Dental implant placement has become a predictable treatment option in recent years due to innovations in biomaterial research and bone tissue engineering. The success of dental implants is directly related to the quantity and quality of the host bone. Vertical and horizontal bone defects of the mandible or maxilla are sequelae of alveolar bone resorption after tooth extraction, infection, or trauma. In the presence of severe alveolar ridge resorption, placement of a dental implant in the planned prosthetic position is not possible, and it always demands a ridge augmentation procedure before or during the implant placement. Nowadays, numerous bone regenerative materials are being used in implant dentistry; however, various degrees of success are reported by different studies. Despite the extensive research on biomaterials in the last 10 years, no ideal substitute has been developed that can completely replace the autogenous bone graft. The regenerative bone procedures in oral implantology are mainly performed for socket preservation, maxillary sinus floor augmentation, and vertical or horizontal defects of the alveolar bone using different bone substitutes with or without a barrier.
membrane. Different bone substitutes that are commonly used are autogenous bone, allograft, xenograft, or alloplasts, such as calcium phosphate or polymers. Different surgical techniques that are employed include autogenous bone blocks, guided bone regeneration, ridge splitting, and distraction osteogenesis. Commerically available bone substitutes are a good alternative due to their excellent biocompatibility and osteoconductive properties. The major drawback of these biomaterials is that these lack osteoinductivity. Therefore, different osteoinductive proteins such as bone morphogenetic protein and platelet-derived growth factor have been used with these osteoconductive biomaterials to trigger and enhance new bone formation.

Bone autograft is still the gold standard in bone regeneration, as it is the only osteogenic, osteoconductive, and osteoinductive material available. Autogenous bone graft harvesting requires a second surgical site and is associated with pain and donor-site morbidity. The amount of required autogenous bone determines the selection of a donor site. In clinical situations where a large quantity of bone is required, autogenous bone grafts are obtained from extraoral locations such as the iliac crest or calvaria. Conversely, whenever a smaller amount of bone is needed, intraoral grafting sites such as the ramus, chin, or tuberosity are preferred. However, for a minor grafting procedure, a second surgical site can be avoided with the use of bone collectors/traps or bone-scraper to collect bone particles during the surgical procedure. Bone collectors/traps consist of bone filters connected to the surgical suction to collect bone debris (bone slurry) generated during implant osteotomy preparation with saline irrigation. It has been demonstrated that despite the use of different disinfection strategies, pathologic bacterial species are always observed in the collected bone. A bone scraper is primarily used to harvest cortical bone from an alveolar process, ramus, maxillary tuberosity, or mandibular symphysis during the implant surgery.

During osteotomy preparation, it has been reported that if host bone is exposed to a temperature of 47°C for 1 minute, host bone resorption or necrosis might occur. Nevertheless, thermal osteonecrosis and implant osteotomy preparation parameters are reassessed, and it has been demonstrated that implant osteotomy preparations can be performed in all types of bone without external or internal irrigation. In the absence of external irrigation, the recorded temperature range was 31.4°C to 36.9°C at the speed of 188 rpm and 35.2°C to 43.0°C at the speed of 462 rpm, respectively. Therefore, it is possible to avoid aqueous irrigation without detrimental effects on bone by adopting a low-speed drilling protocol that does not produce heat exceeding 47°C due to reduced frictional heat generation.

The main advantage of the low-speed drilling protocol is that the bone chips created during the implant osteotomy preparation remain attached to the drill flutes/threads due to the absence of saline irrigation. However, many clinicians do not prefer to adopt low-speed drilling for all the planned implant sites. There is a great need to find alternative techniques to collect bone chips during osteotomy preparation with saline irrigation. A novel method of autogenous bone collection was presented in this study in which implant drills were used to collect autogenous bone particles in the presence of continuous saline irrigation. Therefore, the aim of the present study was to collect autogenous bone during implant osteotomy preparation using two different drilling protocols and to evaluate and compare the proliferation and differentiation ability of the collected bone particles.

**MATERIALS AND METHODS**

The protocol of this study was prepared according to the guidelines of the Helsinki Declaration. The Medical Ethical committee of Vrije University Amsterdam approved the present study (Ethical approval number: 2013/151).

**Study Population**

Twenty healthy patients with a mean age of 57 ± 12 years participated in the study. The patients were recruited from a university teaching institute (Academic Centre for Dentistry Amsterdam, the Netherlands) from October 2013 until July 2016. Sample size calculation was performed with an effect size of 1 at a significance level of .05 and power of 80%. Ten samples were required for each study group. The inclusion criteria were as follows: (1) completely edentulous or partially edentulous patients; (2) enough bone volume at the time of surgery, ie, capable of receiving at least an 8-mm-long implant. Separately, the exclusion criteria were: (1) active periodontal infection; (2) unresolved extraction wounds or inadequate bone at the time of surgery; (3) lack of motivation or compliance; (4) moderate and heavy smoking (> 10 cigarettes per day) or tobacco chewing; (5) severe systemic disease; and (6) pregnancy. Prior to inclusion, patients were informed of all the treatment possibilities, and possible adverse effects. Each patient had given a written informed consent. The patients were randomly assigned to the test or the control group by use of the closed-envelope technique. Prior to the start of the project, 20 envelopes containing both treatment options, equal in number, were sealed. Then, before the start of surgery,
one envelope was randomly picked and opened in front of the patient.

Osteotomy Preparation and Autogenous Bone Collection

Autogenous bone particles were collected from the patients during osteotomy preparation using two different drilling protocols as follows (Fig 1):

- In the standard drilling protocol, the bone preparation was performed according to the manufacturer’s protocol with continuous cooling with sterile saline. Drilling was initiated with a round drill (2.3 mm), and the pilot drill (2.0 mm) at a speed of 800 rpm. Subsequently, the preparation was widened by a consecutive series of drills at different speeds as recommended by the manufacturer, ie, predrill (600 rpm), 3.3 (550 rpm), 3.8 (500 rpm), or 4.3 (400 rpm) depending on the final diameter of the implant.

- In the low-speed drilling protocol, the round drill and a pilot drill were used with aqueous irrigation at 800 rpm. However, all consecutive series of drills were used at a speed of 50 to 150 rpm without irrigation using uninterrupted drilling time < 60 seconds. The bone particles attached to the bur flutes were collected in sterile glass bottles containing phosphate-buffered saline (PBS). All samples were sent immediately to the cell culture laboratory for further preparation and analysis. All collected samples were given a unique code, and the laboratory technician was blinded regarding the nature of the samples. All implant surgeries were performed by a single surgeon (A.T.).

Scanning Electron Microscopy Fixation of samples from each group was done for 5 minutes in 2% glutaraldehyde. Then, samples were washed with Nacocadlylate buffer (0.1 M). After that, dehydration of the samples was performed in an ascending series of ethanol. After dehydration, drying of the samples was done with tetramethysilane. The samples were stored in the dry environment until SEM examination. At the time of analysis, the specimens were mounted on an aluminum stub and sputtered with a thin layer of gold. Scanning electron microscopy (SEM) was used to examine and record the samples.

Cell Culture Analysis

Bone particles for cell culture analysis were washed twice with Dulbecco’s Modified Eagle Medium (DMEM, Thermo Fisher Scientific) and placed into T25 tissue culture flasks (Corning) in normal culture medium containing DMEM, with 1% penicillin, streptomycin, and fungizone (Sigma-Aldrich), 10 IU/mL of heparin (LEO Pharma A/S) and 5% human platelet lysate (Sanquin). The cells were incubated at a temperature of 37°C in a humidified atmosphere containing 95% air and 5% CO₂. The cell culture medium was changed two times in a week. After 2 to 3 weeks of incubation, cells that grew out from the bone grafts were termed as bone explants. These cells were released by using 0.25% trypsin (Invitrogen), and 0.1% ethylenediaminetetraacetic acid (Merck) in PBS and counted (Fig 2a). The number of outgrown cells was calculated per gram of wet bone tissue. Cells (concentration: 20,000 cells/well/400 µL) from passage 3 were placed in 24-well plates. After the overnight attachment of the cells, cells were cultured for 4, 7, and 20 days in the normal medium and in the osteogenic culture medium, which was supplemented by 10 mM of Na β-glycerophosphate, 10⁻⁷ M of dexamethasone, and 50 µg/mL ascorbic acid. The day of the culture of the cells after the overnight attachment was considered as day 0. The culture medium was removed at each time point (ie, days 0, 4, 7, and 20), and the cells were washed with PBS. The cells were submerged in 300 µL of MilliQ water (Merck KGaA) and frozen at −20°C until analysis for DNA measurements. For calcium measurements, the cells were stored dry in the freezer until analysis. The cells were fixed in 4% formaldehyde for alizarin red staining.

Cell Viability Measurements

The Muse Cell Analyzer (Millipore) in conjunction with Muse Count & Viability Kit (MCH 100103) was employed to count the cells and analyze cell viability. Cell viability was determined for cells from passage 3.

Measurements of Cell Proliferation (DNA Content)

DNA content was determined as a measure of total cell number. It was determined by utilizing the Cell Proliferation Assay Kit (Cyquant, Molecular Probes). In a microplate reader, absorption was read at the
Measurements of Cell Differentiation (Calcium Content)
The calcium phosphate deposition was determined first by adding 1 mL of 0.5 M acetic acid to all the samples of days 0, 4, 7, and 20. Then, calcium present in the mineralized matrix of the samples was dissolved by shaking the samples vigorously overnight. The ortho-cresolphthalein complexone (OCPC) method was utilized to measure the calcium content. Preparation of OCPC solution was done by adding 80 mg OCPC (Sigma) in 75 mL H₂O with 0.5 mL 1 N KOH and 0.5 mL 0.5 M acetic acid. Subsequently, the working solution was made as follows: 5 mL OCPC solution was added to 5 mL of 14.8 M ethanolamine/boric acid buffer (pH = 11), 2 mL of 8-hydroxyquinoline, and 88 mL of MilliQ water. Lastly, 300 μL working solution was added to 5 mL of 14.8 M ethanolamine/boric acid buffer. The calcium-containing mineralized nodules present in the medium were stained red by alizarin staining. Separately, toluidine blue stained nucleic acids blue, increasing the sharpness of images and enhancing the visibility of cells.

Statistical Analysis
Three separate runs of the experiment were carried out to ensure reproducibility. Almost identical results were observed from all three runs. Data were expressed as mean ± standard deviation. Repeated measures analysis of variance (ANOVA) with Bonferroni’s test was used to determine the statistical significance. The statistical significance was set at 5% (P < .05), and GraphPad Prism software (GraphPad Software) was used for all the analyses.

RESULTS
Sample Collection
In the control group, 6 out of 10 samples were from the maxilla (1 from the anterior and 5 from the posterior regions), and 4 samples were from the mandible (posterior region). In the test group, 4 out of 10 samples were from the maxilla (1 from the anterior and 3 from the posterior regions), and 6 samples were from the mandible (2 from the anterior and 4 from the posterior regions). The patient’s age and harvesting site were comparable between the control and test groups (Table 1).

Scanning Electron Microscopy
SEM review of the standard drilling samples showed small fine particles and some fine bone dust in the sample (magnification 100x; Fig 2a). On higher magnification, loss of collagen/fibril network and smooth surface was observed (magnification 2,000x; Fig 2b). Low-speed protocol samples demonstrated a larger particle size compared with the standard drilling (100x; Fig 2c). On higher magnification (magnification 2,000x; Fig 2d), collagen fibrils were observed on the outer surface.

Cell Culture Analysis
After 2 to 3 weeks, cell outgrowth was observed in all 10 samples of the low-speed drilling protocol (Fig 2e). In comparison, only 5 out of 10 samples were able to demonstrate cell outgrowth (Fig 2f). Distribution of patients for standard drilling and low-speed drilling groups is presented in Table 1.
Cell Viability Measurements
The cell viability values of passage 3 cells collected with the standard drilling and low-speed drilling were 97.6 ± 1.2 and 97.4 ± 1.2, respectively. No significant difference was found between the standard drilling and low-speed drilling samples.

Measurements of Cell Proliferation (DNA Content)
In a normal medium, the total DNA content was significantly higher for the low-speed drilling samples vs the standard drilling on day 4 (P < .05), day 7 (P < .01), and day 20 (P < .001). No significant difference was observed on day 0 (Fig 3a). In the osteogenic medium, total DNA content was significantly higher for low-speed drilling samples compared with the standard drilling on day 7 (P < .01) and day 20 (P < .01). No significant difference was observed on day 0 and day 4 (Fig 3b).

Measurements of Cell Differentiation (Calcium Content)
Calcium measurements were performed as an indication of differentiation of cells in the osteogenic lineage (n = 5) and are depicted in Fig 4. No significant difference was found regarding calcium content among low-speed drilling samples compared with standard drilling samples at any time point in the normal medium (Fig 4a). However, in the osteogenic medium, a significantly higher amount of calcium was measured only on day 20 for bone collected with the low-speed drilling vs that collected using standard drilling (P < .01). No significant difference was noticed at any other time points (Fig 4b).

Alizarin Red and Toluidine Blue Staining
The results of alizarin red staining of bone explants for day 20 are depicted in Fig 5a, and the results of alizarin red and toluidine blue staining of bone explants for day 20 are depicted in Fig 5b. The visual inspection of the samples revealed no mineralization for both groups in normal medium. Mineralization could only be seen at day 20 for both the standard drilling and the low-speed drilling samples in the osteogenic medium, and these other results are in line with quantitative calcium analysis. A higher amount of the red mineralized nodule formation was observed in low-speed drilling samples compared with the standard drilling samples (Figs 5a and 5b).
DISCUSSION

In the present study, autogenous bone particles were collected during implant site preparation using two different drilling protocols, and the osteogenic efficacy of the collected bone chips was evaluated. Regarding the selection of the present study model, an in vitro study was designed to evaluate the osteogenic potential of the collected autogenous bone in a controlled environment to exclude additional influencing factors. In vivo studies are most commonly recommended to examine the efficiency of bone substitutes to repair the critical-size bone defects.

In this study, the low-speed drilling group had a significantly higher proliferation rate (total DNA content) and differentiation rate (mineralized matrix and calcium content) compared with the standard drilling group. Calcium content measurements and alizarin red and toluidine blue staining revealed matrix mineralization for both groups only in the osteogenic medium at day 20. The results of the present study are in accordance with other cell culture studies, which demonstrated that in the presence of osteogenic medium, cells start to differentiate after the first week of culture, and cell differentiation and matrix mineralization could only be observed after 12 to 14 days.

In this study, no saline irrigation was utilized during osteotomy preparation for the low-speed drilling group. Conversely, in the standard drilling group, saline irrigation was employed to avoid host bone necrosis due to overheating. It has been observed that the saline irrigation during implant surgery decomposes and takes away the natural proteins and signaling factors in the bone and washes off living cells. These biologic proteins and signaling factors are involved in several cellular
mechanisms such as proliferation, differentiation, and chemotaxis of osteoblasts and osteocytes. Thus, the harvesting method of autogenous bone significantly affects the release of growth factors from autograft, which is vital for the differentiation of osteoblasts and the induction of osteogenesis on the surface of the bone. The results of the present study are in accordance with Liang et al (2017), who concluded that low-speed drilling is an ideal technique for collecting autogenous bone particles compared with bone slurry due to high osteogenic efficacy and low biologic risk factors.

The critical factor for the success of autogenous bone grafts is to harvest the graft with high viability of bone-forming cells, ie, osteoblasts and osteocytes. Many studies have shown that the viability of cells in harvested bone grafts is decreased using electrical instruments. Additionally, damage to cell membranes of the cells that are present on the surface or inside the bone chips might occur due to the vibrations from the drilling equipment. It can be speculated that in the standard drilling protocol, high drilling speed together with saline irrigation resulted in the disruption of cell membranes of the bone cells, and consequently, inferior osteogenic response was observed in this group. The results are in line with the cell culture study conducted by Gruber et al, who showed the cell outgrowth in all samples of porcine cortical bone ground by a bone mill. On the other hand, cell outgrowth was observed only in the 5 out of 10 samples that were collected using a bone trap (bone slurry). These findings are further confirmed by Springer et al, who revealed that autogenous bone chips collected by hardball reamer, implant drill, and diamond ball drill had shown reduced numbers of viable cells compared with the samples collected with the bone mill technique. Furthermore, in an in vitro study, the ability of autografts to stimulate an osteogenic response in cell cultures was evaluated using four commonly used autogenous bone harvesting techniques. The study data have shown a significantly higher proliferation and differentiation in bone samples harvested by a bone scraper or bone mill compared with the bone samples collected by bone slurry or piezoelectric.

Previous studies have confirmed the importance of graft volume, graft surface, and particle size for the success of a grafting procedure. Autografts with a small particle size exhibit higher osteoclastic activity and lower mechanical stability compared to autografts with a larger particle size. In the present study, bone chips collected with saline irrigation were smaller in size and had significantly reduced osteoblast proliferation and differentiation compared with the low-speed drilling group. Miron et al (2011) also demonstrated that the particles harvested by piezoelectric surgery and bone slurry were smaller in size compared with the bone mill and bone scraper. These particles had shown significantly reduced osteoblast attachment and differentiation.

Bone augmentation is still a challenge for clinicians, especially in the posterior maxilla. Various surgical techniques and biomaterials are being employed for sinus floor elevation and augmentation. In the recent randomized clinical trial, a bone scraper was compared with the piezoelectric surgery in lateral window preparation for sinus floor elevation. It was reported that both surgical approaches are effective and safe for performing lateral antrostomy. In addition, the bone harvested during lateral antrostomy preparation with a bone scraper or piezoelectric device is usually mixed with other biomaterials to improve their osteogenic efficacy. Similarly, during implant placement, the collection of autogenous bone attached to drill threads might be considered a promising alternative technique for autogenous bone harvesting, and these bone particles can be mixed with osteoconductive bone substitute to render it osteoinductive.

The key limitation of this study was that the osteogenic potential was evaluated only by performing cell culture experiments. This is simply an in vitro study and may not fully be translated to clinical situations.
Whether these findings are clinically relevant in an in vivo environment is still unclear. Additional animal studies are required to assess the role of bone particles collected with two different drilling techniques in new bone formation. In addition, the sample size used in the study was not quite large. A large sample size is always difficult to achieve in studies in which human bone is utilized due to ethical issues.30

CONCLUSIONS

A significantly higher proliferation (DNA content) and differentiation (mineralized matrix formation) were observed in autogenous bone chips harvested by low-speed drilling without saline irrigation compared to standard drilling speed with saline irrigation. Thus, osteogenic efficacy of autogenous bone particles collected using low-speed drilling was superior in comparison with the standard drilling samples, and these bone chips may be used for minor bone regenerative procedures in implant dentistry.

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