

A comparison of three decontamination protocols for bone collected with a bone collector in terms of osteoblastic cell viability and bacterial decontamination.

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Abstract

Purpose: Osseous coagulum collected from a bone collector usually contaminated with bacteria in the oral cavity that could affect the success of bone grafting procedure. The aim of this study was to determine the most practical protocol to decontaminated osseous coagulum with minimum influence on cell viability. **Materials and Methods:** Total 47 bone samples were collected with an osseous collector using a stringent aspiration protocol. Collected bone was flushed with 30 ml sterile normal saline solution (NSS) first before divided into two groups. A control group was flushed with 20 ml of NSS, while, the experimental group was randomly flushed with 25 mg/ml tetracycline (T25), 0.12% chlorhexidine (CHX12) or 0.06% chlorhexidine (CHX6). Bone samples were immediately transported for aerobic and anaerobic bacteria examination and osteoblastic cell viability assessment. **Results:** All the collected bone samples from the bone collectors were contaminated with both aerobic and anaerobic bacteria. CHX12 showed the most efficacies among the experimental groups to decontaminate collected bone while there was no significant difference between T25 and CHX6. However, none of the antimicrobial solutions could preserve the osteogenicity of the bone particles.

Conclusion: Collected bone particles from the intra-oral site were generally contaminated with aerobe and anaerobe microbial. Using 25 mg/ml tetracycline, 0.06 % or 0.12 % chlorhexidine could only partially reduce the microbial; however, none of the decontaminant solutions could preserve the osteoblastic viability.

KeyWords: decontamination, bone collector, tetracycline, chlorhexidine, osteoblast, cell viability

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Introduction

Autogenous bone grafting has been considered as a gold standard for bone regeneration according to the properties of osteoinduction, osteoconduction and osteogenesis.¹⁻⁵ Additionally, it carries no risk of immunologic reaction or disease transmission and it is more economical to use. One of the most significant advantages of the intraoral harvesting sites is the convenient access afforded by local donor sites. These procedures can be quick and the ischemic time of the bone graft is, therefore, short.^{6,7}

For small amounts of graft, many techniques and devices are available to harvest intraoral autogenous bone grafts, such as bone scrapers, piezoelectric devices, bone chisels, rongeur pliers and rotary instruments with bone collectors.⁸ Bone collectors composed of a filter placed in the surgical suction device to collect the bone particles produced during bone drilling.^{6,7} According to simultaneous techniques, bone particles used for immediate grafting procedures.⁹ Using bone collectors for harvesting autogenous bone eliminate the disadvantages of autogenous bone grafts, such as donor site morbidity, an additional surgical site, and increased surgical duration.³

Oral microbial contamination of collected bone particles may reach up to 10^9 colony-forming unit (CFU). Therefore, the contamination of collected bone particle is a usual occurrence. The previous study reported the various aerobic species included *Enterococcus faecalis*, *Staphylococcus epidermis*, *Staphylococcus aureus*, *Streptococcus α-hemolyticus* and *Streptococcus β-hemolyticus*. Within the anaerobic group, *Actinomyces odontolyticus*, *Prevotella intermedia*, *Propionibacterium propionicum*, *Peptostreptococcus asaccharolyticus*, *Peptostreptococcus micros*, and *Eubacterium species*, were observed.⁶ Moreover, when bone collectors are used,

there is a high risk of iatrogenic contamination of the collected bone particles, which may possibly lead to infection and conceivable failure.^{11, 12} Many studies proposed the decontamination methods of the collected bone particles, such as using a stringent aspiration protocol¹⁰, using a preoperative chlorhexidine oral rinse¹⁰, taking an antibiotic prophylaxis^{11, 13}, and treating collected bone debris with various antimicrobial agents.^{14, 15} However, none of the decontamination methods in the literature could completely decontaminate the collected bone particles¹⁶, nor has any of the studies reported cell viability after decontamination. The aims of the present study were to determine the most practical protocol to decontaminated osseous coagulum with minimum influence on cell viability.

Materials and Methods

The study protocol was approved by the ethics committee of the Faculty of Dentistry, Prince of Songkla University, HatYai, Songkhla in Thailand. The subjects considered eligible for the study were 20 years or older, all physically healthy, with no underlying systemic disease as determined by medical history records, and who required an osseous surgery procedure where bone reduction or bone removal was planned. Patients with clinical evidence of periodontal diseases, long-term antibiotic usage, smoking, and pregnancy were excluded. All included subjects provided informed consent before participation.

Surgical protocol

All surgeries were carried out in a minor surgery room under sterile conditions. During surgery, a stringent aspiration protocol was used with 2 separated sterile suction tips. The first

suction tip was attached to an osseous collector (OTA osseous collector100®, Switzerland, Figure 1), which was restricted to collecting bone. While the blood, saliva and irrigant within the surgical field was removed by the second suction tip.



Figure 1 The osseous collector (OTA osseous collector100®, Switzerland)

Decontamination protocol

Tetracycline solution (25 mg/ml; T25), chlorhexidine solutions (0.12 %; CHX12, 0.06%; CHX6) or normal saline solution (NSS) were randomly used for decontaminating the bone particles in each case. After the bone removal process was complete, 30 mL of NSS was aspirated into the bone collector. Then, the collected bone was divided into 2 groups and randomly flushed with 20 ml of one of the decontaminant solutions (T25, CHX12, or CHX6) or NSS (control). Bone particle was carefully removed from the osseous collector with a sterile Molt periosteal elevator and placed in a sterile tube (1.5 ml) containing 500 µl sterile alpha-MEM (Gibco, Invitrogen Co., USA) as a transport medium.

Samples of both groups reached the laboratory within 1 hour where processing of samples commenced immediately. 0.1 g of bone was used for assessment of osteoblastic cell viability. Partial amounts

of bone from the control and experiment groups were sent to the microbiology laboratory for culturing the quantity of aerobic and anaerobic bacteria.

Microbiologic examination

A culture of aerobic bacteria using blood agar and culture for anaerobic bacteria using brain heart medium were composed. After 2 days, the colony count grading was done as follows: no growth (0), few or 1 - 10 colonies (1), moderate or more than 10 colonies (2) and numerous (3).

Osteoblastic-like cell viability assessment

0.1 g of bone collected from the control and experimental groups was cultured in a 6-well culture plate (Nunclon, Nunc, USA) using proliferating medium (alpha-MEM, 10% FBS, 1% penicillin-streptomycin, 0.1% fungizone, all from Gibco, Invitrogen Co., USA) in 5% CO₂ at 37° C. The cultures were left undisturbed for 7 days and thereafter the medium was replaced with an equal fresh volume taking care not to dislodge the bone particles. From this point on, the medium was changed twice a week. After 21 days of culture, the numbers of vital osteoblastic cells were quantified by a haemocytometer.

Osteoblastic phenotypes assessment

To confirm the type of cells growing from the collected bone, osteoblastic phenotypes were assessed for cell differentiation (Alkaline phosphatase staining test; ALP staining) and mineralized nodule formation (Alizarin red staining). On the 21st day, the medium for cultured bone cells in the 6-well plate was replaced with osteogenic medium

(proliferating medium, 0.2% dexamethasone, 0.1% ascorbic acid and 0.1% beta-glycerophosphate) and was changed twice a week thereafter. After 7 days of culture, an ALP staining test and Alizarin red staining test were performed. Alkaline phosphatase staining was carried out using the cytochemical staining for Alkaline phosphatase activity kit (Sigma-Aldrich, St.Louis, MO.). Briefly, cells were washed with phosphate-buffered saline solution and fixed with 4% para-formaldehyde for 5 minutes and were then rinsed with distilled water. ALP staining was performed by using 0.1% fast red violet B salt and 0.3% naphtol AS-BI phosphate. The cell staining was examined under a light microscopic. For alizarin red staining, cells were stained with 0.5% Alizarin Red solution and were then examined under a light microscope.

Statistical analysis

The statistical analysis was performed using SPSS software (version 13, Chicago, USA.) The Wilcoxon Signed Ranks test was used to calculate comparisons within the group. The significant differences among groups were identified by the Kruskal-Wallis test. Significant differences between groups were identified by the Mann-Whitney U test. ($\alpha = 0.05$)

Results

A total of 47 patients (32 women, 15 men) were included. The bone samples were randomly assigned to 3 decontamination protocols (T25, CHX12, CHX6), and NSS which served as control (n=16). The average bone weights from the osseous procedures (alveoloplasty, corticotomy or torectomy) were 4.28 ± 2.51 g.

Microbiologic examination

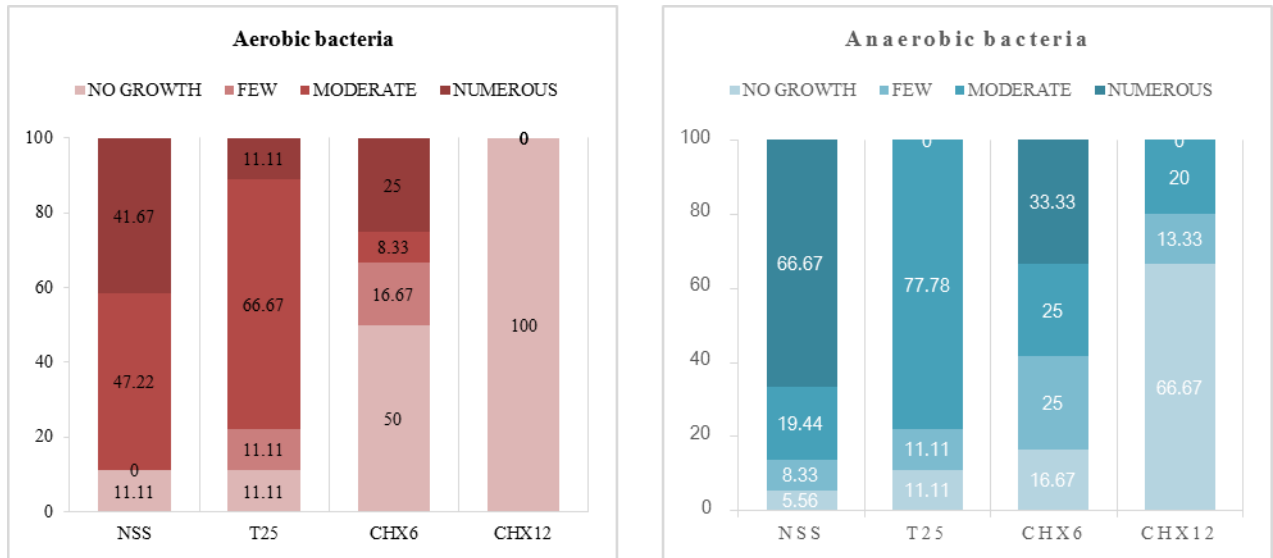
All of the collected bone particles were contaminated with both aerobic and anaerobic bacteria. The effectiveness of T25, CHX12, and CHX6 in reducing the number of aerobic and anaerobic bacteria was shown in Figure 2. Decontamination using T25, CHX6 and CHX12 revealed a significant difference ($p < 0.05$) in reducing both aerobic and anaerobic bacteria colony compared with NSS. For aerobic bacteria, CHX12 decreased the number of bacteria significantly compared to T25 and CHX6, while for anaerobic bacteria, three protocols revealed no significant difference.

Osteogenicity and osteoblastic phenotype of osteoblastic cells

The first evidence of cellular proliferation from the bone particle was observed after 14 days in the NSS group. The mean of osteoblastic-like cells was $5.46 \pm 1.34 \times 10^4$ cells. The osteoblastic phenotype of cells from the bone particle was confirmed by the positive result of the ALP and Alizarin red test. However, until day 21, the osteoblast cells could not be identified in all the decontaminant solution groups (T25, CHX) as shown in Figure 3.

Discussion

Autogenous bone collected during osseous surgery using bone collector can be used to graft bony defects. However, the potential for regeneration decreases when bacteria contaminated the grafting material.¹¹ Therefore, decontamination methods should be considered to decrease the risk of augmentation failure due to bacterial contamination. Contamination



was shown to be significantly reduced by judicious precautions. The use of a

Figure 2 Aerobic and anaerobic bacteria contamination after 3 decontamination protocols

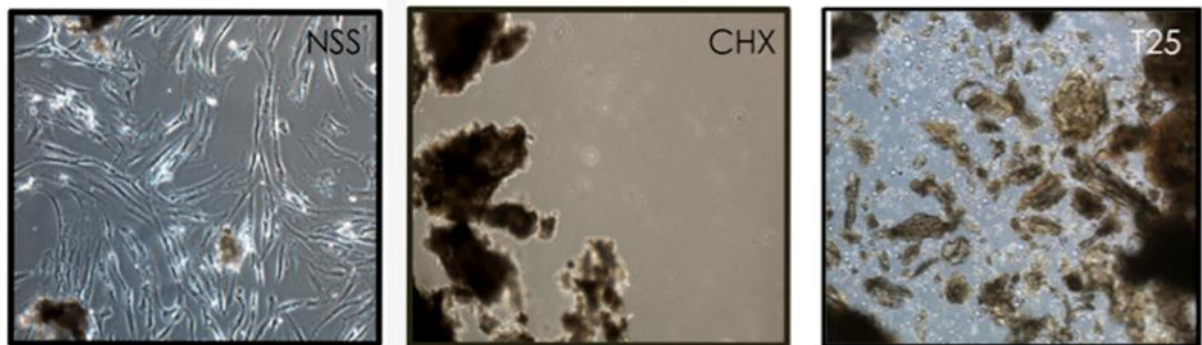


Figure 3 The osteoblastic cells proliferation after culture for 14 days after decontaminating with NSS, CHX (CHX6 and CHX12) and T25

stringent aspiration protocol, which involved a dedicated salivary suction device, achieved a reduction in the bacterial count of 58%.¹⁷ In the present study, the stringent aspiration protocol was implemented for collecting bone particles. Nonetheless, the control group samples were still contaminated with bacteria. In selecting the antimicrobial agent of choice

for decontamination, it should be based on safety of the agent toward osteoblasts, eliminating targeted pathogens known for impairing osteogenesis, and a short exposure time.¹⁸

Previous studies used various antimicrobial solutions such as chlorhexidine^{3, 14, 19, 20}, tetracycline¹⁴ or clindamycin³ to reduce or decontaminate

graft materials. However, none of the studies revealed the osteoblastic viability after the decontamination. Cytotoxicity of the antimicrobial solution depended on two important factors: concentration and exposure duration.¹⁸ The higher levels of antimicrobial solution led to a dose-dependent decrease in cellular viability and activity.²¹

Chlorhexidine is a cationic bisbiguanide antiseptic active against gram-positive and gram-negative bacteria, facultative anaerobes and aerobes, moulds, yeasts and viruses.²² However, recent studies indicated that chlorhexidine was cytotoxic to human alveolar bone cells²⁰ and human osteoblastic cell lines²³, which corresponds to the present study where human osteoblasts could not retain their viability after flushing collected bone with 20 ml of 0.12 % or 0.06 % chlorhexidine. Tetracyclines are a broad-spectrum of antimicrobial activity against gram-positive and gram-negative aerobic bacteria as well as anaerobic organisms.²⁴²⁵ A recent study showed that tetracycline inhibited the proliferation of primary human osteoblasts and MG63 from a concentration of 180 µg/ml (50% Inhibitory Concentration; IC).²⁶ Tetracycline (dose range 0.1 - 2 µg/ml) also revealed both cytotoxic and a moderated genotoxic effect in cultured human blood lymphocytes.²⁷ The cytotoxicity of tetracycline to human osteoblasts was confirmed in this study from non-viable osteoblast cells after flushing collected bone with 20 mL of 25 mg/ml tetracycline.

According to the present study, flushing collected bone first with 30 ml of NSS and 20 ml of 0.12%, completely eliminate aerobic bacterial and partially

eliminate anaerobic bacteria compared to 20 ml of 25 mg/ml tetracycline or 0.06% CHX, which hardly ever eliminated the contaminant bacteria. However, neither CHX nor tetracycline could retain the viability of osteoblasts in collected bone particles. Although bone particles collected from a bone collector is classified as an autogenous bone graft, if the bone was treated with antimicrobial solutions, the property of osteogenesis would be destroyed. To maintain the ideal characteristic of an autogenous bone graft from collected bone, the appropriate technique to eliminate contaminant bacteria while still preserving the osteogenic potential should be identified.

Conclusion

Bone collected from a bone collector showed contamination for both aerobe and anaerobe microbials. The stringent protocol that used two separate suction could not eliminate the tendency of bacterial contamination. Flushing the collected bone with 30 ml of NSS followed by 20 ml of 0.12% chlorhexidine was the most effective protocol to reduce the bacterial contamination. While the use of 0.06% chlorhexidine or 25 mg/mL tetracycline partially reduced the number of contaminant bacteria. However, none of the above decontaminants could preserve vitality of the osteoblasts in the collected bone. Further study needs to identify a suitable technique that can eliminate decontaminant microbials as well as preserve the osteoblast potential of the collected bone particles.

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การศึกษาเพื่อเปรียบเทียบวิธีการทำความสะอาดเพื่อลดปริมาณเชื้อโรคใน กระดูกที่เก็บได้จากเครื่องมือผ่าตัดต่อการมีชีวิตของเซลล์

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บทคัดย่อ

วัตถุประสงค์ เพื่อทำการศึกษาประสิทธิภาพของวิธีการทำความสะอาดเพื่อลดปริมาณเชื้อโรคในชั้นกระดูกที่เก็บได้จากเครื่องมือผ่าตัดต่อการมีชีวิตของเซลล์

วิธีการทดลอง ชิ้นตัวอย่างเป็นกระดูกที่เก็บได้จากเครื่องมือผ่าตัดจำนวน 47 ชิ้นตัวอย่าง โดยนำชิ้นตัวอย่างมาทำความสะอาดด้วยน้ำเกลือ ก่อนจะทำการแบ่งเป็นกลุ่มตัวอย่างดังนี้ คือ กลุ่มควบคุม เป็นกลุ่มที่ทำการล้างด้วยน้ำเกลือเพียงอย่างเดียว, กลุ่มทดลอง คือกลุ่มที่ทำความสะอาดด้วยสารละลายเตตราไซคลีน ความเข้มข้น 25 มิลลิกรัมต่อมิลลิลิตร (T25), กลุ่มที่ทำความสะอาดด้วยคลอเฮกซิดีน 0.12% (CHX12), และกลุ่มที่ทำความสะอาดด้วยคลอเฮกซิดีน 0.06 % (CHX6) หลังจากนำเข้าไปทดสอบความมีชีวิตอยู่ของเซลล์เพื่อทำการประเมิน

สรุปผลการทดลอง จากชิ้นตัวอย่างที่เก็บได้จากเครื่องมือผ่าตัดพบว่าการปนเปื้อนเชื้อแบคทีเรีย และวิธีการปราศจากเชื้อที่มีประสิทธิภาพมากที่สุดคือกลุ่ม CHX12 ในขณะที่กลุ่ม T25 และ CHX6 ไม่มีความแตกต่างกันอย่างมีนัยสำคัญทางสถิติ

คำสำคัญ การทำความสะอาดเพื่อลดปริมาณเชื้อ, เตตราไซคลีน, คลอเฮกซิดีน, การมีชีวิตอยู่ของเซลล์

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