

The effect of rhBMP-2 on canine osteoblasts seeded onto 3D bioactive polycaprolactone scaffolds

B. Rai^{a,*}, S.H. Teoh^{b,c}, K.H. Ho^a, D.W. Hutmacher^{b,d}, T. Cao^a, F. Chen^c, K. Yacob^b

^a Department of bioengineering, Faculty of Dentistry, National University of Singapore, Singapore, Singapore

^b Division of Bioengineering, Faculty of Engineering, National University of Singapore, Singapore, Singapore

^c Department of Mechanical Engineering, Faculty of Engineering, National University of Singapore, Singapore, Singapore

^d Department of Orthopedic Surgery, Faculty of Medicine, National University of Singapore, Singapore, Singapore

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Abstract

Our strategy entails investigating the influence of varied concentrations (0, 10, 100 and 1000 ng/ml) of human recombinant bone morphogenetic protein-2 (rhBMP-2) on the osteogenic expression of canine osteoblasts, seeded onto poly-caprolactone 20% tricalcium phosphate (PCL-TCP) scaffolds in vitro. Biochemical assay revealed that groups with rhBMP-2 displayed an initial burst in cell growth that was not dose-dependent. However, after 13 days, cell growth declined to a value similar to control. Significantly less cell growth was observed for construct with 1000 ng/ml of rhBMP-2 from 20 days onwards. Confocal microscopy confirmed viability of osteoblasts and at day 20, groups seeded with rhBMP-2 displayed heightened cell death as compared to control. Phase contrast and scanning electron microscopy revealed that osteoblasts heavily colonized surfaces, rods and pores of the PCL-TCP scaffolds. This was consistent for all groups. Finally, Von Kossa and osteocalcin assays demonstrated that cells from all groups maintained their osteogenic phenotype throughout the experiment. Calcification was observed as early as four days after stimulation for groups seeded with rhBMP-2. In conclusion, rhBMP-2 seems to enhance the differentiated function of canine osteoblasts in a non-dose dependent manner. This resulted in accelerated mineralization, followed by death of osteoblasts as they underwent terminal differentiation. Notably, PCL-TCP scaffolds seeded only with canine osteoblasts could sustain excellent osteogenic expression in vitro. Hence, the synergy of PCL with bioactive TCP and rhBMP-2 in a novel composite scaffold, could offer an exciting approach for bone regeneration.

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1. Introduction

Scientists are seeking for the ideal bone regenerating device that requires interplay between three components: (i) a 3D scaffold as a pillar of support for the cells, (ii) the cells that secrete bone and (iii) bioactive factors that guide the cells to form the desired tissue [1]. A rapid prototyping process called fused deposition modeling (FDM) allows the design and fabrication of highly reproducible bioresorbable 3D polycaprolactone scaffolds with a fully interconnected pore network [2–4]. Cell culture study showed that polycaprolactone scaffold allows proliferation and differentiation of fibroblasts and osteoblasts [2,5].

A new tissue engineering approach is to seed osteoprogenitor or osteoblastic cells onto matrices to increase its bioactivity [6]. The most important function of osteoblasts is to form mineralized tissue that is regulated by many local factors. Among these, a bone morphogenetic proteins (BMP) are one of the most potent factors [7].

BMPs have the unique functions of inducing the differentiation of cells of the osteoblastic lineage, therefore increasing the pool of mature cells, and of enhancing the differentiated function of the osteoblasts [7,8]. BMPs were originally identified as growth factors that induce bone and cartilage formation when implanted at ectopic sites in rats [9–11]. In accordance with their in vivo effects, BMPs regulate growth and differentiation of chondroblast and osteoblast lineage cells in vitro. Given the unequivocal evidence for a role

*Corresponding author. Tel.: +65-64450046; fax: +65-67773537.

E-mail address: g0201957@nus.edu.sg (B. Rai).

of BMPs in bone development there has been substantial interest in incorporating this cytokine into tissue engineering scaffolds [12–14]. Several points need to be considered when incorporating growth factors for release from such scaffolds: loading capacity, load distribution, binding affinity, release kinetics and long-term stability [15]. Recently, several studies of β -TCP as the carrier for extracted rhBMP-2 have been reported [6,16]. Hence, the combination of BMP with calcium phosphate may make bone repair more effective from a clinical point of view [17,18].

Our strategy entails investigating the influence of varied concentrations (0, 10, 100 and 1000 ng/ml) of human recombinant bone morphogenetic protein-2 (rhBMP-2) [2] on the osteogenic expression of canine osteoblasts, seeded onto poly-caprolactone 20% tricalcium phosphate (PCL-TCP) 3D scaffolds in vitro. We hypothesized that both osteoconductive and osteoinductive properties would be inherent in PCL-TCP scaffolds and that rhBMP-2 could further ameliorate its bioactivity.

2. Materials and methods

2.1. Scaffold manufacturing

Scaffold specimens were fabricated with PCL-TCP filaments using a FDM 3D Modeler RP system from Stratasys Inc. (Eden Prairie, MN). Blocks of $50 \times 50 \times 3$ mm were created directly in Stratasys Quickslice (QS) software. A lay-down pattern of $0/60/120^\circ$ was used to give a honey-comb-like pattern of triangular pores. After manufacture, the specimens were cut with an ultrasharp blade into smaller cuboids of dimensions, $8 \times 8 \times 3$ mm.

These cuboids were surface treated in 5 M-NaOH for 1 h to enhance their hydrophilicity. After rinsing $3 \times$ with phosphate buffered saline (PBS, 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4 , 1.8 mM KH_2PO_4 , pH 7.4) they were sterilized in 70% ethanol for 24 h. This was followed by rinsing twice in PBS with centrifugation at 1000 rpm for 10 min. The scaffolds were dried in humidified atmosphere at 37°C and 5% CO_2 for 1 h and soaked for 3 h in Dulbecco's modified Eagle's medium (DMEM, Sigma, Deisendorf, Germany) prior to cell seeding.

2.2. Porosity calculation

The porosity was calculated through the following method: (1) measuring the weight and volume of each sample, (2) from these measurements, the apparent density of the scaffolds was calculated using the following formula: $\rho^* = m$ (g)/ V (cm^3) and (3) finally,

the porosity was obtained using the following porosity = $\varepsilon = 1 - \rho^*/\rho \times 100\%$ ($\rho = 1.17 \text{ g/cm}^3$).

2.3. Bone morphogenetic protein

The BMP used in the present study was recombinant human BMP-2 (rhBMP-2). It was a kind gift from Dr. Chen Fulin from the division of Bioengineering, National University of Singapore. RhBMP-2 was used in various concentrations: 10, 100 and 1000 ng/ml. Cell seeded scaffolds without rhBMP-2 served as controls. Hence four experimental groups were created.

2.4. Cell culture experiments

2.4.1. Dog osteoblast culture and cell seeding on PCL-TCP scaffold

Bilateral extraction of mandibular premolars was performed on a 1, $\frac{1}{2}$ -year-old female dog (Mongrel) weighing 14.8 kg. The removed premolar explants were rinsed in PBS containing 2% penicillin–streptomycin (PS, Sigma, Eschenstr, Germany) and then diced into smaller bone chips. Cell culture flasks were filled with the bone chips and incubated with DMEM supplemented with 10% FBS (Hyclone, USA) and 1% PS in a humidified atmosphere at 37°C and 5% CO_2 . Medium was replaced every 3 or 4 days. Cell migration from explant cultures was observed after one week. Primary cultures were maintained until they reached 80–90% confluency and were passaged three times. At this stage, cells were trypsinized with 0.25% trypsin/EDTA (Hyclone, USA), centrifuged, resuspended in culture medium and counted with a Neubauer-hemocytometer.

Canine osteoblasts (third passage, 3×10^5 cells/ml), rhBMP-2 of various concentrations and fibrin glue (Tisseel kit, Immuno, Austria) were simultaneously seeded onto the top of the porous scaffolds, which were placed in 24-well culture plates. After cell seeding, 1 ml of fresh culture medium was added to each well and cells were incubated in a humidified atmosphere at 37°C and 5% CO_2 for 4 weeks with medium changes every 3 days. After seven days, the osteoblastic phenotype as well as the production of bone extracellular matrix were stimulated by supplementing the culture medium with 10 mM β -glycerophosphate (Sigma, USA), 100 μM ascorbic acid (Sigma) and 10^{-7} M dexamethasone (Sigma).

2.4.2. Cellular viability and proliferation assays

Confocal laser microscopy (CLM) and a biochemical assay, the AlamarBlue test assessed cellular viability and proliferation. The AlamarBlue (Biosource International Inc, USA) test is a non-toxic, water-soluble, colorimetric redox indicator that changes color in response to chemical reduction of growth medium as a result of cell growth. Scaffold/cell constructs ($n=4$) were placed in culture medium with 5% AlamarBlue solution and

incubated in a humidified atmosphere at 37°C and 5% CO₂. After 3 h of incubation, 100 µl of solution from each scaffold was transferred to 96-well plates and the optical density was measured with a reference filter of 595 nm and measurement filter of 562 nm.

For CLM, samples were stained with two fluorescent dyes: fluorescein diacetate (Sigma, Germany), which stains viable cells green, and propidium iodide (Sigma), which stains necrotic and secondary apoptotic cells red. The scaffold/cell constructs were first incubated with 2 µg/ml FDA for 30 min at 37°C. The samples were then rinsed 3 × in PBS, placed for 2 min in a 100 µg/ml propidium iodide solution, rinsed again in PBS once and viewed under a confocal laser microscope (Olympus 1 × 81, FV500 Fluoview). Depth projection images were constructed from 70 horizontal image sections.

2.4.3. Cellular adhesion assay

Scanning electron microscopy (SEM) and phase contrast light microscopy (PCLM) assessed cellular adhesion. For the SEM experiments, samples were fixed in 2.5% glutaraldehyde (Sigma, Germany) at 4°C overnight. They were then dehydrated in a graded ethanol series of 25%, 50%, 75%, 95% and 100%, air-dried and gold sputtered with JFC-1200 fine-coater for 70 s at 30 mA under high vacuum. The specimens were examined with a Jeol JSM 5600LV SEM operating at 10 kV under high vacuum mode. Samples were examined weekly under SEM for a month. The establishment of osteoblast-like phenotype with intercellular connections were examined daily by PCLM for a month.

2.4.4. Extracellular matrix production

Mineral as well as osteocalcin content is indicative of extracellular matrix production. Von Kossa's method was used to detect the mineral in bone-like nodules. In brief, the specimens were washed in 3 × with PBS, fixed in 3.7% formaldehyde in PBS for 10 min, washed in distilled water 3 ×, then incubated in 0.5% silver nitrate for 1 h. Samples were then washed in distilled water 3 ×, incubated in 0.3% sodium thiosulfate pentahydrate for 3 min, washed in distilled water, dried and viewed under a phase contrast microscope. Osteocalcin production was assessed using an ELISA kit (Takara, Shiga, Japan). Supernatants were collected weekly ($n = 4$) and frozen at -20°C and then thawed prior to analysis. A 100 µl amount of each sample was used and the assay was performed in accordance to the instructions of the manufacturer. Results are presented in ng/ml.

2.5. Statistical significance

Statistical analysis was performed using Student's *t*-test. Data were expressed as mean ± standard deviation (SD). Statistical significance was represented by $p < 0.05$.

3. Results

3.1. Scaffold morphology

SEM analysis revealed the structure of empty PCL-TCP scaffolds. It consists of a fully interconnected network of pores, ranging from 400 to 600 µm in diameter (Fig. 1). The porosity was calculated to be 65%. Further details of these scaffolds can be found in literature [2,3].

Cellular viability and proliferation were assayed via the semi-quantitative AlamarBlue test (Fig. 2). Cell proliferation causes the redox indicator to change from oxidized to reduced form; hence, a higher percentage of reduction is proportional to more growth. Interestingly, groups with rhBMP-2 exhibited an initial burst of cell growth between 3 h and day one. This burst was not dose-dependent as groups with 10, 100 and 1000 ng/ml of rhBMP-2 all showed similar percent increase in reduction respectively. Control without rhBMP-2 demonstrated no such initial burst of cell growth.

However, at day 13, cell growth from all groups declined to a value not significantly different from

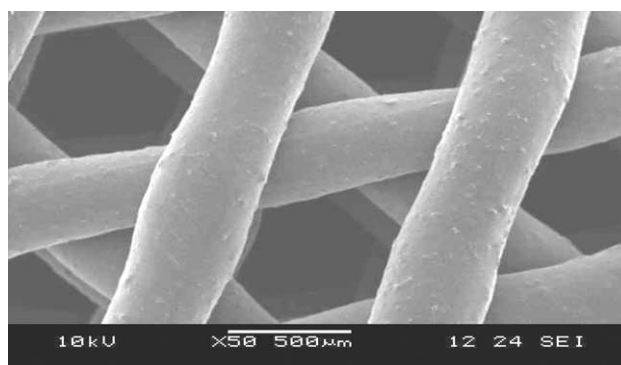


Fig. 1. SEM of empty PCL-TCP scaffolds revealed interconnecting pores of 400–600 µm in diameter.

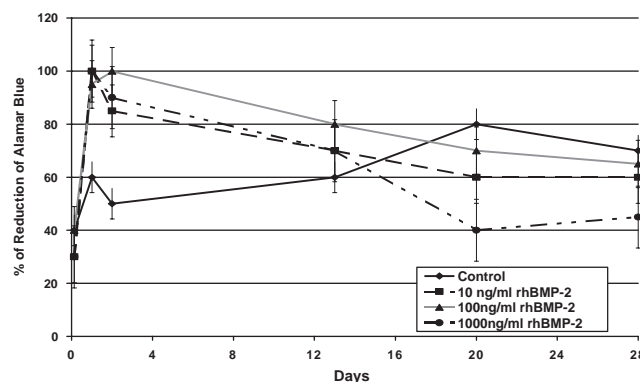


Fig. 2. Cell viability and proliferation was assayed by the AlamarBlue test. Cell density used was 3×10^5 cells/scaffold. Control was scaffold/cell constructs without rhBMP-2. The other groups constituted of scaffold/cell constructs with 10, 100 and 1000 ng/ml of rhBMP-2, respectively ($n = 4$, ±SD).

control. Control and groups with 10 and 100 ng/ml rhBMP-2 stabilized to about 65% reduction of Alamar-Blue, i.e. a 35% increase in cell growth as compared to 3 h. Whereas, the cell/scaffold constructs with 1000 ng/ml of rhBMP-2 showed only a 40% reduction after 13 days, i.e. only a 10% increase in cell growth. Notably, at the end of the experiment, at day 28, there was no significant difference ($p < 0.05$) between control and groups with 10 and 100 ng/ml of rhBMP-2.

Confocal microscopy confirmed cellular viability. Viable osteoblasts as identified by their green fluorescence, were observed to attach onto the bars of the scaffolds as early as 3 h. However, cells were of low density and dispersed (Fig. 3a). This was consistent for all experimental groups. At day one, cell density was greatly increased for groups with rhBMP-2. Cells initiated spreading and spanning across the pores of the scaffolds (Fig. 3b). At day 20, viable osteoblasts from all groups appeared to form aggregates that covered the bars and pores of the scaffolds entirely. Intriguingly, control showed minimal cell death in comparison to groups with rhBMP-2, which evinced high cell death, as indicated by enhanced red fluorescence (Fig. 3c).

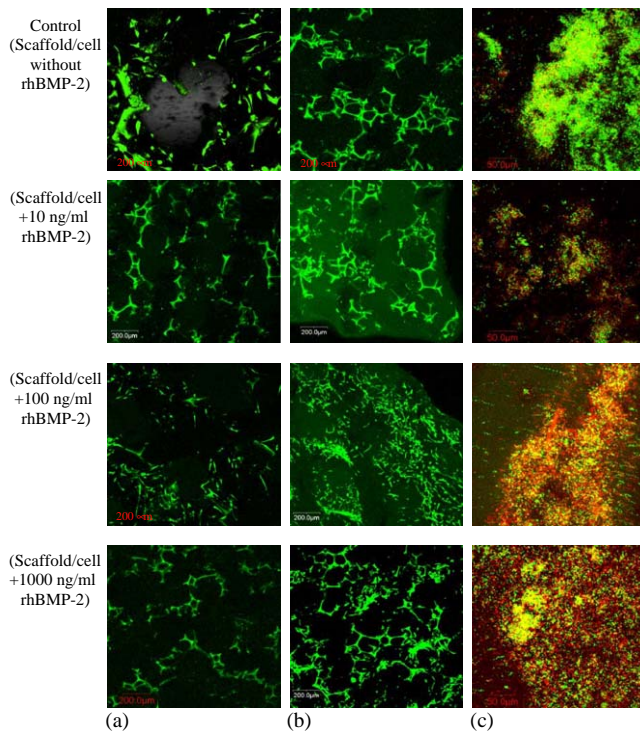


Fig. 3. Confocal micrographs of osteoblasts stained with 2 $\mu\text{g/ml}$ of FDA and 100 $\mu\text{g/ml}$ of PI after (a) 3 h, (b) 1 and (c) 20 days. FDA stained viable cells green while PI stained nuclei of necrotic cells red. At 3 h, cells were attached to the bars of the scaffolds. They were dispersed and in low density. At day 1, significant spreading and proliferation of cells were observed. Viable cells began spanning across the pores. At day 20, aggregates of cells covered both pores and bars of scaffolds. However, a significant fraction of cells underwent apoptosis, especially in the groups with rhBMP-2.

3.2. Cellular adhesion

Under PCLM, at 3 h, it was observed that the inoculated cells from all groups were round and distributed singly or in clumps in the fibrin matrix (Fig. 4a). Cells commenced to spread and migrate from day 1 onwards (Fig. 4b). Groups with rhBMP-2 achieved better confluency in fibrin glue than control. By the second week, $\frac{3}{4}$ of the pore structure of the scaffolds were filled with aggregates of cells for all groups. At day 20, all groups revealed extensive extracellular matrix with black colored mineralized nodules within the pores of the scaffolds (Fig. 4c).

SEM confirmed the adhesion of cells onto scaffolds. At week 1, cells from all groups demonstrated focal contact formation to the surface of the bars and were observed to branch to adjacent bars and struts. Some of the pores were completely filled with cells after only 2 weeks (Fig. 5). Both the surfaces and the pores of the scaffolds were heavily colonized. At the end of the experiment, the surfaces and pores were entirely overwhelmed by cells and what seems to be extracellular matrix. Notably, globular accretions were detected. These observations are consistent for all experimental groups.

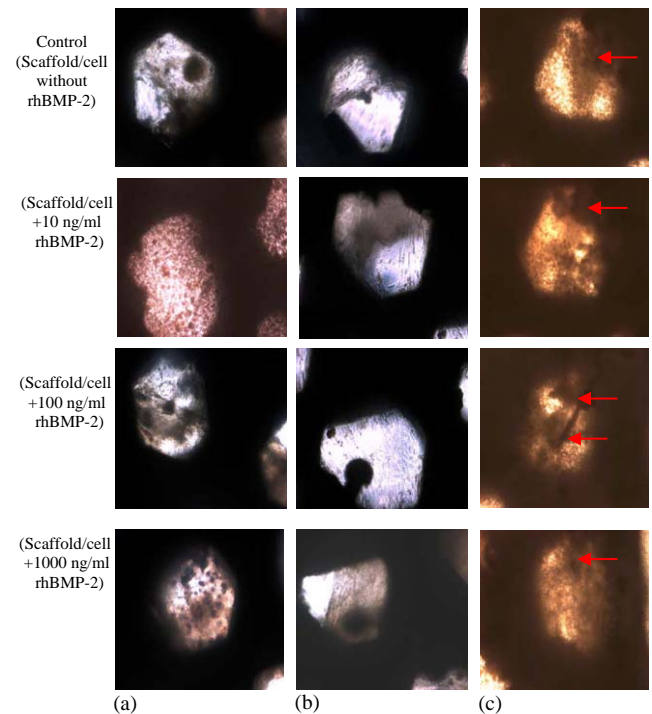


Fig. 4. Phase contrast light microscopy showing osteoblasts seeded PCL-TCP scaffolds at (a) 3 h, (b) 1 and (c) 20 days. All pictures were reproduced at $4\times$ magnification. At 3 h, cells had a round shape and in small clumps. At day one, the cells began to spread and migrate across the pores. By day 20, the pores were completely filled with cells and extracellular matrix. The formation of black-colored mineralized nodules was also observed (arrows).

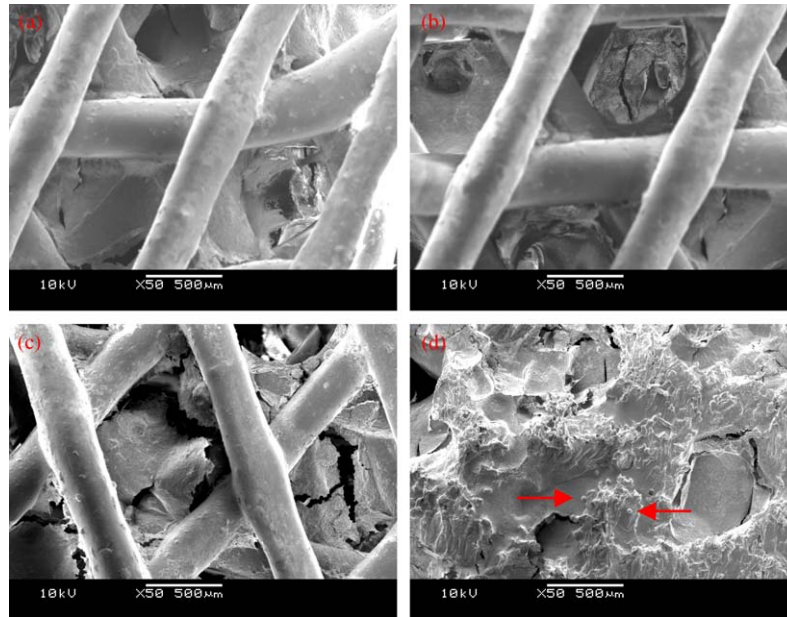


Fig. 5. Scanning electron micrographs of osteoblast adhering onto PCL-TCP scaffolds 2 weeks after cell seeding. (a) Control (scaffold/cell constructs without rhBMP-2) (b) Scaffold/cell constructs with 10 ng/ml rhBMP-2 (c) Scaffold/cell constructs with 100 ng/ml of rhBMP-2 (d) Scaffold/cell constructs with 1000 ng/ml of rhBMP-2. Fig. (a–c) shows the porous space of the scaffolds completely filled with cell/fibrin glue suspension. Fig. (d) depicts both porous surface and bars overwhelmed with cells and the presence of globular accretions (arrows).

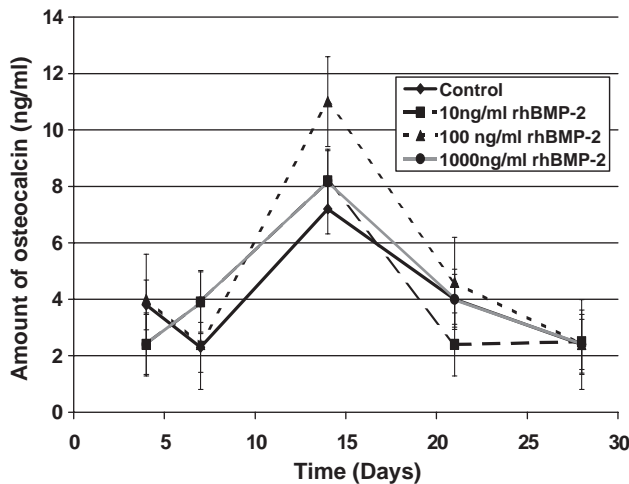


Fig. 6. Extracellular matrix production as assessed by osteocalcin assay. The results are shown as osteocalcin (ng/ml) as a function of days. On day 7, cells were stimulated with differentiation medium (refer to text). Control was scaffold/cell constructs without rhBMP-2. The other groups constituted of scaffold/cell constructs with 10, 100 and 1000 ng/ml of rhBMP-2, respectively ($n=4$, \pm SD).

3.3. Extracellular matrix production

In Fig. 6, the progression of osteocalcin is shown. For all groups, osteocalcin heightened 7 days after stimulation with differentiation medium, and peaked at day 14. Only cells/scaffold constructs with 100 ng/ml rhBMP-2 showed significant increase in levels of osteocalcin in comparison to control ($p < 0.05$). However, after reach-

ing the pinnacle, osteocalcin levels plummeted for all groups. At day 28, the amount of osteocalcin attained a value similar to when non-stimulated for all groups.

The Von Kossa assay ascertains mineralized structures that resemble calcium nodules, an indication of woven bone formation. The scaffolds used for this study has tricalcium phosphate, hence to make accurate comparisons, an additional control i.e. PCL-TCP scaffold alone, was included. A few, small, mineralized nodules were observed on the scaffolds on day 11, just 4 days after stimulation, for groups seeded with rhBMP-2. This was not observed for control. On day 14, the mineralized nodules were large enough to be observed on both culture wells and scaffold surface (Fig. 7). Mineralized nodules were apparent for control as well but in lesser amounts. The number of mineralized nodules did not differ significantly between day 14 and day 21 for all groups.

4. Discussion

Numerous studies have investigated the effect of BMP on various types of scaffolds both in vitro and in vivo. The rhBMP-2 encapsulated in biodegradable poly (DL-lactide-co-glycolide) (PLGA) capsules allowed healing of a segmental defect in rabbit radius and the regenerated bone displayed excellent mechanical properties [19]. It was also discovered that rhBMP-2 could induce bone regeneration in close apposition to dental implant surfaces, however bone fill and osseointegration

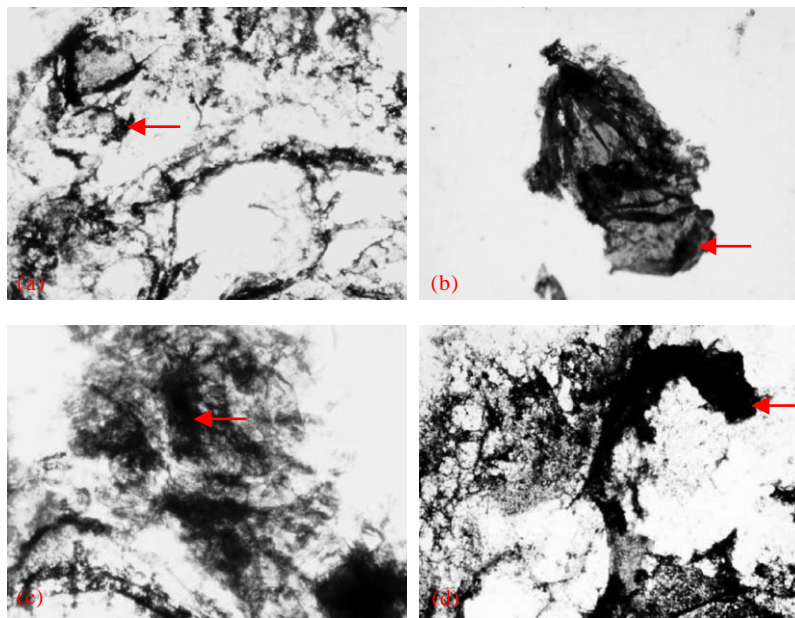


Fig. 7. Extracellular matrix production as assessed by Von Kossa assay at day 20. Dark brown deposits indicate extent of mineralization on the surfaces of culture wells (arrows). (a) Control (scaffold/cell constructs without rhBMP-2), (b) Scaffold/cell constructs with 10 ng/ml, (c) 100 ng/ml and (d) 1000 ng/ml of rhBMP-2. All pictures were reproduced at $4 \times$ magnification.

values from groups with rhBMP-2 were similar as control [20]. An evaluation of the effect of rhBMP-2 on rat bone marrow stromal cells cultured on titanium fiber mesh in vitro disclosed that rhBMP-2 increased cell proliferation from 4 to 8 days but after that DNA content decreased [21].

Our work shows that rhBMP-2 stimulated an initial burst of cells, followed by a decline to a value similar as control. This corresponds with the findings of the above literatures. This behavior could be explained by the binding affinity of rhBMP-2 for the PCL-TCP scaffold and its in vitro release characteristics. It was hypothesized that growth factor release exists in two stages [22]. The initial burst of cells is due to elution of loosely bound protein. Nevertheless, the initial burst could serve to augment the in vivo wound healing response. The second phase of release presents a specific pattern dependent upon the type of material and growth factors used.

For example, the rhBMP-2 release profiles from hyaluronic acid-based scaffolds in vitro, demonstrated that approximately one third of the initially loaded rhBMP-2 diffused out of the scaffolds over the course of 28 days, unlike the rapid release observed in collagen and polylactic scaffolds [23]. Our present study did not investigate the in vitro release kinetics of rhBMP-2 from PCL-TCP scaffolds; hence it is recommended as future work. The identification of a quantitative release carrier model is pertinent for clinical application.

Our work did not reveal any dose-dependent effect of rhBMP-2 on cell proliferation. This corroborates with

the findings of Vehof et al. [21]. However, there is a possibility that the range of rhBMP-2 concentrations used were too wide. Perhaps a narrow range of 100–500 ng/ml of rhBMP-2 could exhibit dose-dependent effects on cell proliferation.

In the present study, osteocalcin levels heightened at the second week, followed by deposition of calcified matrix. This pattern of osteocalcin expression and mineralization coincides with reported sequence of osteoblast differentiation observed by others [24]. However, our strategy of combining PCL-TCP scaffold with rhBMP-2 seemed to accelerate the process of mineralization. Mineralized nodules were observed as early as 4 days after stimulation for cell seeded scaffolds augmented with varied concentrations of rhBMP-2. Additionally, it was observed that 100 ng/ml of rhBMP-2 was the most stimulative of differentiation as it produced the most amount of osteocalcin 7 days after stimulation. It was reported that rhBMP-2 and glucocorticoids such as dexamethasone, which was a component of the differentiation medium, could act synergistically on osteoblast differentiation [7,21]. This verifies the above observation.

An important observation of our research was that PCL-TCP scaffolds proved to be osteoconductive, bioactive and non-toxic to cells in vitro. Cells adhesion and colonization of the scaffold was proof of its osteoconductiveness. Extracellular matrix production verified its osteoinductiveness and confocal microscopy confirmed cell viability, hence indicating its non-toxic nature. This implies great clinical applications for this novel scaffold.

Intriguingly, groups with rhBMP-2 exhibited escalated cell death at day 20. This is perhaps due to a change in cell signaling pathway but needs to be investigated further. It is known that as osteoblasts undergo terminal differentiation and after the cellular matrix mineralizes, they undergo apoptosis. This programmed cellular death is an expected result of cell maturation and the blocking of rhBMP-2 actions not only arrest osteoblast differentiation but also prevents apoptosis [8]. Hence, since the canine osteoblasts used in this study were already differentiated, the addition of rhBMP-2 could have expedited maturation into terminally differentiated state, resulting in quickened death of cells.

In light of the plethora of growth factors currently known, we propose investigation of the effect of other growth factors with the PCL-TCP scaffolds in hope of further enhancing its osseointegration properties. Alternatively, rhBMP-2 could be used in combination with other growth factors to stimulate a microenvironment as close as possible to the *in vivo* condition. Recently, a combination of platelet-rich plasma (PRP) and autogenous bone graft was advocated as a means of enhancing bone formation. PRP is an autologous source of concentrated platelets, when combined with thrombin and calcium chloride, leads to release of platelet derived growth factors and transforming growth factors [25,26]. It proved effective for maxillary sinus augmentation [27] and treatment of bone defects around titanium implants [28]. Hence, this indicates that growth factors in combination can be more effective.

5. Conclusion

In conclusion, rhBMP-2 enhances the differentiated function of canine osteoblasts in a non-dose dependent manner. This resulted in accelerated mineralization, followed by death of osteoblasts as they underwent terminal differentiation. Notably, novel PCL-TCP scaffolds seeded only with canine osteoblasts could sustain excellent osteogenic expression *in vitro*. Hence, the synergy of a purely osteoconductive PCL scaffold with bioactive TCP and rhBMP-2, could offer an exciting approach for bone regeneration.

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