A new bone adhesive to fix mandible fractures in New Zealand rabbits: cytotoxicity assay and comparison of bone formation with conventional plate and screw method

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Statement of the Problem: Using plate and screws as the conventional bone fixation method in maxillofacial fractures leads to many complications as plate exposure, infection or unpleasant feeling on touching. Finding a substitute fixation method has been a far desire for many years.

Purpose: This study compared the new bone formation using an experimental bone adhesive containing a functional monomer (benzophenone tetracarboxylic di-methacrylate, BTDMA) and the conventional plate and screw in fractured mandibles of rabbit.

Materials and Method: This is an experimental animal study. The artificial fractures were induced at the mandibular angles of three male New Zealand rabbits. Screw and plate were used as control and titanium mesh with the resin-based bone adhesive containing 15 wt. % BTDMA monomer were applied as treatment. The mandible radiography were obtained and the density of the fracture line was compared to the control. The newly formed bone was assessed by a microscope.

Results: The results obtained from the MTT cytotoxicity assay showed that 70% of cells were able to grow in the presence of the adhesive. The radiographic density of mesh-adhesive specimens was 119.88±76.29, while conventional plate specimens' density was 120.38±73.89. The average new bone formation score in the mesh specimens and plate specimens was 3.67±4.62 and 7±4.36, respectively. There was no significant difference between the two groups. The application of bone adhesive containing 15% BTDMA monomer in a group of the rabbits showed lamellar bone formation.

Conclusion: Using bone adhesives containing BTDMA could lead to a new bone formation with high density in the case of adequate bonding to the fractured area.

Keywords: Fractures; Bone; Bone cements; Osteogenesis.

Introduction

Trauma on the facial area may lead to damage to soft tissues, teeth, and facial skeletons, including mandible, maxilla, zygomaticomaxillary complex, naso-orbital-ethmoid complex, and ophthalmic structures [1]. The conventional method of treating these fractures is to limit the fractured parts to their original anatomical

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position and then fixing those using conventional screws and plaques. In this method, plaques and screws are necessary to remain at the place until the repair is completed [2]. The plate and screws are made of stainless steel, vitalium, titanium and titanium alloys. These materials have advantages such as high biocompatibility, low allergic potential, and excellent corrosion resistance [3]. The use of the screws and plaques, however, can lead to unfavorable reactions, infections, screw loosening, and sensitivity to heat and cold [4]. Also, in fragile areas of the skin or mucosa, the plates or screws may become noted, touched, or even exposed, which is associated with a higher risk of infection [5]. It has been shown that the application of screws, whose length is more than bone thickness, damages internal anatomical structures such as the vessels, nerves, and the root of the teeth [6].

The maxillofacial bones and structures are tenuous, so their fractures are relatively small. Screws in these areas with thin and fragile bones where the fractures are small can break the bony segments apart and cause further complications such as new fractures while inserting [7,8]. Due to their easiness and quickness, using adhesives is a simple approach to substitute screws [9]. The advantages of using adhesives include better distribution of force between two bone fragments [10]. In this method, force spreads through a large adhesion surface rather than concentrating on the small stress-full screwed contact points [11]. Furthermore, bone adhesives can avoid stress and strain on the fracture area while inserting screws [12]. Adhesives are designed to repair various body tissue damage, including bone and cartilage fractures and also tendon and ligament rupture [13].

Amongst the materials which are used in tissue adhesives, due to its non-biocompatibility and high risk of infection, cyanoacrylate is not confirmed, yet [14]. Methyl methacrylate and fibrin also do not have enough durability under the considered conditions [15]. Other adhesives, such as fibrin and protein-aldehyde, are used for soft tissue and are not well-applied to bones [16]. In orthopedic surgeries to achieve proper bonding between the adhesive and bone, the dense and cortical outer bone layer is removed to reach the cancellous bone structure [10]. The spongy structure of the cancellous bone provides better penetration of the adhesives and increases its strength [17]. In maxillofacial surgery, because of the difference and importance of the surface structure of the facial bones, it is not possible to remove the cortical bone, so the bonding between adhesive and bone must be obtained directly on the smooth not-permeable cortex. However, the hydrophilic nature of the bone does not allow the bone to be wetted and impregnated with the hydrophobic monomers such as methyl methacrylate [18]. It indicates the necessity of an amphipathic binding agent. In addition to the bond’s strength, other factors such as biocompatibility, repair induction ability, neo-osteogenesis, infection rate, immune response, chronic inflammation, and tissue necrosis should be determined. In this study, controlled artificial mandible fractures in New Zealand rabbits were prepared. The fractures were then fixed using two methods; i: applying an experimental bone adhesive containing a functional monomer (BTDMA) and, ii: the conventional screw and plaque method. The amount and quality of the formed bone was then assessed and compared. The cytotoxicity of the adhesive was also evaluated through MTT test.

Materials and Methods

Three male New Zealand rabbits with an average weight of 3 kg were obtained from the animal laboratory of Baqiyatallah University of Medical Sciences. The research protocol was approved under IR.TUMS.DENTISTRY.REC.1396.4156. Our protocol was designed and conducted based on the ethical guidelines for research on experimental animal studies available at http://ethics.research.ac.ir.

3,3′,4,4′ benzophenone tetracarboxylic dimethacrylate (BTDMA) monomer was synthesized according to reference [19]. Briefly, 0.01 mol of 3,3′,4,4′-benzophenone tetracarboxylic dianhydride (BTDA, Aldrich) and reacted with 0.025 mol of 2-hydroxyethyl methacrylate (HEMA, Aldrich) in 30 mL of N, N-dimethylformamide (DMF; Merck) as solvent in a three-necked flask equipped with amagnetic stirrer, reflux condenser, nitrogen inlet tube and 0.008 g of HQ as an inhibitor. The reaction was performed at 60°C for 16 h. After completion of the reaction, the product was washed with distilled water and separated by a centrifuge. The product was then confirmed using FTIR (Equinox 55, Bruker, Germany) and 1H-NMR (Avance 400, Bruker, Germany) techniques. Figure 1 shows the reaction route.

A base adhesive containing di- and tri-methacrylate monomers and ethanol as solvent was first prepared. To prepare a dual-cure adhesive, the base adhesive was separated in 2 parts. In one part, benzoyl peroxide (Merck, Germany) as a chemical initiator and camphorquinone (Aldrich, Germany) as a photo initiator were added. In the second part, N, N, dimethyl-p-toluidine (Aldrich, Germany), and N, N dimethyl amino-
ethyl methacrylate (Aldrich, Germany) were added as amine co-initiators. Our previous study showed that the highest mechanical properties of the adhesive were obtained when 15 wt.% BTDMA was added to adhesive [20]. Therefore, 15 wt. % of the synthesized BTDMA was incorporated into both parts of the adhesive. The prepared dual-cure adhesive could be light-cured when exposed to the visible blue light source (wavelength≈470nm) or chemically cured, in the absence of light exposure, 4min after mixing. The dual cure nature of the adhesive provides the following advantages: having applied the adhesive on the fractured surface, the adhesive is cured through visible light irradiation; the deep areas and the surfaces which are not exposed to the light source, are chemically cured through the redox initiator system of the adhesive.

To evaluate the cytotoxicity of the resin-based bone adhesive by MTT assay, disc-shaped specimens were prepared by light-curing the adhesive in a mold (diameter 5mm, height 1mm) while covered with a transparent film to prevent oxygen inhibition. The specimens were then subjected to the MTT assay. The MTT assay is a colorimetric assay for measuring cell metabolic activity. It is based on the ability of nicotinamide adenine dinucleotide phosphate (NADPH)-dependent cellular oxidoreductase enzymes to reduce the tetrazolium dye MTT to its insoluble formazan, which has a purple color [21]. The test results showed that approximately 70% of neo-osteoblasts were able to live and grow (Table 1).

The surgical procedure was performed under deep sedation with ketamine and xylazine intramuscular injection. Local anesthesia and hemostasis induced by injection of lidocaine 2% + epinephrine 0.00001. After skin preparation and draping, we approached both mandibular angle areas with two separate extraoral incisions. Vertical bilateral mandible osteotomy was performed by microsurgical motor and saw handpiece behind the last posterior tooth and front of the masseter muscle attachment site to induce the fracture. (Figures 2 and 3a). The buccal cortex and spongy bone, as well as the lower mandibular border, were cut. In order to protect the lingual tissues, the lingual cortex was broken by elevator twisting (Figure 2) (Figure 3a). In each rabbit, we conducted a conventional plate and screw fixation method on one side and resin adhesive on the other side. Four hole mini plates and mini-screws (0.5mm) were used in all three samples. For each fracture, one plate was used. Two screws were applied at each side of the fracture line (Figure 2). Then the incision was sutured with silk 3-0 cotton. On the other side of each mandible, a titanium mesh (2×1cm) (MON-DEAL, Germany) was applied to the fracture site. The BTDMA containing bone adhesive was placed below and above the mesh. A portable LED device was used to cure the adhesive with blue light (Figure 3b). Both skin incisions in each animal were then sutured by the same technique and material (Simple interrupted stitches by 3-0 silk), and local tetracycline was sprayed on the location (Figure 3b). Cefazolin (50mg/kg/day with 6h intervals) was injected into all animals intramuscually for one week. The wounds were cleaned daily with a 3% hydrogen peroxide solution and saline. The sutures were taken off on day 7. The same postoperative nutritional and medical care was provided for all animals. For the first three days after surgery, the rabbits were treated with dextrose syrup 50%, and after three days, regular feeding started.

After six weeks, the animals were sacrificed by Ketamine and Xylazine injection. The mandible bones were completely resected and were placed in a 10% buffered formalin solution then transferred from the animal research center of Baqiyatallah Hospital to the dental school of Tehran University of Medical Sciences in order to conduct radiography and histology examinations (Figure 4a, b, c). Before decalcification, we took digital radiographs from each side of each mandible (Projection distance of 4 cm, projection angle of 90 degrees, 65 kvp, 300 mA, and 0.12 msec) (Figure 5).

The radio-opacity of new bone formed in both fracture lines of each mandible was then compared by Image J software. (Table 2). The samples were decalcified using 10% and 5% nitric acid and 10% formic acid solutions. 12 histopathologic slides (2 slides from each side of each mandible) of Hematoxylin and Eosin were provided and evaluated by an expert oral pathologist under the light microscope, and the following grades were assigned to each slide:

- Grade 1 indicated the fibrous union.
- Grade 2 indicated predominantly fibrous tissue with some cartilage.
- Grade 3 indicated equal amounts of fibrous tissue and cartilage.
- Grade 4 indicated all cartilage.
- Grade 5 indicated cartilage predominantly with some woven bone.
- Grade 6 indicated equal amounts of cartilage and woven bone.
- Grade 7 indicated predominantly woven bone with...
some cartilage.

- Grade 8 indicated woven bone.
- Grade 9 indicated woven bone and some lamellar bone.
- Grade 10 indicated lamellar bone.

The average score was calculated for each sample. The final data were analyzed using a t-test by SPSS 20 software. (Figure 6a, 6b, Figure 7, Figure 8, Figure 9).

Table 1. Histologic grading of ossification in each sample, the highest ossification grade belongs to the plate side of rabbit three and the lowest grade belong to the adhesive side of rabbit 2 and 3.

<table>
<thead>
<tr>
<th>Rabbit number</th>
<th>Fixation method</th>
<th>Microscopic ossification grade</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Adhesive and mesh</td>
<td>9</td>
</tr>
<tr>
<td></td>
<td>Plate and screw</td>
<td>2</td>
</tr>
<tr>
<td>2</td>
<td>Adhesive and mesh</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>Plate and screw</td>
<td>9</td>
</tr>
<tr>
<td>3</td>
<td>Adhesive and mesh</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>Plate and screw</td>
<td>10</td>
</tr>
</tbody>
</table>

Table 2. The radiographic result showing the radio-opacity calculated by image J at each sample’s fracture line.

<table>
<thead>
<tr>
<th>Rabbit number</th>
<th>Adhesive</th>
<th>Plate</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>206.20</td>
<td>35.86</td>
</tr>
<tr>
<td>2</td>
<td>91.96</td>
<td>152.54</td>
</tr>
<tr>
<td>3</td>
<td>61.49</td>
<td>172.75</td>
</tr>
</tbody>
</table>

Figure 1. Synthesis of BTDMA.
**Figure 2.** Bone adhesive two bottle composition. Bottle A and B had the same amounts then we dropped out of each bottle and mixed on a glass slab. Then it was transferred to the fracture site.

**Figure 3.**

- **a:** vertical osteotomy of one mandible side.
- **b:** fixation of this side fracture by one titanium plate and four 0.5 mm screws.

**Figure 4.**

- **a:** vertical osteotomy of one side of mandible.
- **b:** blue visible light exposure to the adhesive.
- **c:** fixation of this side by dual-cure bone adhesive applied by a passive titanium mesh.

**Figure 5.** The resected mandible was prepared for histological assay.

**Figure 6.**

- **a:** digital radiograph of one animal “plate and screw” side of mandible.
Results

Cytotoxicity test

The results of the MTT cell toxicity test are presented in Figure 1. At zero time point, the level of cytotoxicity in each group was 100%, which continuously reduced (chart 1). It became 64% 72 hours. It was estimated 80% and 75% in samples 1 and 2, respectively.

Histopathologic analysis

Three New Zealand rabbit jaws were evaluated. After six weeks, the radiographs were taken from both sides of the jawbones, and the images were evaluated using ImageJ software. The evaluation of the new bone formation on the fracture line was carried out by a histologic scale from 1 to 10. According to the results of the study, about 33% of the samples responded positively to the bone adhesive, while the rest on the plate side showed evidence of lamellar bone formation.

The average grade of ossification at the mesh and adhesive side was 3.67, indicating the formation of cartilage. The average grade on the plate and screw side was 7, which indicates the formation of lamellar and cartilage bones. The highest level of ossification was reported on the plate side of the third rabbit. The results of the paired t-test showed that there was not any significant difference in the degree of ossification on both sides (plate and adhesive) (p=0.59). Table 1 shows the histological grading analysis, which is by the pathologist. The grading of bone tissue in rabbit number 1 showed lamellar bone formation on the side treated with adhesive and the presence of fibrous tissue on the...
plate surface. Also, the presence of mature lamellar bone in the side plate in rabbits 2 and 3 and the presence of fibrous tissue on the side in which was treated with adhesive was evident. The average total ossification at the plate side was seven, and at the adhesive side was approximately four. Grade 7 indicates the presence of bones and cartilage, and grade 4 shows the presence of cartilage and lack lamellar bone. In general, the presence of bone marks on the side treated with adhesives was evident, but its amounts were higher in the conventional method.

The radiographic density of mesh and plate samples was 119.98±76.29, and 120.38±73.89, respectively. Also, the average oscillation size of the locations with mesh and plate were 3.67±4.62 and 7±4.36, respectively. In other words, the amount of bone on the locations treated with the conventional method was better than that of treated with adhesive. Furthermore, there was not any significant difference in terms of average oscillation and also radioactivity levels on both sides with the plate and mesh.

**Discussion**

In this study, the amount and quality of new bone formation in the conventional method of fixing bone fragments by screw and plates on the jaw of three rabbits were compared to the application of bone adhesive containing BTDMA monomer. Based on previous pieces of evidence, the application of glue has a significantly more impact on bone formation and also in the prevention of tissue-fabricating. Furthermore, according to radiographic evidence, the lamellar bone was formed on the plate side, while fibrous tissue was observed on the mesh side.

Al-Jandan et al. [22], in their study, examined bone regeneration following the application of complementary adhesives. They observed evidence of bone formation in both jaw regions. They showed that stronger bonds and bone volume are present on the side of intervention, indicating the effects of demineralized bone matrix (DBM). Despite the different protocols used, their observations are consistent with the findings of the present study. In another study, Dadas et al. [23] evaluated the effects of butyl cyanoacrylate for the prognosis of zygomatic bone as well as histologic toxicity and body responsiveness (as a foreign body). They reported that tissue responses are present in both control and treatment groups. The BTDMA monomer used in this study contains methacrylate C=C double bonds in its structure which enable the monomer to be copolymerized with the di- and tri-methacrylate monomers in the adhesive and provide a strong cross-linked network. The presence of carboxylic acid functional groups in the structure of BTDMA provides its possible ionic interactions with the Ca\(^{2+}\) of the bone resulting in a strong and durable adhesion [19]. Furthermore, the presence of rigid benzyl rings in the BTDMA structure provides higher cohesive properties for the cured adhesive. Due to the presence of the carboxylic acid groups in the structure of the functional monomer, it exhibits higher hydrophilicity which helps the adhesive to better wet the bone surface and penetrate the bone porosities. BTDMA can potentially be used instead of the Bis-GMA or as one of the compounds of monomeric resin systems. Due to its mechanical properties and high water absorption capacity, this monomer can be added to polyacid-enhanced resins [24]. This article is extracted from a research project (Code: 36688) sponsored by Cranio Maxillo Facial Research Center (CMFRC), Tehran University of Medical Sciences (TUMS).

**Conclusion**

The bone adhesives containing BTDMA in some samples of our study have produced mature lamellar bones. Therefore, the application of polymeric bone cement and adhesives in optimal situations can lead to high-density bone formation.

**Conflict of Interest**

There is no conflict of interest to declare.

**References**


