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Enhancing in vivo vascularized bone formation by cobalt chloride-treated bone marrow stromal cells in a tissue engineered periosteum model

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ABSTRACT

The periosteum plays an indispensable role in both bone formation and bone defect healing. This study constructed in vitro an artificial periosteum by incorporating osteogenic differentiated bone marrow stromal cells (BMSCs) and cobalt chloride ($CoCl_2$)-treated BMSCs. The engineered periostea were implanted both subcutaneously and into skull bone defects in SCID mice to investigate ectopic and orthotopic osteogenesis and vascularisation. Micro-CT, histomorphometrical and immunohistochemical methods were used to assess the ectopic and orthotopic osteogenesis and vascularisation of the implanted constructs after two weeks in subcutaneous and four weeks in bone defect areas. The results showed that $CoCl_2$ pre-treated BMSCs induced higher degree of vascularisation and enhanced osteogenesis within implants in both ectopic and orthotopic areas. This study provided a novel approach using BMSCs sourced from the same patient for both osteogenic and pro-angiogenic purposes in constructing tissue engineered periosteum to enhance vascularized osteogenesis.

INTRODUCTION

Bone defects, especially large bone defects, remain a major challenge in orthopedic surgery [1]. Autologous bone transplantation is considered the most effective treatment, but insufficient donor tissue, coupled with concerns about donor site morbidity, has hindered this approach in large-scale applications. On the other hand, immunological reactions that would occur in recipients against allo or xenogeneic bone grafts impose a major restriction on the clinical application of these approaches.

In both physiological and pathological processes, the periosteum plays an indispensable role in both bone formation and defect healing alongside the involvement of various growth factors and mechanical loading [2-4]. Periosteum is a dense, dual-layered and highly vascularized connective tissue membrane covering cortical bone surfaces [2, 5, 6]. The cambial layer of periosteum contains osteoblasts and osteogenic progenitor cells, whereas in the fibrous layer there are collagen fibers, fibroblasts and blood vessel networks. It is the osteogenic function of periosteum that determines the rate of *de novo* postnatal periosteal bone formation and consequently the bone strength during the process of growth or defect healing [4]. Transplantation of autogenous or allogenous periosteum has been applied successfully in the restoration of various-sized bone defects, especially large bone defects [7-9]. Bone healing induced by periosteum has several advantages compared to direct bone or bone substitute transplantation, such as healing with a natural bone structure, optimal bone integrity in the defect area, appropriate degree of vascularization, and minimal ectopic ossification through the defect encasement [7, 8]. However, major concerns in the case of autogenous periosteum transplantation include insufficient autologous donor tissues and donor site morbidity; and in the case of allogenous transplants, immunological rejection reactions. Therefore, *in vitro* tissue-engineered periosteum, designed to mimic the anatomical structure of natural periosteum, may provide a novel approach in the treatment of large bone defects.

Bone marrow stromal cells (BMSCs) are progenitor cells with the capacity to be differentiated into osteogenic lineage following ex vivo expansion and osteogenic induction. It has been demonstrated that these cells are able to regenerate bone tissues upon in vivo transplantation in bioactive scaffolds containing hydroxyapatite/tricalcium phosphate (HA/TCP)[10, 11]. However, tissue engineered bone substitutes, especially BMSCs delivery constructs, show some difficulties in forming functional bone tissues due to the limitation of blood supply after implantation. A critical

problem in using the bone tissue engineering approach to restore large bone defects is that the nutrient supply and cell viability at the centre of the scaffold is severely hampered since the diffusion distance of nutrients and oxygen for cell survival is limited to 150-200μm [12]. Indeed, numerous studies have recently identified that tissue engineered products could only form a limited tissue layer on the scaffold surface. Moreover, in the centre of the scaffold, cell density is typically low and necrosis may occur. Therefore, the desired tissue engineered bone constructs should contain certain pro-angiogenic factors in order to recruit blood vessels into the bone substitutes. It is known that hypoxia will increase the expression of pro-angiogenic factors, such as VEGF, by BMSCs, which can help restore the vascularization in ischemic tissues [13, 14]. VEGF is also thought to be one of the key factors coupling both vascularization and osteogenesis [15]. $CoCl_2$ is a hypoxia mimicking agent, which can activate the hypoxia inducible factor-1 (HIF-1) in mesenchymal stem cells and subsequently activate HIF-α target genes including VEGF, EPO, and p21[16]. In this study, a dual-layered periosteum was constructed using BMSCs treated by $CoCl_2$ and osteogenic differentiated BMSCs in a type I collagen scaffold. The osteogenesis and vascularization of the tissue engineered periosteum were evaluated subcutaneously and in a critical size defect of SCID mice.

MATERIALS AND METHODS

Cells and cell cultures

BMSCs were isolated and characterized base on the protocols developed in our previous studies [17-19]. Briefly, bone marrow was obtained from hip and knee replacement patients in orthopedic department of the Prince Charles Hospital in Brisbane with informed consent and ethics approval from the Ethics Committee of Queensland University of Technology. Mononuclear cells (MNCs) were isolated from the bone marrow by density gradient centrifugation over Lymphoprep (Axis-shield PoC AS, Oslo, Norway) according to the manufacturer's protocol. The MNCs were plated into the culture flasks and cultured in growth media containing Dulbecco's Modified Eagle Medium (DMEM; Invitrogen Pty Ltd., Mt Waverley, VIC, Australia), 10% (v/v) fetal calf serum (FCS; InVitro Technology, Noble Park, VIC, Australia) and 1% (v/v) penicillin/streptomycin (Invitrogen). The unattached hematopoietic cells were removed through media changes. When

reaching the 70-80% confluence, the attached mesenchymal cells were subcultured using 0.25% trypsin (Invitrogen) with 1mM EDTA (Invitrogen). Only passages 2-5 (P2-P5) mesenchymal cells were used in this study. The medium was changed every three days until the cells were confluent.

Cobalt chloride treatment of BMSCs

BMSCs were firstly cultured in DMEM until reaching 70-80% confluence in cell culture flask, then the media was changed to the DMEM containing 10% (v/v) FCS and 100μM $CoCl_2$ (Sigma Aldrich Pty Ltd, Castel Hill, NSW, Australia) one week before the implantation. To observe the VEGF expression by the treatment, the real-time polymerase chain reaction (real-time PCR) and western blot methods were used on both treated and untreated BMSCs (n=4). Briefly for the real-time PCR, total RNA was collected from treated or untreated BMSCs and cDNA was generated using a cDNA reverse transcription kit (Invitrogen). A 12.5μl SYBR green q-PCR master mix (AB Applied Biosystems, Melbourne, Australia) was mixed with 5 μl Ultrapure water (Invitrogen), 2.5 μl reverse and forward primers (Sigma-Aldrich Pty. Ltd**,** Castle Hill, NSW, Australia) and 2.5 μl cDNA to make a 25 μl final reaction system in a 96-well PCR clear plate. The gene expressions of VEGF and 18s (house keeping gene) were detected by a real time-PCR machine (7300 sequence detection system, AB Applied Biosystems, Melbourne, Australia). The sequences of forward and reverse primers of each gene are detailed in Table 1. As for the western blot, total cell proteins were obtained and 10μg proteins from each sample were used to do western blot analysis. Briefly, the protein samples were loaded onto SDS-page gels and run at 125V for 1h. Then the proteins were transferred to a nitrocellulous membrane (Pall Corporation, East Hills, NY, USA) using semi-dry transfer method. The VEGF and α-tubulin were recognized by VEGF (1:2000, rabbit anti-human, Thermo Fisher Scientific, Fremont, CA, USA) and α-tubulin (1:5000, rabbit anti-human, Abcom Inc., Cambridge, MA, USA) primary antibodies which then bound to the HRP-conjugated anti-mouse or rabbit secondary antibodies (1:10,000, Thermo Fisher Scientific, Fremont, CA, USA). Super-signal substrate (Cata.No.34095, Thermo Fisher Scientific, Fremont, CA, USA) was used to react with HRP to generate chemiluminescence which left band images on X-ray films. All operations were performed under the same conditions.

Periosteum construction

Collagen scaffolds were 1mm-thick bovine type I collagen sheet (Medtronic Australia Pty Ltd, North Ryde, NSW, Australia) and customized to a size of 5mm in diameter, then sterilized by soaking in 75% ethanol for 10mins, and washed at least three times in PBS prior to cell seeding. The scaffold was placed in a well of 96-well cell culture plate. A total of 6×10^4 BMSCs were seeded on top of the scaffolds and cultured in growth media for 24hrs, then changed to osteogenic differentiation media containing 10mM β-glycerophosphate, 50µM ascorbic acid and 100nM dexamethasone (Sigma) for 2 weeks. The $CoCl_2$ treated BMSCs (1×10^5) were then seeded onto the other side of the collagen scaffolds. The tissue engineered periosteum was incubated in growth media for another three hours, and then implanted in SCID mice subcutaneously or in bone defect sites.

The cell seeding efficiency onto the scaffolds was evaluated by using a NucleoCounter (ChemoMetec A/S, DK-3450 Allerød, Denmark) to count the cells attached on the scaffold in comparison to the total cells seeded. The distribution of osteogenic differentiated BMSCs on scaffolds was observed by cell nuclei staining. Briefly, the scaffold was fixed in 4% paraformaldehyde, embedded in paraffin and 5µm-thick serial sections were cut perpendicular to the cell seeding surface using a microtome (Leica Microsystems GmbH, Wetzlar, Germany). Cell nuclei were stained with DAPI (Molecular Probes, Invitrogen) and observed under a microscope (Carl Zeiss Microimaging GmbH, Gottingen, Germany) at 100 × magnification. The distribution of $CoCl_2$-treated BMSCs in the scaffolds was observed by labelling these cells with intra-cellular green fluorescent nano-particles (Q-tracker 565; Invitrogen). After labelled cells were seeded onto the scaffolds, a 60µm thick slice was cryosectioned and stained with DAPI and observed directly under a fluorescent microscope (Carl Zeiss Microimaging GmbH, Göttingen, Germany) at 100 × magnifications.

Subcutaneous implantation of tissue engineered periosteum in SCID mice

Animal ethics approval for the use of severe combined immunodeficient (SCID) mice in this experiment was granted by the Animal Ethics Committee of Queensland University of Technology.

Each group contained four 5-week old male SCID mice (Animal Resources Centre, Canning Vale, WA, Australia). The animals were anesthetized with 1mL of ketamine (100mg/mL) and 0.15 mL of xylazine (20mg/mL) injected intraperitoneally. Three small incisions were made longitudinally along the central line of the shaved dorsal area, approximately 1cm apart, and subcutaneous pockets were made on each side of the incision with a pair of surgical scissors. Each individual pocket had one scaffold. Incisions were closed with surgical clips. All animals recovered fully from the surgery by the following day and were sacrificed after two weeks. The implants were retrieved for following analyses.

Skull defect implantation of tissue engineered periosteum in SCID mice

A total of twenty-eight 5-week old male SCID mice (Animal Resources Centre) were used. Skull defect model was generated according to our previous studies [20, 21]. Briefly, small incision was made longitudinally along the centre line. After the skull bone surface was exposed, the periosteum covering the bone surface was totally removed using a dental scraper. A Ø2.5mm defect was then created using a dental bur. Care was taken during the drilling to avoid entering the dura. The phosphate buffered saline (PBS) was applied onto the drilling site to prevent overheating-related damages to local tissues. The defect was filled with tissue engineered periosteum, covered with skin and closed using surgical clips. All animals recovered fully from the surgery by the following day and were sacrificed after four weeks. The skull defect area together with implants was harvested.

Assessment of mineralization by micro-CT scanning

All implants were fixed in 4% paraformaldehyde solution overnight at room temperature and then washed in PBS. Cross-sectional scans parallel to the cell seeding surface of subcutaneous or skull defect implants were performed in a micro-CT scanner (μCT40, SCANCO Medical AG, Brüttisellen, Switzerland) at a resolution of 12μm and three dimensional (3D) images of implants were reconstructed from the scans by the micro-CT system software package. To validate the in vivo mineralization of tissue engineered periosteum, scaffolds cultured in vitro or implants without pre-seeded cells or carrying only untreated BMSCs were also scanned. For in vitro or subcutaneously implanted scaffolds, total volume and average density against hydro-apatite (HA) of

mineralization were measured and recorded by the micro-CT system software package for statistical analysis. For scaffolds in the skull defect, volume and average density of mineralization within the implants (defined by encircling the implant area on each micro-CT slice as area of interest) were measured and recorded for statistical analysis.

Mineralization detected by von Kossa staining in subcutaneous implants

Following micro-CT scanning, subcutaneous implants were embedded in paraffin and 5μm-thick serial sections were cut perpendicular to the implants using a microtome (Leica Microsystems GmbH). Slices near the central area of the implants were used for von Kossa staining. In brief, after slice dewaxing and hydration, Mayer's haematoxylin (HD Scientific Pty Ltd., Kings Park, NSW, Australia) were used for nuclear staining. 5% silver nitrate (Sigma-Aldrich) was added onto each slice and exposed to light for at least 30 minutes. Then the slices were briefly washed in distilled water and dehydrated in ethanol before being mounted with cover slides. The mineralization was observed under a microscope (Carl Zeiss Microimaging GmbH).

Assessment of new bone formation by histological H&E staining

Following micro-CT scanning, skull defect implants were decalcified in 10% EDTA and embedded in paraffin and 5μm-thick serial sections were cut perpendicular to the implants using a microtome (Leica Microsystems GmbH). Slices near the central area of the implants were used for the H&E staining. Briefly, following slice dewaxing and hydration, Mayer's haematoxylin (HD Scientific Pty Ltd.) were used for nuclear staining. After dehydration in alcohol with up-grading concentrations, cell plasma and extracellular matrix were stained by eosin (HD Scientific Pty Ltd.). Based on H&E staining, *de novo* bone formation within the implants was observed and the area of new bone tissues on each slice was measured using AxioVision software (Carl Zeiss Microimaging GmbH). Three measurements from each implant were averaged and used for statistical analysis.

Assessment of vascularization and new bone formation using immunohistochemical staining

Slices near the central area of the implants were used for the immunohistochemical staining. A von Willebrand factor (vWF) antibody (mouse anti-human, Chemicom International Inc., Temecula, CA) was used to identify the presence of endothelial cells in the implants. An alkaline phosphatase

(ALP) antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) was used to confirm newly formed bone matrix in implants. In brief, following slice dewaxing and hydration, endogenous peroxidase activity was quenched by incubating the sample slices with 3% H_2O_2 for 20 minutes then blocked with 10% swine serum for 1 hour. The samples were incubated with the vWF primary antibody (1:300) or ALP antibody (1:200) overnight at 4^oC, followed by incubation, at room temperature, with a biotinylated swine-anti-mouse, rabbit, goat secondary antibody (DAKO Multilink, CA, USA) for 15 minutes, and then with horseradish perioxidase-conjugated avidin-biotin complex (DAKO Multilink, CA, USA) for another 15 minutes. The antibody complexes were visualized by the addition of a buffered diaminobenzidine (DAB) substrate for 4 minutes. Mayer's haematoxylin (HD Scientific Pty Ltd.) were used for counter staining. The number of vWF^+ blood vessels were counted and normalized to the area of the implant on each slice. Three measurements from each implant were averaged and used for statistical analysis.

Double staining of both vWF and calcium on subcutaneous implants

Double staining with vWF antibody and von Kossa method (calcium staining) was performed on subcutaneous implants to determine the relationship between osteogenesis and vascularization. In brief, following vWF staining, 5% silver nitrate (Sigma-Aldrich) was added onto each slice and exposed to light for at least 30 minutes and the slices briefly washed in distilled water and dehydrated in ethanol before being mounted with cover slides.

Statistical analysis

Analysis was performed using SPSS software (SPSS Inc, Chicago, Il, USA). All the data were analyzed using one-way ANOVA or Friedman test for group differences. The significance level was set at $p \leq 0.05$.

RESULTS

VEGF expression in $CoCl_2$-treated BMSCs

The VEGF gene expression of $CoCl_2$-treated BMSCs was assessed by real time q-PCR. BMSCs cultured in $CoCl_2$-induced hypoxia condition expressed significantly higher level of VEGF than that

in normal culture condition ($p<0.05$)(Fig.1A). The intracellular VEGF proteins identified by western blot further confirmed the upregulation of VEGF in $CoCl_2$-treated BMSCs (Fig.1B).

Tissue engineered periosteum

Collagen sponges were used as scaffold for the construction of tissue engineered periosteum. A total of 6×10^4 BMSCs were firstly seeded onto the collagen scaffolds (Fig. 2A). The cell seeding efficiency was $92.3 \pm 2.5\%$ (Fig. 2C). After two weeks of osteogenic differentiation, $CoCl_2$-treated BMSCs were seeded onto the other side of the scaffolds (Fig.2B). The cell seeding efficiency of $CoCl_2$-treated BMSCs was $85.3 \pm 5.0\%$ (Fig. 2C). The collagen scaffolds with both osteogenic differentiated and $CoCl_2$-treated BMSCs formed a generally dual-layered cell and matrix construct similar to periosteum structure with one layer of osteogenic cells and the other layer of pro-angiogenic cells. The histology of the tissue engineered periosteum revealed that after the initial cell seeding, cells attached to the collagen fibres and distributed evenly within the scaffold (Fig. 2A). Slices from cryosectioning revealed a dual-layered structure with most fluorescence-labelled $CoCl_2$-treated BMSCs on one side of the construct and osteogenic cells on the other side of scaffold (Fig. 2B).

Enhanced vascularisation and mineralization by $CoCl_2$-treated BMSCs in subcutaneously implanted cell constructs

General observation on the subcutaneous implants showed that the construct containing $CoCl_2$-treated BMSCs attracted more blood vessels growing around the construct compared with other constructs (Fig.3A-C). Further vWF staining on implant slices confirmed that scaffolds containing $CoCl_2$-treated BMSCs had higher degree of vascularisation (Fig.3D-H, $p<0.05$, Friedman test).

Micro-CT scanning and 3D reconstruction revealed that substantial amount of mineralization was found in the implants containing osteogenic differentiated BMSCs (Fig.4 B-D). The constructs containing both osteogenic differentiated and $CoCl_2$-treated BMSCs showed the highest volume of mineralized areas (Fig.4D & I, $p<0.05$, Friedman test), although no significant differences were found in the average density of mineralized areas between groups (Fig.4J, $p>0.05$, Friedman test).

No mineralization was found in the scaffolds without pre-seeded cells or containing undifferentiated BMSCs (Fig.4I). Very limited mineralization was detected in vitro-cultured constructs (Fig.4A&I). von Kossa staining on implants confirmed $CoCl_2$-treated BMSCs significantly enhanced the mineralization within the implants (Fig.4E-G). However, despite the mineralization in the implants, no histological bone tissue was detected in all subcutaneously transplanted constructs based on the H&E staining at this time point (Fig.4H).

In subcutaneous implants, von Kossa and vWF double staining showed a close relationship between the mineralization and blood vessel distribution (Fig.5). It was noted that micro-blood vessels grew around and into the mineralized areas (Fig.5).

Enhanced new bone formation and vascularization by $CoCl_2$-treated BMSCs in skull defect

In skull defects, general observation revealed that the defects filled with scaffolds carrying osteogenic differentiated BMSCs (alone or together with other cells) showed much better repair than control groups of collagen scaffold alone or impregnated with undifferentiated BMSCs(Fig.6). Micro-CT scanning and 3D reconstruction showed that the implants carrying osteogenic differentiated BMSCs plus $CoCl_2$-treated BMSCs had significantly higher volume of mineralized areas than other implants (Fig.7K, $p<0.001$, one-way ANOVA test), while no significant difference was found in the average density of mineralized areas among different groups (Fig.6L, $p>0.05$, one-way ANOVA test). The 3D reconstructed defect area (Fig.6 A-D) showed that the defects filled with implants carrying osteogenic differentiated BMSCs plus $CoCl_2$-treated BMSCs healed much better than other groups. H&E staining on slices revealed that the implants carrying osteogenic differentiated BMSCs plus $CoCl_2$-treated BMSCs had significantly more newly formed bone tissues than other implants (Fig.7E-G&M, $p<0.001$, one-way ANOVA test), and the defects were almost bridged by the newly formed bone tissues (Fig.7G). Interestingly, the *de novo* bone tissue formed along and incorporated the collagen fibers of the scaffold (Fig.7 H&I). Immunohistochemical staining against ALP further confirmed the newly formed bone tissue within implants was surrounded by ALP^+ osteoblasts (Fig.7J).

General observation also showed that the implants carrying both osteogenic differentiated BMSCs and CoCl2-treated BMSCs attracted blood vessels to grow into the defect area (Fig.6E&F). Further vWF staining on implant slices confirmed that scaffolds containing $CoCl_2$-treated BMSCs had

higher degree of vascularisation (Fig.8, $p<0.001$, one-way ANOVA test). vWF^+ micro-blood vessels were found gathering around the *de novo* bone tissues and involved into the newly formed bone tissues (Fig.8 D-F).

DISCUSSION

As recently pointed out in a review by Zhang et el, periosteum engineering could assist in structural *de novo* bone formation and is therefore a promising method for bone defect restoration [22]. Although much work has focused on the structure, cell populations and osteogenic mechanisms within the periosteum [4, 23-26], few, if any, references were found which addresses engineered periosteum containing different cell populations such as osteogenic and pro-angiogenic cells, not to mention an *in vivo* assessment of any such constructs. To the best of our knowledge, this is the first attempt to construct an artificial bi-layered periosteum with BMSCs dedicated into two different cell functions – osteogenic and pro-angiogenic. Previous studies concerning artificial periosteum have described structures with a single cell population, and therefore differ considerably from the natural structure of real periosteum [27-29]. The obvious drawback of these approaches is the lack of information about osteogenic and pro-angiogenic cell interactions in forming a vascularised bone. In the experiment presented here, both osteogenic (BMSCs derived osteoblasts) and pro-angiogenic cells ($CoCl_2$-pre-treated BMSCs) were seeded onto either side of a collagen membrane, trying to mimic the distribution of cell populations in physiological structure of periosteum.

Although there are many other cell populations residing within the real periosteum, osteogenic and endothelial cells are the major and most important cell populations [30, 31] and the interactions between the osteogenesis and vascularisation determine the osteogenic function of periosteum to a large extent. To enhance the vascularisation of engineered tissues and organs, three different approaches are most often used: i) autogenous or allogenous endothelial cells which could be directly incorporated into the host blood vessel networks[32, 33]; ii) impregnate pro-angiogenic growth factors, such as VEGF, bFGF, and PDGF into the scaffold to incur the new blood vessel ingrowth[15, 34]; iii) incorporate gene vectors (plasmid or virus) encoding pro-angiogenic growth factors or cells transfected by those gene vectors within scaffolds to attract new blood vessels[35].

All these approaches show their disadvantages in the potential in vivo applications, such as the difficulties in isolation of endothelial cells from the host and immunological reactions to allogenous endothelial cells, the low growth rate of autologous endothelial cells in vitro, biological safety concerns about the gene vectors or transfected cells and unstable temporal and spatial release of pro-angiogenic growth factors in defect areas. Recently studies have tried to induce autologous bone marrow-derived mesenchymal stem cells to differentiate towards endothelial cells, but the results so far are still elusive and uncertain[36, 37]. Interestingly, it has been reported that either low oxygen pressure or hypoxia-mimic agents, such as $CoCl_2$ or desferrioxamine, can upregulate the expression of pro-angiogenic growth factors, VEGF in particular, in a variety of cells including BMSCs[38, 39]. The hypoxia pre-treated BMSCs had been successfully used to help re-vascularize the ischemic or infarcted muscles in animal models[10, 13]. In this study, BMSCs were pre-treated with hypoxia-mimic agent $CoCl_2$ and the VEGF expression after treatment was found to be increased by around 5 folds. VEGF is the most important growth factor involved in both angiogenic and osteogenic processes [40-42], Therefore, in this study $CoCl_2$ pre-treated BMSCs were incorporated together with BMSCs-derived osteoblasts into the engineered periosteum model for the generation of vascularized bone in vivo.

Two different animal models are often used to assess the in vivo osteogenic ability of engineered tissues: subcutaneous (ectopic) and bone defect (orthotopic) osteogenesis models. In this study, both models were included. During pathological and physiological osteogenesis, two distinct patterns of bone formation are involved – intramembranous and endochondral ossification. Intramembranous ossification predominantly takes place in the periosteum during normal bone formation in which osteoprogenitor cells differentiate directly into osteoblasts laying down new bone tissues [43]. Although much has been reported about both *in vivo* and *in vitro* interactions between osteogenesis and vascularization [15, 32], the knowledge of *in vivo* interactions between osteogenesis and vascularisation, especially those during early osteogenic stages and related to periosteal intramembranous ossification, is still very much patchy. Early stage histological and ultrastructural changes in microvessels of periosteal calluses after bone facture, using the light and electron microscope, has been described previously, but no quantitative data about the interaction between osteogenesis and vascularization was provided [44]. In the present study, observations and assessments about the osteogenesis and vascularisation at a relatively early stage in the

engineered periosteum were performed two weeks after subcutaneous implantation and four weeks after skull defect implantation. Osteogenesis and vascularization were then quantified using micro-CT scanning, histological and immunohistochemical methods [45]. Our findings showed that subcutaneously implanted scaffolds presented more extensive calcification compared with scaffolds prior to in vivo implantation, while in skull defects *de novo* bone tissues were evident. Another noteworthy point is that in the skull defect naked collagen implants or implants carrying only BMSCs were almost degraded and generated very limited new bone tissues within the defect when compared with implants carrying BMSCs-derived osteoblasts. This phenomenon suggested that new bone formation may require the orchestration of an osteogenesis-favouring environment, appropriate calcium precipitation on the collagen fibres and osteogenic cells. As for the vascularisation, $CoCl_2$ pre-treated BMSCs helped significantly increase blood vessel density in both subcutaneously and skull defect implanted scaffolds compared with others. The upregulated VEGF expression by $CoCl_2$ pre-treated BMSCs could stimulate the osteogenesis of BMSCs-derived osteoblasts after the cells were seeded and this osteogenic process could be even more enhanced by subsequently enhanced vascularization. The calcium-vWF double staining and vWF^+ micro blood vessels surrounding *de novo* bone tissues also revealed the close relationship between the osteogenesis and vascularization. To confirm the pro-osteogenic and pro-angiogenic effects of $CoCl_2$ pre-treated BMSCs, untreated BMSCs were used together with BMSCs-derived osteoblasts as an internal control. The findings in this study showed that the addition of untreated BMSCs did not significantly help the in vivo ectopic or orthotopic osteogenesis by BMSCs-derived osteoblasts and vascularization.

Conclusion: Based on the findings of this experimental study, it could be concluded that $CoCl_2$ pre-treated BMSCs can enhance the vascularization in both ectopic and orthotopic osteogenesis in a periosteum model. This study provides a novel approach using BMSCs sourced from the same donor for the regeneration of vacularized bone tissue.

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