

In vitro study on the influence of strontium-doped calcium polyphosphate on the angiogenesis-related behaviors of HUVECs

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Abstract Angiogenesis is of great importance in bone tissue engineering, and has gained large attention in the past decade. Strontium-doped calcium polyphosphate (SCPP) is a novel biodegradable material which has been proved to be able to promote in vivo angiogenesis during bone regeneration. An in vitro culture system was developed in the present work to examine its influence on angiogenesis-related behaviors of human umbilical vein endothelial cells (HUVECs), including cell adhesion, spreading, proliferation and migration. The effects of microtopography, chemical property and the ingredients in the degradation fluid (DF) on cell behaviors were discussed. The results showed that cells attached and spread better on SCPP scaffold than on calcium polyphosphate (CPP), which might partially result from the less rough surface of SCPP scaffold and the less hydrogel formed on the surface. In addition, cell proliferation was significantly improved when treated with SCPP DF compared with the treatment with CPP DF. Statistical analysis indicated that Sr^{2+} in SCPP DF might be the main reason for the improved cell proliferation. Moreover, cell migration, another important step during angiogenesis, was evidently stimulated by SCPP DF. The improved in vivo angiogenesis by SCPP might be assigned to its better surface properties and strontium in the DF. This work also provides a new method for in vitro evaluation of biodegradable materials' potential effects on angiogenesis.

1 Introduction

Tissue engineering has emerged as a promising approach to treat the loss or malfunction of a tissue or organ without the limitations of current therapies [1]. This approach co-cultures tissue-specific cells with a three-dimensional (3-D) scaffold in vitro which is then transplanted into the patient. Right after the transplantation, angiogenesis occurs to establish the blood vessels, which is necessary for the growth and development of the cells into the desired function of replaced tissue or organ [2, 3].

A great number of biomaterials, permanent or biodegradable, naturally occurring or synthetic, have been examined as scaffolds for bone tissue engineering. Recently, calcium polyphosphate (CPP), an inorganic polymer, has drawn much attention due to its good biocompatibility and controllable degradability in biological environments. CPP has been shown to be an effective substrate for the in vitro growth of a variety of cells, including osteoblasts, dental pulp cells, human gingival fibroblasts, chondrocytes and marrow cells etc [4–8]. And it has been proved to support bone ingrowth in vivo [9]. The controllable degradability makes CPP be a prominent substitute for bone tissue engineering since the undegradation or uncontrollable degradation has emerged as the main problem of other calcium phosphate materials, such as hydroxyapatite (HA) and tricalcium phosphates (TCP) [10]. Pilliar and Grynopas et al. [9, 10] initially studied the structures and degradation characteristics of porous CPP. The authors found out that the degradation rate of porous CPP could be controlled through varying the particle size. Our previous work showed that the in vitro degradation of CPP is dependent on the polymerization degree and crystalline structures [11].

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Strontium (Sr) is a bone-seeking element which presents a beneficial effect on bone growth [12]. It has been proved to be able to decrease bone resorption and to enhance bone formation in vivo [13–15]. Strontium (Sr) was introduced into CPP to form strontium-doped calcium polyphosphate (SCPP), a novel hybrid material. It is supposed that with the degradation of SCPP, calcium ions (Ca^{2+}), phosphorus (Pi) as well as strontium ions (Sr^{2+}) are released out and benefit the bone regeneration. Our previous work demonstrated that with addition of Sr, SCPP could provide enough mechanic strength for bone regeneration and promote the growth of osteoblasts in vitro [16], 1/99 molar ratio of Ca/Sr (1% SCPP) giving the best biocompatibility. Furthermore, in vivo experiments showed that SCPP could stimulate bone formation, and better angiogenesis was observed in SCPP-repaired canine thighbone compared with CPP [17]. However, the mechanism of improved angiogenesis remains unknown.

The aim of the present study was to elucidate how the SCPP scaffold influences the angiogenesis process. An in vitro culture system was developed to examine the behaviors of endothelial cells related to angiogenesis, including cell attachment, spreading, proliferation and migration. The effects of microtopography, chemical property and degradation products on cell behaviors were discussed.

2 Experimental

2.1 Preparation of porous CPP and SCPP scaffolds

Porous 1% SCPP was prepared using a method described earlier [16]. The samples were press-formed as a disc of 5 mm in diameter and 1 mm in thickness. The structure and topography of the discs was examined by scanning electron microscopy (SEM, JSM-5900 LV, JEOL) prior to cell culture.

Porous CPP scaffold was prepared according to the procedures described previously [11], and served as a control.

2.2 In vitro aseptic degradation

Before degradation, all the discs were sterilized in a steam sterilizer at 134 °C for 25 min, and dried at 60 °C for 4 h. Each disc was then placed in a vial filled with sterile physiological saline solution ($w/v = 1/50$), and incubated at 37 °C. The discs were withdrawn after 7, 14, 30, 60, 90 days' incubation, and the corresponding degradation fluids were kept at 4 °C before analysis. All the operations above were performed under aseptic conditions.

The concentrations of Ca^{2+} , Pi and Sr^{2+} in the degradation fluid at different time intervals were measured by Inductively Coupled Plasma Atomic Emission Spectrometry (ThermoElemental Company, USA).

2.3 Cell culture

Endothelial cells are generally used in tissue engineering for angiogenesis studies [18, 19]. ECV304, a unique spontaneously transformed human umbilical vein endothelial cell line, was used in the present study in order to minimize the biological variation of endothelial cells isolated from different donors and to establish a constant supply of cells [20]. The culture medium was a HEPES-buffered RPMI-1640 solution, supplemented with 10% fetal bovine serum, 1% penicillin and 1% streptomycin (all ingredients obtained from Gibco). The cells were cultured in 25-cm² flasks at 37 °C in a humidified atmosphere containing 5% CO₂, harvested with the use of 0.25% trypsin (Invitrogen), and resuspended in medium.

2.4 Visualization of HUVECs growth on the porous scaffolds

For SEM observation, the HUVECs were seeded on the SCPP scaffolds (5×10^4 cells/disc), and cultured for 7 days. During this period, the culture medium was changed every 2 days. On the 7th day, the samples were fixed in 3% glutaraldehyde in 0.1 M phosphate buffer solution (PBS) at 4 °C overnight, washed twice with PBS for 10 min, and then dehydrated by increasing the concentration of alcohol (30, 50, 70, 80, 90, 95, 99 and 100%). The critical point drying of specimens was undertaken with liquid CO₂. Finally, the samples were sputter-coated with gold, and the morphology of cells was examined under SEM.

2.5 Proliferation assay of HUVECs treated with degradation products

The colorimetric 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT) assay was performed to quantify the effect of degradation products on cell proliferation [21]. Briefly, 4×10^3 cells per well were seeded in a 96-well microplate (Corning, USA) in a final volume of 100 μL . After being cultured overnight, the cells were treated with the degradation fluid of SCPP and CPP for 1, 2, 3, 4, 5 days, respectively. After the treatment, 20 μL MTT (Sigma, USA) solution (5 mg/mL) were added to

each well and the cells were incubated at 37 °C for 4 h. The MTT solution was then removed and replaced by 150 μ l DMSO, and the microplates were shaken for 10 min. The optical density (OD) of each well was determined using a microplate reader at a wavelength of 490 nm, thus allowing evaluating the cell numbers which are proportional to the OD value. The physiological saline was used as a negative control.

2.6 Migration assay of HUVECs treated by the degradation products

The migration activity of HUVECs was tested with the transwell chamber system [22]. The transwell (PCF, 8 μ m in pore size, Millipore, USA) was lodged into a well of a 24-well plate. HUVECs (1×10^4 cells) were added to the upper chamber, and the medium with 1% FBS and 50% degradation fluid was added to the outer well. After incubation at 37 °C for 24 h, the residual cells in the upper side of the filter were wiped off with cotton swabs. The membrane was fixed with 100% methanol for 10 min. The cells that migrated to the lower side of the membranes were stained with Giemsa for 15 min and were counted under an optical microscope. Physiological saline served as a negative control.

2.7 Statistical analysis

Statistic analysis was performed with SPSS12.0. Data of cell proliferation and migration were expressed as mean \pm SD. Significant difference of cell proliferation and migration between SCPP and CPP or physiological saline group was determined by independent-samples *t* test. *P* values lower than 0.05 were considered statistically significant for tests.

Stepwise multiple regression analysis was performed to determine the main factor (Ca^{2+} , Pi or Sr^{2+}) influencing cell proliferation. Corresponding *P*-values were considered significant if inferior to 0.01.

3 Results and discussion

3.1 Porous structure and surface topography of scaffolds

It is well known that the 3-D structure and the surface topography of biomaterials strongly influence endothelial cells' attachment, spreading, growth and eventually the angiogenesis [23–27]. The porous structure and surface topography of 1% SCPP and CPP scaffolds were examined by using SEM as shown in Fig. 1. Both SCPP and CPP exhibit three-dimensionally interconnected porosity with a pore size of about 100–400 μ m. On the other hand, the porosity was found to be about 65 vol.% for both scaffolds.

However, a major difference was observed at a magnification of 5000 (Fig. 1c and f). The crystal grain size of SCPP (about 5 μ m) was larger than that of the CPP (about 2 μ m). Moreover, it seems that the crystal grains of SCPP are more intimately connected with each other, resulting in a smoother surface and a more compact bulk.

3.2 Degradation products and degradation rate

CPP is an inorganic polymer with a linear P–O–P backbone which can be cleaved by hydrolysis in aqueous media, with the release of Ca ion and phosphorus as the result [10]. In the case of SCPP degradation, Ca^{2+} , Pi and Sr^{2+} are released into the degradation fluid.

As shown in Fig. 2a and b, Ca^{2+} and Pi were detected both in CPP and in SCPP degradation fluids. The

Fig. 1 SEM of CPP and SCPP porous scaffolds. CPP: (a) 20 \times , (b) 200 \times , (c) 5000 \times ; SCPP: (d) 20 \times , (e) 200 \times , (f) 5000 \times

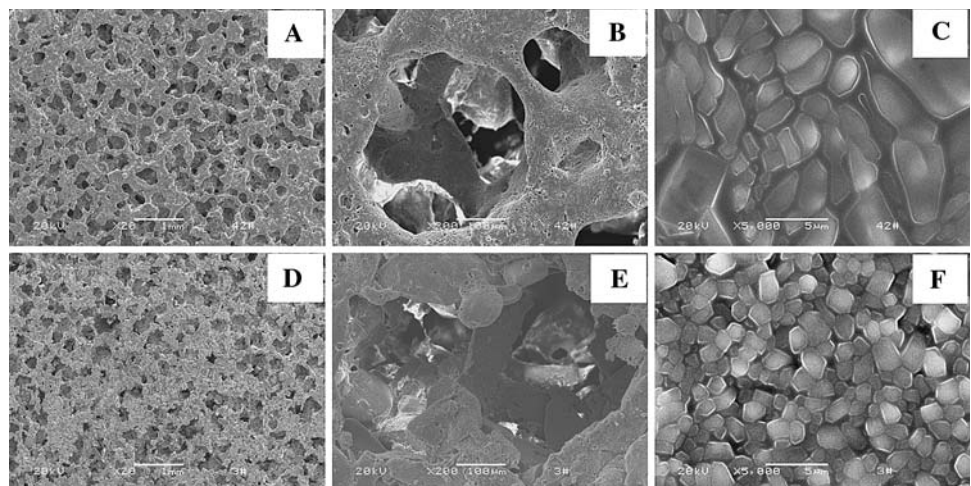
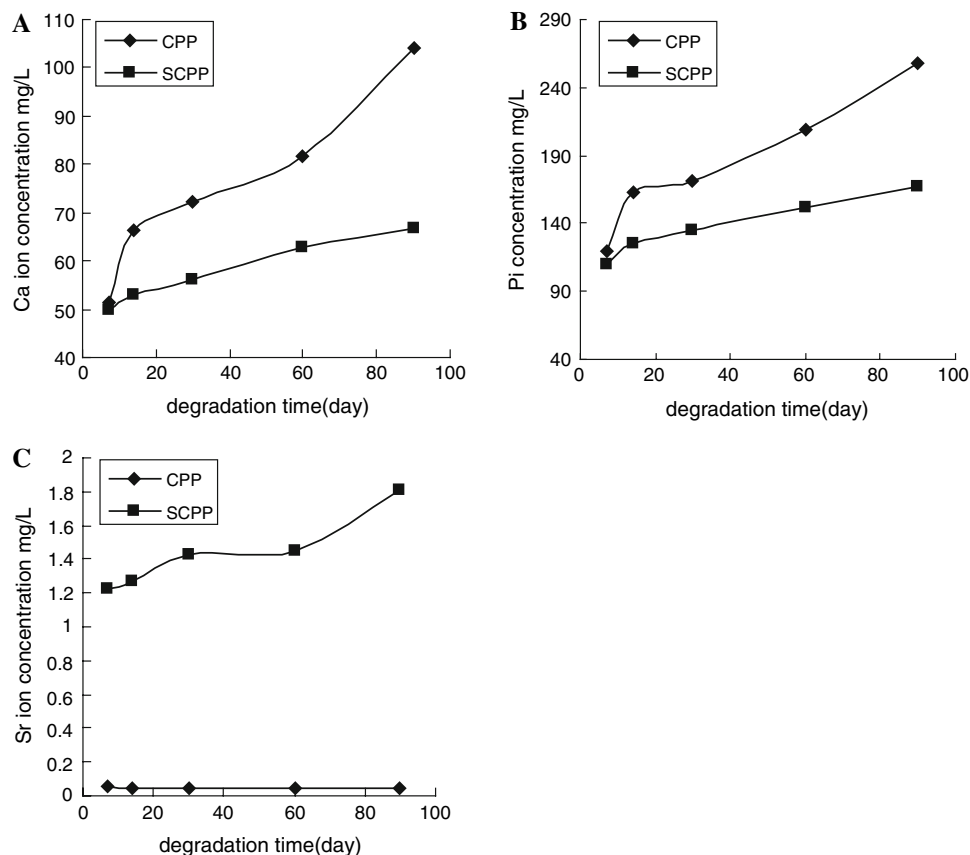


Fig. 2 Concentration of degradation products and degradation rate of CPP and S CPP in vitro. (a) Ca ion; (b) Phosphorus (Pi); (c) Sr ion



concentration of Ca^{2+} and Pi increased with time. Sr^{2+} was detected only in S CPP degradation fluid. The concentration of Sr^{2+} increased from 1.222 mg/L on the 7th day to 1.808 mg/L on the 90th day. Surprisingly, a very small amount of Sr^{2+} (about 0.05 mg/L) was detected in CPP degradation fluid (Fig. 2c), which may result from the impurity of the raw materials. These results suggest that S CPP is degradable in physiological saline. On the other hand, the concentrations of Ca^{2+} and Pi in the S CPP degradation fluid at 14, 21, 30, 60, and 90 days were lower than those in CPP degradation fluid, indicating that the degradation rate of S CPP was lower than that of CPP. This is probably due to the difference of surface roughness between S CPP and CPP. In fact, degradation rate is correlated with the size of interface between degradable scaffold and degradation medium. The smoother surface of the S CPP scaffold leads to a smaller interface area, and subsequently slows down the degradation process.

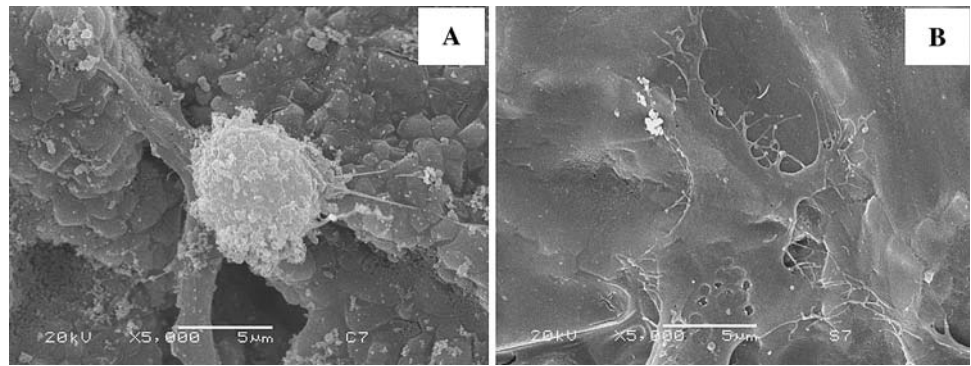
3.3 Effect of S CPP topography on adhesion and spreading of HUVECs

Endothelial cells are often seeded on porous scaffold with tissue-specific cells to improve angiogenesis [28, 29]. The

adhesion of HUVECs on the S CPP and CPP surfaces is evaluated by using SEM. Cells loosely attached onto the CPP scaffold with a round shape (Fig. 3a). And the crystal grains of the CPP bulk were clearly visible. In contrast, the cells tightly attached to the S CPP scaffold, fully spread and developed a confluent monolayer which covered the whole surface (Fig. 3b). These results suggest that the surface of S CPP could promote the adhesion and spreading of the HUVECs, probably due to the smoother surface. Chung et al. [26, 27] reported that increased surface roughness of biomaterials at 10–100 nm scale could enhance the adhesion and growth of HUVECs. However, the situation seems different at micron scale. Xu et al. [25] studied the function of human vascular endothelial cells on poly (L-lactic acid) with various surface roughnesses at micron scale. The attachment of cells was better on the smooth surface than on the rough surface. This is in agreement with our results.

It was also noted that the two scaffolds exhibited different surface behaviors in the culture medium. After immersion in RPMI 1640 culture medium with 10% FBS and incubation at 37 °C for 24 h, the two scaffolds were observed under phase contrast microscope and scanning electronic microscope (SEM). Phase contrast microscope showed the presence of hydrogel around CPP scaffold, while little hydrogel was detected around S CPP.

Fig. 3 SEM of HUVECs growing on the porous scaffolds after 7 days. 5000× (a) CPP; (b) S CPP



Furthermore, a lot of flocs were observed by using SEM on the surface of CPP scaffold, in contrast to S CPP. The Ca/P ratio of the flocs derived from EDAX analysis was 1/2, i.e. almost the same as that of CPP. It is thus assumed that the flocs might be dehydrated hydrogel of CPP and the addition of strontium diminishes the hydrogel formation on the surface of CPP.

It is well known that cell adhesion onto biomaterials is mediated by proteins adsorbed to the surface. In the case of calcium/phosphate ceramics, the acidic carboxyl and alkaline amino groups on the protein's surface bond to the Ca^{2+} on the ceramic surface by ionic bonding. In addition, the proteins' alkaline amino groups bond to the oxygen in PO_4^{3-} by hydrogen bonding and electrostatic attraction [30[r1]]. This indicates that protein adsorption depends on the amount of Ca^{2+} and PO_4^{3-} on the surface of ceramics. The number of Ca^{2+} and PO_4^{3-} is larger on S CPP surface because of the absence of flocs. Therefore, it is suggested that the enhanced cell adhesion and spreading on S CPP scaffold might partially result from the increased protein adsorption due to the larger amount of Ca^{2+} and PO_4^{3-} on the surface.

3.4 Effect of the degradation products of S CPP on HUVECs proliferation and migration

Synthetic biodegradable materials have been extensively used as scaffolds in tissue engineering because of their good mechanical properties and controllable degradability. Degradation products are continuously released out, accumulate to a high concentration and subsequently alter the peri environment for cell growth. The biocompatibility of degradation products and other species is thus of major importance. However, attention has rarely been paid to the in vitro biocompatibility of degradation products [31–35]. Little has been done to investigate the effects of degradation products on angiogenesis in vitro. In the present study, we evaluated the influence of S CPP degradation products on the behaviors of endothelial cells intimately related to

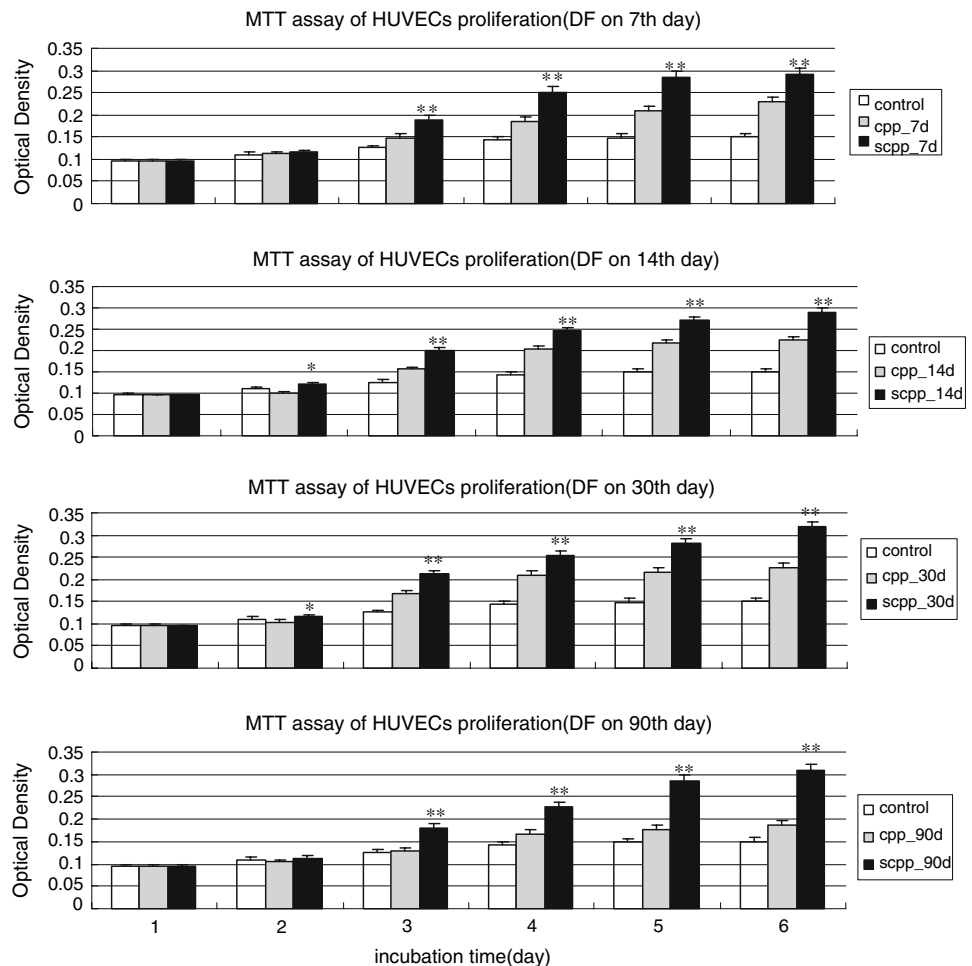
angiogenesis, which may provide new routes in the selection and design of biodegradable materials with improved angiogenesis.

The effects of the degradation products on cell proliferation were tested at first since a large amount of cells are necessary for vascular development [36–38]. As shown in Fig. 4, proliferation of HUVECs treated by S CPP or CPP degradation fluid within 90 days did not exhibit any significant difference from that of the control after 1 day's cultured. However, remarkable promotion in cell proliferation by both of S CPP and CPP degradation fluid occurred in the following 4 days, compared with the negative control. Moreover, the S CPP group showed significantly higher OD values than the CPP group. This means that the degradation products in S CPP degradation fluid could significantly promote the proliferation of HUVECs compared with those of CPP.

This result led us to further investigate the mechanism of the improved cell proliferation and to find out which is the main factor leading to the increase of OD value: strontium whose concentration is far higher in S CPP degradation fluid than that in CPP fluid, or the difference of Ca and Pi concentrations between the two degradation fluids. Stepwise multiple regression analysis, with the aim of learning about the relationship between several independent or predictor variables and a dependent or criterion variable, was performed. Sr^{2+} was found to exert the most influences on the OD value ($R^2 = 0.670$, $P < 0.01$) among the three variables. It is thus concluded that Sr^{2+} plays the utmost role in the cell proliferation promotion. This conclusion has been confirmed by our recent results showing that strontium (100 nmol/L–1 mmol/L) could stimulate the proliferation of HUVECs after treatment with strontium chloride (SrCl_2) as compared to the blank control.

Strontium has been gradually recognized as a bone-seeking trace element in the research to treat osteoporosis. It enhances the replication of preosteoblastic cells, and simulates bone formation in calvarial cultures in vitro [13]. Furthermore, it has been demonstrated that strontium decreased bone resorption in vivo [14, 15]. However, little

Fig. 4 The proliferation of HUVECs treated by degradation fluid (DF) on 7, 14, 30, 90 day, * $P < 0.05$ vs. CPP group, ** $P < 0.01$ vs. CPP group, $n = 5$ per group



has been done on the influence of strontium on HUVECs' growth and on angiogenesis. In this study, it was found for the first time, that strontium (concentration: 1.222–1.808 mg/L) could significantly promote the proliferation of HUVECs although the mechanism is not clearly identified. In a recent report, researchers at Harvard Medical School found that Sr^{2+} is a full calcium-sensing receptor (CaR) agonist in two kinds of cells, HEK293 cells stably transfected with the bovine CaR (HEK-CaR) and rat primary osteoblasts (POBs). Therefore, the anabolic effect of Sr^{2+} on bone in vivo could be mediated, in part, by the CaR. On the other hand, it could be assumed that strontium may act via a cell surface CaR to stimulate the proliferation of HUVECs.

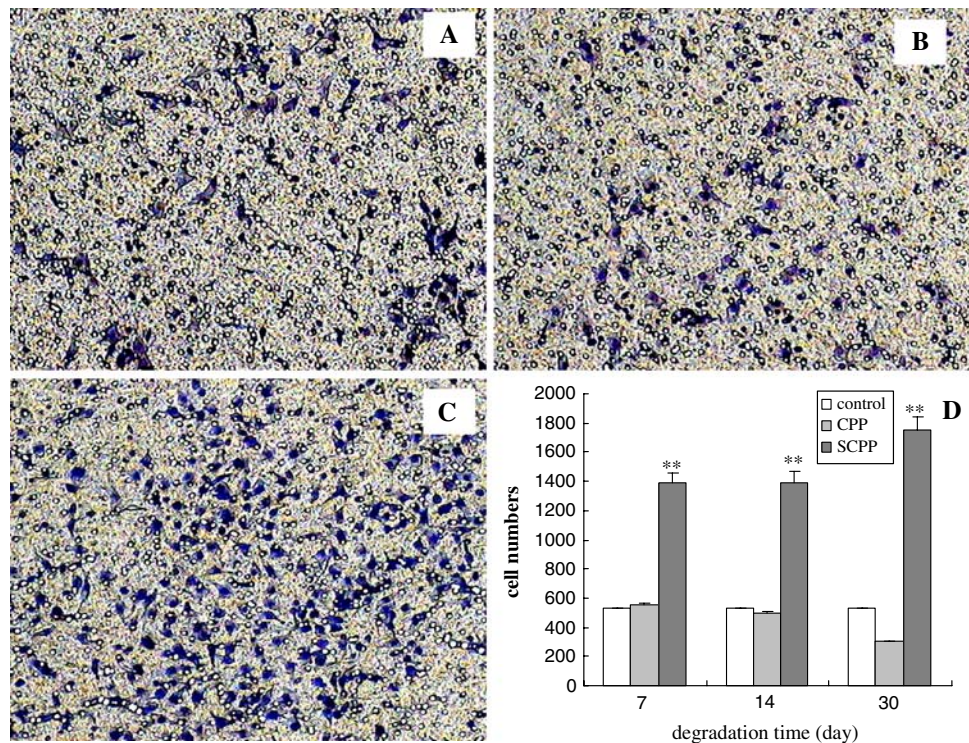
The potential of SCPP degradation products to induce cell migration was investigated since the ability of endothelial cells to migrate from the existing blood vessels to organize new capillaries is an important step in angiogenesis [37, 39–42]. Figure 5a–c show the stained HUVECs treated by degradation fluid on the 14th day. Much more migrated cells (in purple) are observed in SCPP group (Fig. 5c) than in CPP (Fig. 5b) and physiological saline

(Fig. 5a) group, in agreement with cell counting results as shown in Fig. 5d. The cell number of the SCPP group was much higher than those of the CPP and the control, thus indicating that the degradation products in SCPP degradation fluid could significantly promote the migration of HUVECs.

4 Conclusion

HUVECs attached and spread better on SCPP scaffold than on CPP, which might partially result from the less rough surface and less hydrogel formed on the surface of SCPP scaffold. Cell proliferation was significantly improved when treated by SCPP DF compared with the treatment by CPP DF. Statistical analysis indicates that Sr^{2+} in SCPP DF might be the main reason for the improved cell proliferation. Moreover, cell migration, another important step during angiogenesis, was evidently stimulated by SCPP DF. Therefore, the improved in vivo angiogenesis by SCPP might be assigned to the improved angiogenesis-related cell behaviors due to better surface properties and to

Fig. 5 The migration of HUVECs treated by degradation fluid migrated cells stained by Giemsa 40×, (a) physiological saline, (b) DF of CPP on 14th day, (c) DF of SCPP on 14th day; (d) Numbers of migrated cells, ** $P < 0.01$ vs. CPP group, $n = 3$ per group



strontium in the DF. Last but not least, this work may provide a new route for in vitro evaluation of biodegradable materials' potential effects on angiogenesis.

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