Gentamicin-loaded borate bioactive glass eradicating osteomyelitis of *Escherichia coli* in a rabbit model

Zongping Xie\(^a\), Xu Cui\(^b\), Cunju Zhao\(^a\), Wenhai Huang\(^b\), Jianqiang Wang\(^c\), Changqing Zhang\(^*a\)

\(^a\)Department of Orthopaedic Surgery, Shanghai Sixth People’s Hospital, Jiaotong University, Shanghai, 200233, China

\(^b\)Institute of Bioengineering & Information Technology Materials, School of Material Science and Engineering, Tongji University, Shanghai 200092, China

\(^c\)Department of Microbiology Laboratory, Shanghai Sixth People’s Hospital, Jiaotong University, Shanghai, 200233, China

\(^*\)Corresponding-Author: Department of Orthopaedic Surgery, Shanghai Sixth People’s Hospital, Jiaotong University, Shanghai, 200233, (China); Tel: +8613003104089; Fax: +86-21-64701361;

E-mail: x91034@yahoo.com.cn.

Running Title: Gentamicin-loaded borate bioactive glass and *Escherichia coli*
Abstract: The treatment of osteomyelitis induced by Gram-negative bacilli is rarely reported in the literature. This study established a rabbit tibia model of osteomyelitis induced by Gram-negative bacilli *Escherichia coli*. Using this model, pellets composed of a chitosan-bonded mixture of borate bioactive glass and gentamicin were evaluated in vitro and in vivo for treating osteomyelitis induced by *Escherichia coli*. Our results showed that the pellets in phosphate buffered saline released gentamicin continuously over 26 days. Without co-using systematic antibiotic, the implantation of the gentamicin-loaded pellets into the osteomyelitis region of the tibia resulted in the eradication of 81.82% infections based on microbiological, histological and radiographic evaluation, and supported the ingrowth of new bone into the tibia defects after 6 weeks of implantation. The results indicate that the gentamicin-loaded borate bioactive glass implant, combining sustained drug release with the ability to support new bone formation, could provide a method for treating osteomyelitis induced by Gram-negative bacilli.

Keywords: Borate glass; Osteomyelitis; Gentamicin; *Escherichia coli*;
1. Introduction

Osteomyelitis needs a prolonged administration of systemic antibiotics, multiple surgical procedures and a long-term hospitalization in China. While an uncertain prognosis and high relapse are present, a huge therapeutic cost remains challenge to the orthopaedic surgeon. Theoretically, local antibiotic delivery system is a reasonable solution, which is capable to offer a high dosage to the infected region without systemic toxicity and benefit to the management of the dead space [1, 2]. The first product on the market for local treatment of infected bone is polymethylmethacrylate (PMMA) antibiotic beads (such as Septopal®). However, over 90% of the trapped drug remain inside the cement beads. Furthermore, bone cement has to be removed by a second surgical procedure [3]. Biodegradable polymers such as polylactic/polyglycolide (PLGA), chitosan or collagen were also used as antibiotic-loaded systems. Except they cannot support osteoconductive bone ingrowth into the void, the systems cannot maintain a constant release of antibiotics to cure the infection [3]. To overcome these major limitations, antibiotics were added to osteoconductive bone fillers such as calcium sulfate hemihydrates pellets [4] and tricalcium phosphate (TCP) [5]. A limitation of TCP is that it degrades too slowly to match the bone regeneration rate [6]. Calcium sulfate has disadvantages such as transient cytotoxic effect to lead to inflammatory reactions, the insufficient ability to stimulate bone regeneration, a high resorption rate, quick elution in vitro and friability, and the low mechanical strength [7].

Borate glasses were developed by replacing larger amounts of silicon dioxide (SiO₂) in 45S5 bioactive glass with boric oxide (B₂O₃) and were found to have controlled and complete
degradation properties, fair biocompatibility and bioactivity, and the ability to support the growth and differentiation of human mesenchymal stem cells[8-13]. Our previous works have shown that borate glasses are successful as vancomycin and teicoplanin carriers in eradicating osteomyelitis induced by methicillin-resistant *Staphylococcus aureus* (MRSA) in a rabbit model, showing excellent biocompatibility and compressive strength, full osteointegration with direct apposition of the newly formed bone, and stimulating bone regeneration as its degradation[7,13].

*Staphylococcus aureus* remains the most common causative pathogen in osteomyelitis; however, Gram-negative bacilli are more common in adults with chronic or nosocomially acquired infections [14-17]. In view vancomycin and teicoplanin are not active against Gram-negative bacteria, the purpose of this study was to establish a model of osteomyelitis induced by *Escherichia coli* and to further evaluate the effects of borate glasses as gentamicin carriers on treating osteomyelitis induced by Gram-negative bacteria in rabbits.

2. Materials and Methods

2.1 Fabrication of gentamicin-loaded borate bioactive glass (GBBG) pellets

Borate bioactive glass with the composition (mol%) of 6Na₂O, 8K₂O, 8MgO, 22CaO, 54B₂O₃, 2P₂O₅ was prepared by mixing the required amounts of Na₂CO₃, K₂CO₃, MgCO₃, CaCO₃, H₃BO₃ NaH₂PO₄, (analytical grade, Sinopharm Chemical Reagent Co., Ltd. China) and melting the mixture in a platinum crucible for ~2 h at 1200°C. After the melting was quenched between cold steel plates, the glass was crushed, ground, and sieved to give particle size <50 μm.
The preparation of drug-loaded pellets: Gentamicin powder [Hualuan Ltd. Of North China Pharmaceutical Group Corporation (NCPC), China], glass particles, and chitosan solution (98% deacetylated), citric acid and glucose (Sinopharm Chemical Reagent Co., Ltd. China) were mixed according to a ratio of 11/23/48:500:200 by weight respectively, while the amounts of chitosan, citric acid, and glucose in the solution was 1:10:20 by weight. The mixture was filled into a polyethylene mold, allowed to harden for 30 min, and dried for 24 h. Three kinds of pellets containing respectively 16, 32 and 64 mg/g gentamicin were obtained. All pellets were prepared under sterile conditions. The gentamicin-loaded pellets were designated GBBG.

2.2 In vitro elution study

Three kinds of pellets were weighed and calculated for the amounts of gentamicin theoretically before elution. Each size pellet was placed into 20 ml phosphate buffered saline (PBS, pH 7.4, 37°C) that was collected and replaced every 48 hrs until 26 days. Each collection of PBS elute was stored at −20°C and the gentamicin concentration diffused from the pellets was measured from PBS elute within 7 days after collection. Each elution series was replicated three times.

The antibiotic concentrations in the PBS eluate were determined by a high performance liquid chromatographic (HPLC) from the assay standard curves for gentamicin. The low limit of detection was 0.1 µg/ml of gentamicin. The HPLC analyses were conducted on a Walters 600 Multisolvent Delivery System (Agilent Technologies, Palo Alto, CA, USA). The recovery rates were obtained through dividing theoretical amount by released amount.
2.3 Bacterial preparation

A standard strain of *E. coli* ATCC25922 (Department of Microbiology Laboratory of Shanghai Sixth People’s Hospital) was used, which was sensitive to gentamicin. The bacteria was stored on nutrient agar slopes (Oxoid, Basingstoke, Hampshire, United Kingdom) at 4°C.

The inocula were prepared by subculturing the bacteria in 10 ml of nutrient broth (Oxoid) overnight at 37°C the day before inoculation. Each broth culture was then diluted in sterile PBS, the density of inoculum was 10⁹ CFU/ml of saline.

2.4 Antibacterial activity

The minimum inhibitory concentration (MIC) for gentamicin against *E. coli* was determined by using an antibiotic twofold tube-dilution method yielding 0.05, 0.1, 0.2, 0.4, 0.8, 1.6, 3.2, 6.4, 12.8, 25.6 µg/ml drug concentrations in two sets (non-processed gentamicin and the released gentamicin from pellets,) of 10 tubes with 2 ml sterile cation-supplemented Mueller-Hinton broth (Oxoid). One tube with no gentamicin (2ml PBS) was served as a control.

The tubes were inoculated with *E. coli* prepared as described previously at a density of 10⁸ CFU/ml. The MIC was the lowest concentration of antibiotic that prevented turbidity after 24 hours of incubation at 37°C.

2.5 Animal model

Thirty-five male New Zealand White, specific-pathogen-free rabbits (8 months old, body mass, 2.34 to 3.4kg, average 2.8kg) were used. The study protocol was approved by the Animal Care Committee of Shanghai Sixth People’s Hospital, Shanghai Jiaotong University, China. All
experiments were carried out, in accordance with the guidelines of the local Animal Welfare Committee. During the study, animals were kept in individual cages in standard conditions (room temperature 20±0.5°C, relative humidity 55±5% and illumination with a 12h / 12h of light / darkness photoperiod), and allowed to be fully active. The rabbits were fed a routine diet.

Under intramuscular anesthetization [0.5 ml/kg of ketamine (42.8 mg / ml) and xylazine (0.7 mg/ml)], the right hind leg of the rabbit was shaved from the knee to the ankle and cleaned with povidone-iodine, and draped with sterile sheets. An 18-gauge needle was inserted percutaneously through the lateral aspect of the proximal tibial metaphysic into the medullary cavity. Following extracting 0.3 ml bone marrow, 0.1 ml 5% sodium morrhuate (Eli Lilly, Japan K. K, IN), 0.1 ml E. coli (1×10^8 CFU/ml) in PBS, and 0.1 ml sterile PBS were injected sequentially.

The animals were physically examined weekly. Infectious symptoms and signs such as weakness, loss of appetite, decreased mobility, degeneration of the operated limb or flexion contracture of the affected knee joint were observed and recorded. The infection was allowed to progress for 3 weeks, at which time the severity of osteomyelitis was determined radiographically.

2.6 Treatment groups

Four rabbits died due to septic complications after induction of osteomyelitis, and were excluded from any further study.

The remaining 31 animals were randomly devided into three groups. Animals in Group 1
(n=11) were treated by debridement and implantation of GBBG beads (32 mg/g). Animals in Group 2 (n=10) were treated by debridement and implantation of pure borate glass pellets and 10 mg of gentamicin powder. Animals in Group 3 (n=10) were treated by debridement only. Each group was sacrificed 9 weeks after infection.

2.7 Debridement and device implantation

All animals were anesthetized again. The right hind leg was shaved and cleaned with povidone-iodine skin cleanser, and draped with sterile sheets again. A longitudinal incision with 2 cm in size was made at the proximal tibial metaphysis. A cortical bone window with the size of 1.5 cm long by 1 cm wide was created. Necrotic tissue was debrided through the window and the surrounding soft tissue until bleeding was observed. Purulent marrow was flushed out with sterile, normal saline, using a 16-gauge catheter attached to a 10-ml syringe, inserted distally and proximally into the intramedullary canal. A sample of the debrided tissues was placed into a sterile tube for bacterial examination. If the entire bone was infected, another window was opened in the distal tibial metaphysis to debride. After the surgical site was irrigated completely, the dead spaces were filled with different implants (~10 pellets) as described previously. The muscle flaps were realigned and the deep fascia and skin were sutured in layers.

2.8 Monitoring of the rabbits

Anteroposterior and lateral radiographs of the right tibia were taken immediately after operation. All rabbits were examined clinically at 48 h intervals for the first week after operation, and weekly thereafter for as long as 6 weeks, with particular attention given to the wound.
healing, the limb mobility, the signs of infection, and the rabbit’s ability to thrive. One rabbit from group 2 died due to an unknown reason, at the first week after debridement, and was excluded from any further examination. One from group 2 and two from group 3 died due to septic complications from 4th to 5th week after debridement, and were included in further analysis, because the data could be obtained and had influence on last treatment effects. In the end of the study, the rabbits were sacrificed by an intravenous injection of 2.5 ml of Euthatal (sodium pentobarbitone. 200 mg/ml). The tibia was harvested for further evaluation.

2.9 Microbiological Evaluation

Specimens were collected post mortem from the bone, marrow and, if any, necrotic tissue at the site of infection; placed in tubes; and sent to the laboratory where the specimens were incubated on blood agar for at least 48 h at 37 °C. The plates were evaluated by a microbiologist at 24 and 72 hours for colony growth. The isolates were analyzed by means of SIEMENS MicroScan WalkAway 40SI NC 31 automatic identification system (SIEMENS, German). The low limit of detection was 1 cfu/g of *E. coli* for the infected tissue.

2.10 Radiographic and Histopathologic Evaluation

Anteroposterior and lateral radiographs of the right tibia were taken at pre-operation, after debridement, and before tibia harvest. One investigator (Jia, WT), blind to the results, independently evaluated the radiographs, to confirm the presence of osteomyelitis by using criteria described by Norden et al. [18]. A numerical score was assigned to each variable. All scores have been added, to form a composite radiological score with a maximum of 6
representing radiographic severity.

The harvested tibia was fixed in 10% formol calcium for 7 days and 4% paraform for 3 days. Bone blocks were coded and then cut from each tibia. The bone blocks were routinely tared and embedded. Sections (5µm thick) in the longitudinal direction of the tibia were stained with hematoxylin and eosin (HE). All sections were examined and scored under conventional light microscopy by one pathologist, who was blind to the implantations and the results of the bacterial cultures. Using the criteria described by Smeltzer et al [19], a numerical score was assigned to each variable, and all scores have been added, to form a composite histopathologic score with a maximum of 16 for representing histopathologic severity. The degradation of implants and bone regeneration were also examined.

2.11 The Analysis Methods for the Data

Statistical analysis was performed by using the SAS6.12 software (SAS Institute Inc., Cary, NC 27513-2414 USA). Fisher's exact test was used to compare the posttreatment positive rates of *E. coli* with each other of the three groups. One-way ANOVA was used to compare bodyweights, the radiographic scores and histopathologic scores of the three groups. If any significant difference was observed, then LSD test was applied to compare each other of the three groups. The paired t-test was applied to compare bodyweight and radiographic score changes, after treatment among each group. Data are reported at a significance level of p < 0.05.

3. Results

3.1. In vitro study
After gentamicin was incorporated into borate glass, the MIC for the *E. coli* was 0.4µg/ml, which was the same as that of non-processed gentamicin. Gentamicin in borate glass powder was gradually released into the surrounding medium at concentrations sufficient to inhibit bacterial growth during 26 days (Fig. 1). The recovered amounts of drug at Day 2 over the total recovered amounts during 26 days were 39.6%, 87.03% and 90.12% (w/w) respectively for 16, 32 and 64mg/g pellets. The cumulative percentages of antibiotic release were 46.56%, 55.59% and 85.3% respectively for 16, 32 and 64mg/g pellets. The burst effect became more intensive when the amounts of gentamicin in the pellets increased. The cumulative percentages of antibiotic release markedly increased along with the amounts of gentamicin in the pellets increasing (Table 1). During all 26 days, the pellets kept its original shape, but became more porous along with the immersed time increasing.

3.2. In vivo study

*E. coli* ATCC25922 was detected in all remaining 31 animals at the third week after injection. The bodyweights of the rabbits at the time of infection, debridement and sacrifice showed no significant differences between three groups (P=0.864, 0.367 and 0.070, respectively; one-way ANOVA) (Fig. 2). The average bodyweights decreased in all three groups at week 3 after infection, but increased by 0.37, 0.01 and 0.24kg, respectively for group 1, 2 and 3 at week 6, in which, only the average bodyweight in group 1 exceeded that before infection. There were no significant differences for the bodyweight changes within group 1 and group 3 (P=0.077 and 0.068, respectively, one-way ANOVA). Significant differences were observed within group 2
between before infection and three weeks after infection, and before infection and six weeks
after treatment (P=0.037 and 0.041, respectively, paired t-test).

Table 2 showed the results of microbiological examination following gentamicin treatment.
Significant differences were observed between group 1 and group 2, group 1 and group 3
(p=0.040 and 0.012, respectively, Fisher’s exact method). GBBG cured 81.82\% osteomyelitis in
group 1.

Table 3 showed the radiographic and histopathologic average scores. There were no
significant differences among radiographic scores before treatment of three groups, but
significant differences were observed after treatment (P=0.000, one-way ANOVA). By LSD
multiple comparisons, significant differences were observed between group 1 and group 2, group
1 and group 3 (p=0.001, and 0.003; respectively). Group 1 had the best result of the radiographic
scores after treatment among three groups. The significant improvement of radiographic
scores of group 1 was observed after treatment (p=0.000, paired t-test). The radiographic scores of
group 2 and 3 worsened after treatment, but there were no significant differences. Significant
differences were observed among histopathologic scores between group 1 and 2, group 1 and
group 3 (p=0.014, and 0.004, respectively, LSD test). Histopathologic average score of group 1
was 3.82±2.75, showing better treatment result than group 2 and group 3.

The typical radiographs of three groups were shown in Fig.3. Three weeks after injection of
*E. coli* into the tibia cavity, radiographic images showed the presence of osteomyelitis in all
animals, along with bone destruction, new periosteal bone formation, and sequestral bone
formation. Six weeks after treatment, most of implants in group 1 had been reabsorbed and replaced by new bone formation, infection were cured. Few implants in group 2 had been absorbed and some were rejected due to infection worsening and poor blood supply. The inflammation and bone destruction obviously deteriorated for both group 2 and 3.

The typical histological findings were shown in Fig. 4. For the most rabbits of group 1, the treated tibia did not show macroscopic enlargement, consisted of resorption of implanted pellets and new bone formation around and inside the implanted pellets. Osteogenic activity characterized by the presence of newly formed bone trabecules with a continuous coating of the osteoblastic cells were clearly observed. There were also some macrophages surrounding the implants, indicating a kind of foreign body reaction. Histopathologic findings in group 2 and 3 consisted of marked infiltration of polymorphonuclear leukocytes, fibrosis with many proliferative lymphocytes and some plasma cells, fat necrosis, necrotic debris with pus cells in the center, sequestral bone formation, destruction of bone and subperiosteal new-bone formation, showing obvious osteomyelitis.

4. Discussion

Although *Staphylococcus aureus* are still the main pathogens causing osteomyelitis, Gram-negative organisms also commonly cause such infections. Burt et al reported that osteomyelitis due to Gram-negative bacteria represented 28% of all cases of osteomyelitis [20]. In one study of 55 patients, 32 patients with chronic osteomyelitis were induced by Gram-positive and/or Gram-negative organisms [14]. But there are very few experimental
models of osteomyelitis induced by Gram-negative organisms. In this study, we have developed a rabbit model of colibacillary osteomyelitis with pathophysiological, clinical, radiographic, and histological characteristics similar to those of human disease. By using our model, osteomyelitis has been developed in all 35 rabbits, with a low rate (11.43%) of mortality and a high rate of recovery of the infecting organism (100%). Then, we have further evaluated the efficacy of borate glass as antibiotic vehicle on our model.

Gentamicin was selected as the antibiotic because it has a strong antibacterial activity against all Gram-negative organisms and most Gram-positive organisms, especially for Enterobacteria, and can keep its activity over six months at room temperature [21]. It also has a joint action with teicoplanin and simultaneously redeems the deficiency of teicoplanin and vancomycin that are not active against all Gram-negative bacteria. Accordingly, while treating combined bone infection caused by Gram-negative and Gram-positive organisms, especially with MRSA, two kinds of pellets respectively loaded with teicoplanin and gentamicin could be used.

As an antibiotic carrier, the borate bioactive glass provides advantages. It can convert rapidly and completely into a hydroxyapatite (HA)-type material when immersed in PBS or simulated body fluid (SBF). This HA-type material has a porous structure that is an important requirement for scaffolds capable of supporting transport of nutrients and new bone ingrowth. After loaded with drug, it therefore served as a multifunction device, initially providing a local drug release function and later serving as a scaffold for bone repair. At the same time, it can keep
its high compressive strength after immersion in SBF, which had been proved by our previous works [7, 13]. It also could be used at the location where load bearing is required.

The MIC of the released gentamicin against the standard *E. coli* strain was identical to that of non-processed gentamicin, which indicates that the antibacterial activity of the gentamicin was not altered following incorporating into the bioactive borate glass. In an attempt to decrease the burst effect of drug release, chitosan was used to bond the mixture of borate bioactive glass particles and gentamicin powder. In vitro, the released gentamicin maintained a concentration sufficient to inhibit bacterial growth during the whole study period. But at the second day, the burst effect of drug release seemed more intensive while the loaded gentamicin increased. This may be due to the drug release mechanism was simultaneously controlled by diffusion and matrix degradation. After 26 days immersion, there were still 53.44%, 44.41% and 14.7% drugs respectively trapped in the 16, 32 and 64 mg/g pellets. To balance the burst effect and the cumulative released amounts of drugs, the 32 mg/g pellets were chosen in this study.

Six weeks after implanting of GBBG, 2 out of 11 rabbits were positive for *E. coli* culture with bad radiographic and histopathologic scores. In the remaining 9 rabbits, all radiographic and histopathologic images and microbiological investigations have shown that osteomyelitis was cured. In contrast, only 1 out of 9 rabbits showed the infection was cured for groups 2 and 3. The implanting of pure borate glass pellets and gentamicin powder had the same bad result of treatment with the debridment only. It proved that the GBBG had also a good delayed release effect in vivo for gentamicin, and the local implanting of gentamicin powder had no efficacy for
the osteomyelitis treatment. The fact that the treatment with GBBG pellets alone acquires a 81.82% cure rate in this study implies the better results if co-using systematic antibiotic in the future study.

The radiographic and histopathologic images showed the GBBG pellets were reabsorbed and replaced by new bone formation, which is consistent with our previous studies [7, 13]. However, some foreign body reaction around the material has also been observed (Fig. 5), which is inconsistent with our previous studies [7]. The foreign body reaction may be due to the adding of chitosan, an organics that degrades through the phagocytosis of macrophage other than converting borate glass to a hydroxyapatite (HA)-type material in the body fluids. In contrast, the area of implants and surrounding tissue in group 2 has deteriorated bone infection and abscess formation, but no any sign of new bone formation. Our results have demonstrated that the GBBG pellets first cure the infection by releasing the gentamicin loaded in it, and then repaired the bone defect by new bone formation promoted by the borate glass degradation.

5. Conclusions

In conclusion, the chitosan-bonded gentamicin and borate glass pellets cured the bone infection induced by *E. coli* and repaired the bone defects within 6 weeks of implantation in a rabbit tibia osteomyelitis model. This study has further demonstrated that borate bioactive glass could be used as a carrier of gentamicin not only to eradicate the osteomyelitis caused by Gram-negative bacteria, but also to repair the bone defect caused by infection along with the borate glass degradation.
Acknowledgements

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References


Fig. 1 Release of gentamicin in vitro from three kinds of pellets (16, 32, 64mg/g).

Fig. 2 The bodyweight changes of three groups before and after treatment. The mean of group 1 are 2.75±0.27, 2.48±0.39, 2.85±0.46 respectively. The mean of group 2 are 2.81±0.25, 2.27±0.49, 2.28±0.48 respectively. The mean of group 3 are 2.76±0.26, 2.23±0.37, 2.47±0.67 respectively. (P<0.05, one-way ANOVA)

Fig. 3. Radiographs of three groups showing experimental osteomyelitis three weeks after infection (1a-3a), bone windows and implants after debridement (1b-3b) and deteriorative osteomyelitis (2c, 3c), control of bone infection and reabsorbed implants, new bone formation (1c) six weeks after treatment.

Fig. 4. Typical histopathologic samples 11 weeks after infection (hematoxylin and eosin): The above left picture (group 1, ×40) showed new bone formation (white arrow) around and inside the material (M), Haversian canals (black left arrow) and some foreign body reactions (black right arrow) around the material. The above right picture (group 2, ×100) showed abscess formation (white arrow) around material (black arrow). The left picture (group 3, ×200) showed a sequestrum (white arrow) surrounded by abscess formation.
Table 1 The antibiotic releases of three kinds of pellets in vitro

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<tr>
<th>Kinds of Pellets</th>
<th>16mg/g</th>
<th>32mg/g</th>
<th>64mg/g</th>
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<td>The recovered rates at the second day</td>
<td>39.6%</td>
<td>87.03%</td>
<td>90.12%</td>
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<tr>
<td>The cumulative percentages of antibiotic release</td>
<td>46.56%</td>
<td>55.59%</td>
<td>85.3%</td>
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<th>TABLE 2. Results of E. coli examination after treatment</th>
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<td>Negative</td>
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<tr>
<td>Positive</td>
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<tr>
<td>Total</td>
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<td>Clearance</td>
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Significant differences were observed between group 1 and 2, group 1 and 3 after treatment (p=0.040 and 0.012, respectively, Fisher’s exact method).

<table>
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<th>TABLE 3. Radiographic and histopathologic average scores of three groups</th>
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<td>Groups</td>
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<td>Radiographic score before treatment</td>
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<td>Radiographic score after treatment</td>
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<td>histopathologic score after treatment</td>
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Significant differences were observed among radiographic scores after treatment (P=0.000, one-way ANOVA). By LSD multiple comparisons, significant differences were observed between group 1 and group 2, group 1 and group 3 (p=0.001, and 0.003; respectively). Significant differences were observed among histopathologic scores between group 1 and 2, group 1 and group 3 (p=0.014, and 0.004, respectively, LSD test).