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Original article

Bone formation and mineralization around the implant in osteoporotic animal models enhanced by mesenchymal stem cells

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ABSTRACT

Background: *Poor bone quality, sometimes caused by osteoporosis, can lead to dental implant failure. Human umbilical cord mesenchymal stem cells (hUCMSCs) can differentiate into osteoblasts and offer an alternative therapy for poor bone conditions.* ***Purpose:*** *This study aims to evaluate the effect of hUCMSCs induction on the extent of osseointegration by the new bone formation area of dental implants in osteoporotic animal models.* ***Methods:*** *The samples were divided into two groups, i.e., control and induced hUCMSCs groups at different times. An ovariectomy was performed to assess the osteoporosis condition. The control group was injected using gelatin, and the treatment group was administered hUCMSCs. Terminations were conducted at 2 and 4 weeks, respectively. The collected samples were then subjected to histological examination to analyze new bone formation and its proportion. In addition, alkaline phosphatase staining was also performed to evaluate the mineralization area. The data was analyzed using the one-way analysis of variance (ANOVA).* ***Results:*** *Both for new bone formation area as well as the proportion of new bone, it was observed that the best results were in the group with induction of hUCMSCs at 2 weeks. Alkaline phosphatase staining also confirmed that the highest mineralization was observed in the same group.* ***Conclusion:*** *hUCMSCs induced new bone formation in the implant placement in osteoporotic animal models.*

Keywords: *bone regeneration; human umbilical cord mesenchymal stem cells; implant; medicine; osteoporosis*
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INTRODUCTION

Menopause is a hormonal condition that affects all women over the age of 50. It is characterized by the cessation of menstrual bleeding for at least 12 consecutive months.[1] Decreased estrogen secretion affects parathyroid hormone activity and vitamin D absorption during menopause. This condition leads to reduced bone development and disrupts the balance of osteoclast and osteoblast activity.[2]

One of the most common pathological diseases in postmenopausal women is osteoporosis, characterized by a loss of bone density, tissue damage, and bone microarchitecture disorders that induce brittle and easily fractured bones.[3] According to research conducted in Europe and the United States, 30% of women worldwide have osteoporosis, and 40% of them are postmenopausal women.[4] The prevalence of osteoporosis in older women aged 50–70 years in Indonesia is 23%.[5] Osteoporosis has a significant impact on the dental and oral fields, particularly in the treatment of dental implants in prosthodontics.[6]

Dental implants have become an alternative for replacing one or more missing teeth or even the entire tooth in the edentulous jaw.[7,8] Several factors contribute to the successful integration of implants with bone, including the implant's surface, shape and topography, surgical technique, implant loading, and the quality and quantity of the implant recipient's bone.[9] Bone quality is the most critical factor in successful osseointegration

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and alveolar bone retention. Osseointegration is defined as a direct structural and functional relationship between the bone and the load-bearing surface of the implant without disturbing the soft tissue between the implant and the bone.[10] In the early stages of osseointegration, erythrocytes and fibrin are present, accompanied by the proliferation of vascular structures and the migration of mesenchymal cells.[11] A cell-rich immature bone (woven bone) begins to cover the blood vessels and comes into contact with the implant surface at 2 weeks. The newly formed mineralized bone extends from the cut bone ends and covers most of the implant surface between 2 and 4 weeks of healing.[12] At 4 weeks after implantation, osteoid and woven bone are formed on implant surfaces.[13] In patients with osteoporosis, the bone mass and density are lower than normal. Therefore, it causes difficulties in achieving osseointegration, resulting in failure of dental implant treatment.[9] Several therapies have been proposed to enhance osseointegration in osteoporosis patients, including the use of human umbilical cord mesenchymal stem cells (hUCMSCs).[14]

In vitro studies regarding the use of hUCMSCs have been previously conducted.[15–17] These cells have potential for use in regeneration.[18–20] They act as osteoprogenitors and promote bone formation.[21] In previous studies, hUCMSCs successfully promoted osteoblast differentiation in osteoporotic models.[14] Other studies have also proved that hUCMSCs increased the number of osteoblasts, the expression of tumor growth factor-β1, runt-related transcription factor 2 (Runx2), alkaline phosphatase, collagen type I, osteocalcin, osterix (Osx), and bone morphogenetic protein 2 (BMP-2).[8] However, it is not yet known how extensive the effect of hUCMSCs induction is on the osseointegration process for osteoporosis patients using dental implants. Therefore, this study was conducted to evaluate the effect of hUCMSCs induction on the extent of osseointegration by the new bone formation area of dental implants in osteoporotic animal models.

MATERIALS AND METHODS

This research was approved by the Ethical Commission of Health Research, Dr. Soetomo General Hospital, Surabaya (No. 547/Panke.KKE/IX/2017) and Ethical Commission of the Faculty of Veterinary Medicine, Universitas Airlangga (No. 2.KE.152.09.2018). Samples were collected from 28 three-month-old female Wistar strain rats of the *Rattus norvegicus albinus* species, weighing 180 to 200 g. The samples were divided into four groups: an ovariectomy group injected with gelatin solvent for 2 weeks (K2) and 4 weeks (K4), and an ovariectomy group injected with hUCMSCs and gelatin for 2 weeks (P2) and 4 weeks (P4).

The umbilical cord was taken from the placenta of a healthy, full-term baby, born by Caesarean operation with elective indications without any medical complications. The umbilical cord was then cut to about 1 cm, and the artery, vein, and adventitia were separated to obtain Wharton's jelly. Afterward, the Wharton's jelly was sliced with a scalpel to approximately 1 mm^3 and used for the primary culture of hUCMSCs.

The phenotype characterization of hUCMSCs was performed using flow cytometry. In the sixth passage, hUCMSCs were seeded in wells with alpha minimum essential medium (αMEM). Afterward, the cells were fixed with 10% formaldehyde and incubated using the Human MSC Analysis Kit (BD StemflowTM, BD Biosciences) with the addition of mouse anti-human CD73, CD90, and CD105 primary antibodies, and a negative cocktail containing CD45, CD34, and CD19 antibodies.

The female Wistar strain rats of the *Rattus norvegicus albinus* species weighing 180 to 200 g were placed in a separate cage for 1 week before the ovariectomy. The ovariectomy was performed through a ventral incision from the umbilicus to the pubis. The ovaries and fallopian tubes were ligated separately, and the bilateral ovaries and periovarian fat were completely removed. The peritoneal incision was closed with simple absorbable catgut sutures prior to skin closure. Postoperatively, the rats were allowed to move freely in their cages, with their usual diet for 12 weeks.

The subjects were made to fast for 6 to 8 hours before surgery. For anesthesia, 1 cc of 10% ketamine was administered, and 1 cc of Xyla was injected intramuscularly into the semitendinosus muscle. The hair on the femur where the implant would be placed was shaved, and the skin was cleaned with povidone-iodine and 80% alcohol. The instruments used were sterilized in an autoclave. A 10 mm incision was made from the femur's dorsal surface to the bone surface. The drilling sequence was performed at 7 mm from the distal edge of the femur, based on the length and diameter of the implant (1 mm diameter and 2 mm length), with saline irrigation at a speed of 800 rpm and a torque of 20 N. The implant was placed on the proximal surface of the femur, and primary stability was achieved before suturing the muscles and skin with 4-0 Vicryl. Suturing threads were removed 7 days after implant placement.

Perforation was performed on the femur of rats that had been implanted, using a needle perforator (STABIDENT) to penetrate the bone. Subsequently, hUCMSCs with solvent gelatin (P2 and P4 groups) and only gelatin solvent (K2 and K4 groups) were injected into the perforation holes using 1 mL syringes.

The animals were terminated after completing the duration of the experiment. In experimental animals, anesthesia was administered via intramuscular injections of 1 cc of 10% ketamine with 1 cc of Xyla, followed by perfusion. A 0.5 mm proximal and distal to the implant margin were cut.

The specimens were fixed in 4% paraformaldehyde in 0.1 M phosphate buffer, at pH 7.4, at 4°C, for 48 hours in 2 weeks. Then, they were washed overnight in a 0.1 M phosphate buffer, pH 7.4, containing 10% sucrose for

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1–2 hours at room temperature. The bone samples were dehydrated according to the following schedule: 70% ethanol for 1 day, 80% ethanol for 1 day, 90% ethanol for 1 day, and lastly, 100% ethanol for 4 days at room temperature. Each specimen was vacuumed for the first 30 minutes at every step. After that, the bone samples were processed in xylene for 1 day and 3 days at room temperature, each being vacuumed for the first 30 minutes. The samples were then infiltrated in a solution of 100 ml methylmethacrylate and 1 g V-601 at 4°C for 1 day and vacuumed for the first 30 minutes. Fresh solution replacement is required before infiltrating the specimens for 1 week at 4°C. Finally, the infiltrated tissue samples were placed at the bottom of 25 ml glass vials, with MMA solution (100 ml MMA and 1 g V-601) poured into each glass vial and polymerized within 2 days at 30°C. The specimens were then assessed microscopically using histomorphometric evaluation of new bone formation, proportion of new bone area, and alkaline phosphatase staining. The data was then analyzed using one-way ANOVA with $\alpha = 0.05$.

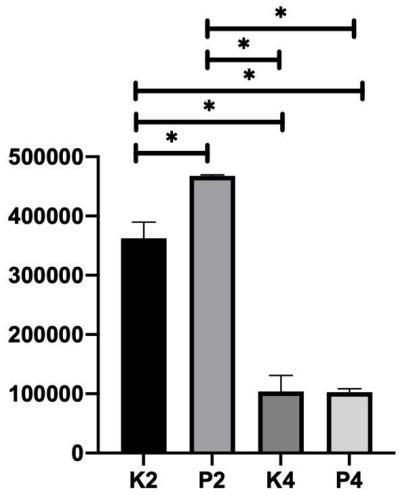

Figure 1. Graph analysis of new bone formation. The * indicates a statistical difference (p-value < 0.05). n = 7; K2: injected with gelatin solvent for 2 weeks, P2: injected with hUCMSCs and gelatin for 2 weeks, K4: injected with gelatin solvent for 4 weeks, P4: injected with hUCMSCs and gelatin for 4 weeks.

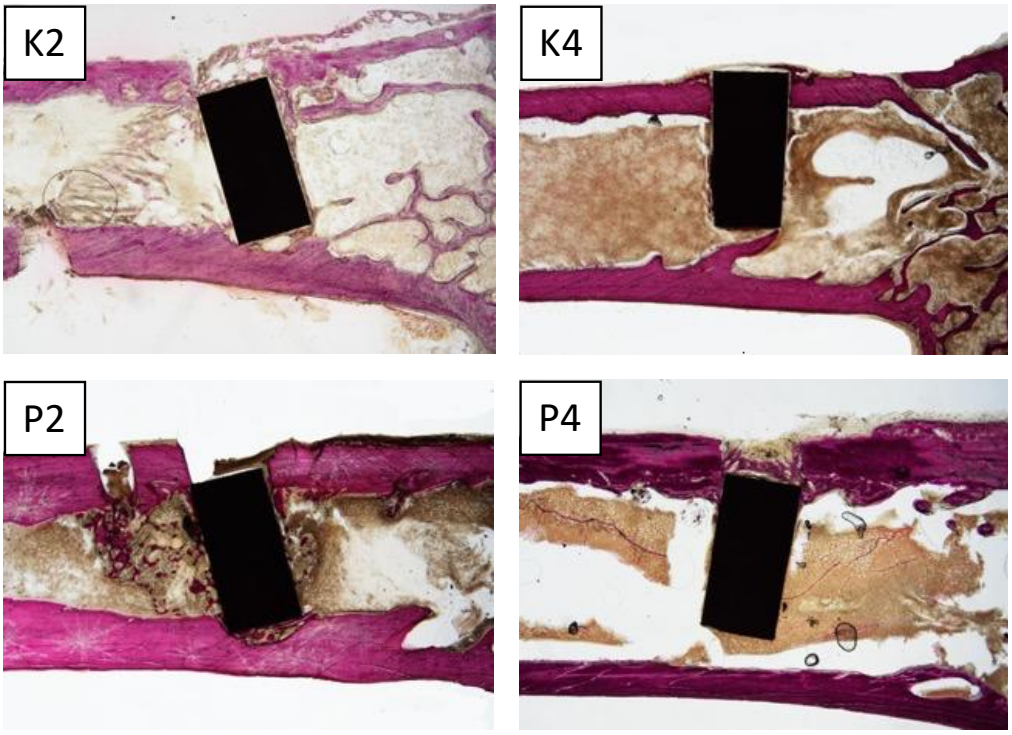

Figure 2. Histological image of each experimental group for new bone formation around implant surface. The black block is an implant placed in the femur.

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RESULTS

The new bone formation area was measured histologically using a microscope in the cortical area around the implant surface. The highest new bone formation was observed in induction with hUCMSCs at week 2 (P2), followed by control at week 2 (K2), and induction with hUCMSCs at week 4 (P4). The lowest new bone formation was observed in the control week 4. The graph of the new bone formation area analysis results can be seen in Figure 1, and the histological image is presented in Figure 2.

The proportion of new bone area result was in line with the previous results, i.e., the highest was found in induction with hUCMSCs at week 2 (P2 group), followed by control at week 2 (K2 group) and induction with hUCMSCs at week 4 (P4 group). The lowest new bone formation was found in the control at week 4. The graph results of the proportion of the new bone area analysis can be seen in Figure 3.

Alkaline phosphatase staining was performed to confirm mineralization in the bone area around implant placement. This analysis was performed qualitatively and indicates that the highest alkaline phosphatase was noted in the P2 group. The histological image is shown in Figure 4.

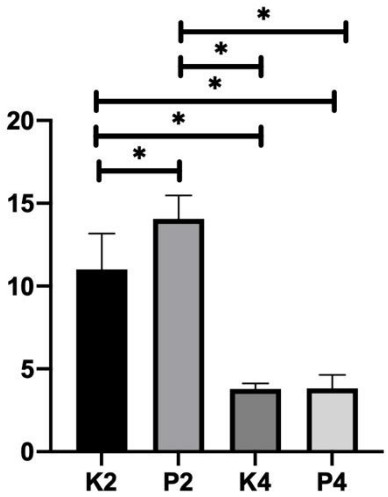

Figure 3. Graph analysis of the proportion of new bone area. The * indicates a statistical difference (*p-value* < 0.05). n = 7; K2: injected with gelatin solvent for 2 weeks, P2: injected with hUCMSCs and gelatin for 2 weeks, K4: injected with gelatin solvent for 4 weeks, P4: injected with hUCMSCs and gelatin for 4 weeks.

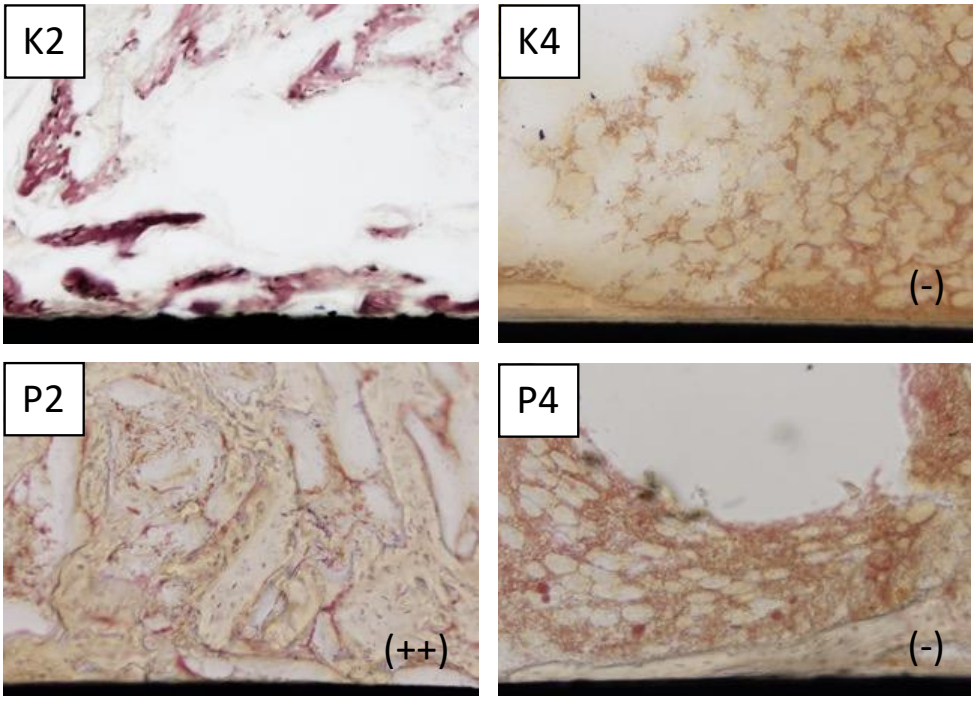

Figure 4. Histological images of each experimental group for alkaline phosphatase staining.

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DISCUSSION

Osseointegration of implants in patients with osteoporosis is difficult to predict and is always an obstacle.[22] In this condition, there is an imbalance between bone formation and resorption, resulting in a weaker structure, especially in the cancellous bone.[23] This is because the differentiation, proliferation, and osteogenic capabilities of mesenchymal stem cells are inhibited, which impedes bone formation in the early phase of implant osseointegration. Although dental implants are a viable treatment, osteoporosis deleteriously affects peri-implant long-term stability and bone loss.[24]

In this study, an increase in new bone formation and the proportion of new bone were observed. Similar results were obtained based on the histological assessments. We noted an increased number of woven bone formations on hUCMSCs induction; the woven bones were more even and had a trabecular bone structure, indicating a superior acceleration of bone mineralization. The qualitative results of alkaline phosphatase staining support these results; a more intense staining result indicates increased alkaline phosphatase activity. This increase in alkaline phosphatase indicates the differentiation activity of osteogenic cells that occurs at the beginning of healing and peaks on days 5–14, followed by a gradual decrease.[25] The final stage of osteogenic differentiation (days 14–28) is characterized by high levels of osteocalcin and osteopontin and deposition of calcium phosphate.[26] The biocompatibility capabilities of titanium implants can induce bone regeneration.[27] However, using hUCMSCs accelerates implant osseointegration in the early stages of regeneration, significantly affecting the long-term outcome of implant treatment, especially in patients with osteoporosis.

In the early stages of osseointegration, blood clots produce fibrin as the initial form of healing, followed by the proliferation of the vascular structures and, migration of mesenchymal cells, and the formation of woven bone and bone trabeculae structures. However, during 1–4 weeks, an important osteoimmunological balance exists among host bone cells, immune cells, and implants.[28] Afterward, the large volume of woven bone is remodeled and replaced by the lamellar bone around the implant.[29] At that time, the implant was characterized by a thin seam of newly formed bone tissue lining their surface, and spaces of tissue lining their surface, and space of provisional connective tissue could be observed in the compartments between the threads.[11]

The most significant challenge for this early stage is obtaining the fixation of the implant. Implant fixation is divided into two types: mechanical fixation and biological fixation. Biological fixation is obtained at cancellous bone by forming woven bone, which will become trabecular bone. The trabecular bone fills the gap between the implant and the host bone.[29] While mechanical fixation is achieved at the cortical bone, avoiding excessive micromotions affecting fibrin tissue and impairing vascularity in the initial bone healing is essential. However, balance in osteoimmune regulation is an important factor for dental implant longevity, including protection from bacterial infection.[30] Bacterial infection and byproducts should be considered; for instance, *Porphyromonas gingivalis* and lipopolysaccharides can trigger inflammation and periodontal and alveolar bone destruction.[15,31] Research by Sugiura discovered that maximum micromotion was significantly affected by cancellous bone density. In high-density cancellous bone models, the micromotion was always <15 μm. Moreover, in low-density cancellous bone models, the maximum extent of micromotion was influenced by crestal cortical bone thickness.[32]

Applying hUCMSCs at the implant site in osteoporotic rats increases osteoblastogenesis abilities to achieve faster biological fixation. The potential of hUCMSCs is demonstrated by the increased TGF β1 expression, Runx2, and the number of osteoblasts in osteoporotic rats.[33] The hUCMSCs demonstrate high-osteogenic activity and increased osteoporotic mandibular bone regeneration, as shown by increased expression of Osx and BMP-2 and decreased tartrate-resistant acid phosphatase expression. This indicates that hUCMSCs can promote osteogenic differentiation and increase mineralization and bone formation in the osteoporotic bone.[8] In addition, hUCMSCs are useful for assisting the early-stage process of implant osseointegration in patients with osteoporosis. HUCMSCs have the potential to increase the proportions of direct bone-to-implant contact. They may, indeed, provide an earlier and better anchorage of devices, thus allowing for an earlier functional loading of implants. Furthermore, recent studies about hUCMSCs transplantation with hypoxia pretreatment showed amelioration of these cells, and may be an alternative for future stem cell based research.[34–36]

From this research, it can be concluded that hUCMSCs induced new bone formation in the implant placement in osteoporotic animal models. More in-depth research should be conducted in the future, especially regarding measuring bone-to-implant contact and using a larger OVX animal model, more representative of osteoporosis in humans, so that the rate of cure and effectiveness of hUCMSCs can be examined in more depth.

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