>New bone (%)</th>
<th>The remaining graft material (%)</th>
<th>Reconstructed bone Hounsfield number</th>
</tr>
</thead>
<tbody>
<tr>
<td>Micro</td>
<td>1.85±1.17 MD=2.2</td>
<td>0.15±0.18 MD=0.05</td>
<td>14.36±8.4 MD=13.7</td>
<td>15.41±10.6 MD=17.75</td>
<td>2477±480 MD=2670</td>
</tr>
<tr>
<td>Nano</td>
<td>2.05±0.74 MD=1.8</td>
<td>0.12±0.15 MD=0.05</td>
<td>26.62±10.88 MD=27.5</td>
<td>35.64±18.2 MD=37.5</td>
<td>1387±429 MD=1382</td>
</tr>
<tr>
<td>P-value</td>
<td>0.87</td>
<td>0.87</td>
<td>0.015</td>
<td>0.010</td>
<td>0.001</td>
</tr>
</tbody>
</table>
The values of the variables severity of inflammation, foreign body reaction, and bone vitality did not have significant differences in nano and micro groups. In a study by Motskin et al., it was proved that HA with nano-sized particles is toxic to macrophages and inflammatory cells and leads to their proliferation reduction, and the reason for that is expressed as HA nanoparticles look for easier entry into the cell and lysosomal degradation leads to increased cytoplasmic calcium ion, leading to cell death. This result may be due to different methods of preparing nanoparticles that in their research was as Sol-Gel method and the use of different dispersants that prevents the agglutination of nanoparticles to facilitate entry into the cell.

Bone vitality was reported negative in two cases of micro and one in nano group and positive for the rest. Foreign body reaction in three cases of micro group (weeks 4 and 16) and two cases of nano group (week 12) was positive, all of which have been associated with inflammation.

The possibility of moving of graft materials or other clinical factors as a cause of these reactions is considered. The percentage of residual graft material in both groups has been downward over time, which marks the gradual absorption and replacement by new bone. However, in micro group despite less percent of new bone formation, the percentage of graft material remains was significantly lower than the nano group. Among its causes, dislodging of larger micro-grafting particles from the tissue when cutting for preparing slides due to more hardness and non-cutting of these particles in several slides of micro group can be noted that leads to lower average in micro group.

In this study, to compare the density of regenerative bone in two micro and nano groups of grafting material Hounsfield number from CBCT images is used. Quantity of bone density can be obtained objectively from CT and CBCT images in the form of Hounsfield number. Obtained Hounsfield number related to micro-particle group (2477±480) and about the nano-particle group (1387±429) (p=0.001). The difference values of bone vitality, inflammation, and foreign body reaction, were not significant between the two groups. The amount of new bone formed in samples with nano powder was significantly more than micro group. Inflammation, foreign body reaction, and bone vitality was not significantly different between the two groups. Moreover, radiographic density of the repaired area in the micro group was significantly higher than nano group.

### REFERENCES