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Cytokine release kinetics of concentrated growth factors in different scaffolds

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Abstract

Objectives Concentrated growth factors (CGF) exhibit superior potential for periodontal tissue regeneration; however, little is known about the release pattern of CGF over time. This study was designed to investigate the in vitro release pattern of growth factors and cytokines from CGF in deproteinized bovine bone mineral (DBBM) and intrafibrillarly-mineralized collagen (IMC).

Materials and methods CGF, CGF mixed with DBBM (CGF-DBBM), and CGF mixed with IMC (CGF-IMC) were cultured in vitro for 28 days and media supernatants were collected after 24 h, 72 h, and 7, 14, 21, and 28 days respectively. The factors investigated included platelet-derived growth factor-BB, transforming growth factor beta 1, vascular endothelial growth factor, insulin-like growth factor 1, basic fibroblast growth factor, C3a, and C5a.

Results We found that CGF-IMC released the highest total amount of cytokines compared to CGF and CGF-DBBM ($p < 0.01$). Growth factors were continuously released till 28 days. The release curves of most growth factors and cytokines included two peaks at 24 h and during 14 to 28 days. CGF-IMC released much more growth factors than CGF and CGF-DBBM during 14 to 28 days.

Conclusions Within the limitation of the study, CGF-IMC offers advantages over CGF-DBBM and CGF for sustained release of growth factors and cytokines.

Clinical relevance This study provides strong evidence for clinical use of IMC used as a new carrier for CGF in periodontal regeneration. We need more studies to investigate the effect of sustained release of growth factors in tissue regeneration.

Keywords Concentrated growth factors · Platelet concentrates · Release kinetics · Cytokines · Growth factors · Scaffolds

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Introduction

Periodontitis is an inflammatory disease that leads to the loss of tooth-supporting tissues. The ultimate goal of periodontitis treatment is complete and predictable periodontal tissue regeneration. As signaling molecules, polypeptide growth factors control tissue regeneration at every stage [1]. In vitro recombination of exogenous growth factors is time-consuming and costly, and its safety is still under study. In addition, the complex interactions and networks between endogenous growth factors cannot be simulated in vitro [2]. Therefore, how to obtain abundant endogenous growth factors has become one of the hotspots in tissue engineering field.

Platelets are one of the major resources of autogenous growth factors. Platelet concentrates are derived from autologous blood. Their simple collection process and clinical application without risk have made them increasingly used to enhance tissue healing and regenerative procedures in dental and maxillofacial surgery [3], wound healing [4], and skin

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regeneration [5]. Platelet-rich plasma (PRP) is the first generation of platelet gels for periodontal regeneration therapy [6], while the potential benefits of this procedure have been criticized. Many of the discrepancies are related to the lack of more suitable standardization methods and definition of different PRP preparations, as the protocols and biological and surgical techniques differ widely among different research groups [7, 8]. Furthermore, PRP contains anticoagulants, which interfere with the natural healing process of the human body. Platelet-rich fibrin (PRF), the second generation of platelet concentrates, has shown the ability for enhanced soft tissue regeneration [9]. PRF overcomes the need for exogenous thrombin. Kobayashi et al. have proven that the advantage of PRP is the release of significantly higher proteins at earlier time points, whereas PRF displayed a continual and steady release of growth factors over a 10-day period [10]. However, the species of growth factors in PRF are relatively simple due to the single centrifugal force.

Concentrated growth factors (CGF), a novel generation of platelet concentrates product [11–14], are made by centrifuging blood samples at alternating and controlled speeds. Different centrifugation speeds permit the isolation of a large and dense fibrin matrix with abundant growth factors. Previous studies have demonstrated high levels of growth factors contained in CGF [15, 16]. Theoretically, growth factors in CGF should be released slowly without the addition of exogenous thrombin, close to the natural process of tissue healing. However, little is known about the release pattern of growth factors from CGF over time. Furthermore, CGF are often used in combination with a variety of biomaterials in regenerative medicine and tissue engineering [17, 18]. There is no data available to date on the release pattern of growth factors from CGF mixed with biomaterials. Therefore, the objective of this study was to investigate the in vitro release pattern of growth factors and cytokines from CGF, and CGF mixed with deproteinized bovine bone mineral (DBBM) and intrafibrillarly mineralized collagen (IMC) over time.

Materials and methods

Volunteers

The volunteers of this study were selected from patients referred to the Department of Periodontology, at the First clinical division, Peking University School and Hospital of Stomatology. The study was performed in accordance with the Helsinki Declaration of 1975, as revised in 2000, and the study protocol was reviewed and approved by the university ethical board (Peking University, School and Hospital of Stomatology). The subjects were enrolled to this study based on the following inclusion criteria: (1) age > 18 years, (2) systemically healthy, (3) diagnosed as severe chronic periodontitis, and (4) non-smokers. Exclusion criteria consisted of donors with systemic diseases, pregnant and/or lactating women, patients taking any drug known to affect the number or function of platelets in the past 3 months, and patients with abnormal platelet counts. All the patients received periodontal non-surgical treatment (oral hygiene instructions, full-mouth scaling, and root planning) 1 month before surgery. Nine patients were included in this study. All patients were informed of the nature of this study and signed an informed consent prior to their inclusion.

Preparation of CGF

CGF were produced as follows: 9 mL of blood was drawn from each donor by venipuncture of the antecubital vein under empty stomach in the morning. The blood was collected in sterile Vacuette tubes with white cap (Greiner Bio-One, GmbH, Kremsmunster, Austria) without anticoagulant solutions. These tubes were then immediately centrifuged (Medifuge, Silfradentsrl, Sofia, Italy) using a program with the following characteristics: 30 s acceleration, 2′ 408×*g*, 4′ 323×*g*, 4′ 408×*g*, 3′ 503×*g*, and 36″ deceleration and stop (RCFave) [11].

At the end of the process, two blood fractions were created: a superior liquid phase and the lower red blood cell (RBC) layer. The top 2 mL of the superior phase was platelet-poor plasma (PPP). The remaining interim layer was mainly represented by platelet-rich plasma. Platelets, white blood cells, and CD34-positive stem cells were located at the bottom interface. CGF was composed of the remaining interim layer, the bottom interface, and the top 3 mm of RBC layer. The PPP layer was aspirated and stored at − 80 °C. CGF was aspirated for use.

Biomaterials

Biomaterials are widely used as scaffolds in regenerative medicine and tissue engineering. Two biomaterials were selected in the present study. One was a representative of classical, xenogeneic bone implants DBBM (Bio-Oss, Geistilich, Switzerland); the other one was a bone-like IMC, which was proven to be a good bone graft substitute with a great regeneration potential for treating large bone defects in our previous studies [19–22]. Briefly, IMC was prepared by reconstituting collagen fibrils from a type I tropocollagen (Corning®, 100 mg, 9.46 mg/mL) using simulated body fluid as a phosphate source, white Portland cement (Lehigh Cement Co., Allentown, PA) as a calcium source, and polyacrylic acid (Mw 2000, Millipore Sigma, St. Louis, MO) as a stabilizer of amorphous calcium phosphate [23]. After 7 days, the fibrillized collagen suspension was collected by centrifugation, poured into the plastic molds, and lyophilized to form sponge-like porous scaffolds.

Scanning electron microscopy

To investigate the microstructure, these two types of scaffolds were dehydrated in a graded series of ethanol (50, 70, 80, 85, 90, 95, and 100%), critical point dried, and observed with scanning electron microscopy (SEM) (S-4800, Japan) at 15 kV. Elemental analysis of the scaffolds was performed using energy-dispersive X-ray spectroscopy (EDS) coupled to the SEM.

Grouping

As soon as CGF was aspirated and mixed evenly, it was divided into three groups. The first group was CGF alone. The second group was CGF mixed with DBBM (CGF-DBBM), and the third group was CGF mixed with IMC (CGF-IMC). The volume of each piece of IMC was 0.4 mL. Therefore, the volume of CGF in each group and the volume of DBBM particles were also 0.4 mL. Then CGF and the mixture were allowed to coagulate for 20 min at room temperature (Fig. 1). CGF, CGF mixed with DBBM, and CGF mixed with IMC were placed in separate Eppendorf tubes covered with 2 mL of culture medium (Dulbecco's modified Eagle medium with 1% penicillin/streptomycin), and incubated at 37 °C in a humidified atmosphere with 5% CO_2 for 28 days. During culture, the whole medium was collected at 1, 3, 7, 14, 21, and 28 days post incubation, and an equal volume of fresh medium was added back to each tube. All collected cultures were stored at − 80 °C for further analysis.

Quantification of cytokines

Representative growth factors in platelets including platelet-derived growth factor-BB (PDGF-BB), transforming growth factor beta 1 (TGF-β1), vascular endothelial growth factor (VEGF), insulin-like growth factor 1 (IGF-1), and basic fibroblast growth factor (bFGF) were investigated. Complement C5a fragment has been shown to be involved in the recruitment of dental pulp stem cells (DPSCs) [24, 25]. C3a could promote proliferation of DPSCs and fibroblast, and specifically guide fibroblast recruitment [24]. Therefore, the levels of C3a and C5a at different time points were also investigated in the present study.

When all the samples were collected, the levels of those cytokines were evaluated using double antibody sandwich enzyme-linked immunosorbent assay (ELISA) kits (R&D, Minneapolis, MN, USA; Abcam, Cambridge, Cambridgeshire, UK) according to the manufacturer's instructions: Human PDGF-BB Quantikine ELISA Kit (R&D), Human TGF-β1Quantikine ELISA Kit (R&D), Human VEGF Quantikine ELISA Kit (R&D), HumanIGF-1Quantikine ELISA Kit (R&D), Human FGF basic Quantikine ELISA Kit (R&D), Human Complement Component C5a DuoSet ELISA (R&D), and Human Complement C3a des Arg ELISA Kit (Abcam). To detect the total amount of TGF-β1, the latent form of TGF-β1 was first converted into the active form according to the manufacturer's instructions. OD values were measured at 450 nm absorbance using a microplate reader (Bio-Rad, USA). The concentrations of different cytokines were determined according to the manufacturer's instructions. Standard curves were generated using standards provided by each kit. The absorbance from wells containing medium only were set as the blank and subtracted from the test wells readout. Triplicates were performed for all assays.

Statistical analysis

All reported values are the means of triplicate samples. Data were analyzed using SPSS version 10.0 (Chicago, IL, USA). The levels of total proteins released in different scaffolds were analyzed by one-way analysis of variance (one-way ANOVA). Statistical analysis of the data on release dynamics was performed by two-way analysis of variance (two-way ANOVA) followed by the Turkey post-hoc test for comparisons. The levels of cytokines in PPP were also compared with those in CGF with significance set at the $p \leq 0.05$.

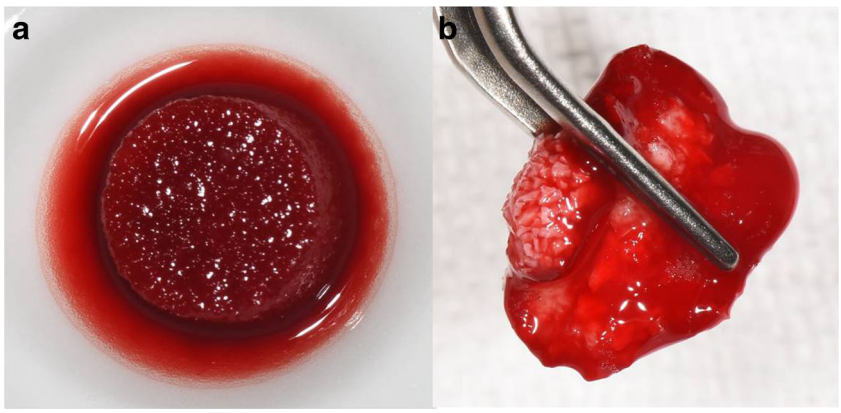

Fig. 1 The gross images of coagulated form of CGF-DBBM (**a**) and CGF-IMC (**b**)

Data availability The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

Results

Characterization of scaffolds

Representative SEM images and EDS curves of DBBM and IMC scaffolds were shown in Fig. 2. The massive DBBM scaffold was composed of micro-sized apatites with Ca/P ratio of 1.54 ± 0.06, whereas the IMC scaffold showed a highly porous structure with interconnected macropores of 128.3 ± 24.7 μm. Under high magnification, the IMC exhibited bone-like subfibrillar nanostructure without any apatites formed around the collagen fibrils. The presence of nanoapatites within the fibrils in the IMC was confirmed by EDS, where the Ca/P ratio was 1.52 ± 0.06, indicating of calcium-deficient nanoapatites.

Total protein release

The total accumulated protein release was calculated for those growth factors and cytokines evaluated (Fig. 3). It was found that CGF-IMC released the highest total amount of cytokines when compared to CGF-DBBM or CGF except for bFGF ($p < 0.01$). CGF-IMC and CGF-DBBM released significantly more bFGF compared to CGF alone ($p < 0.001$), while there was no significant difference found in the levels of bFGF released by CGF-IMC and CGF-DBBM ($p > 0.05$). Furthermore, the accumulated protein of PDGF-BB, bFGF, and C3a in CGF-DBBM was significantly higher than that in CGF alone ($p < 0.01$). While the accumulated protein of TGF-ß1, VEGF, IGF-1, and C5a in CGF-DBBM and CGF showed no significant difference ($p > 0.05$).

Dynamics of accumulated release

Differential dynamics of release of growth factors and cytokines in CGF, CGF-DBBM, and CGF-IMC were observed in Fig. 4.Values are expressed as the cumulative mean quantity of proteins at different time points (24 h, 72 h, 7 days, 14 days, 21 days, and 28 days). Figure 4 showed persistent releases of all the proteins evaluated from CGF, CGF-DBBM, and CGF-IMC during 28 days in vitro.

The levels of growth factors released in CGF rose slowly during 28 days while the levels of TGF-ß1, bFGF, and C5a in CGF rose significantly from 14 days ($p < 0.05$). The levels of growth factors released in CGF-DBBM increased significantly from 72 h (C5a), 7 days (IGF-1 and bFGF), or 14 days (PDGF-BB, TGF-β1, VEGF, and C3a) ($p < 0.05$). The levels of growth factors released in CGF-IMC increased dramatically from 7 days (VEGF, IGF-1, and C5a) or 14 days (PDGF-BB, TGF-β1, bFGF, and C3a) ($p < 0.05$). Comparison between three groups showed that, except TGF-β1, the levels of the other growth factors and cytokines released in CGF-IMC were several times of those in CGF and CGF-DBBM from 14 days and the trend continued to 28 days ($p < 0.01$).

Dynamics of release percentage

The kinetics of percentage of the growth factors released at different time point was displayed in Fig. 5. The trends of the kinetics of percentage of total release were consistent with the kinetics of accumulated release.

Analysis of release kinetics showed that the most popular pattern was bimodal releasing with a low peak value at 24 h

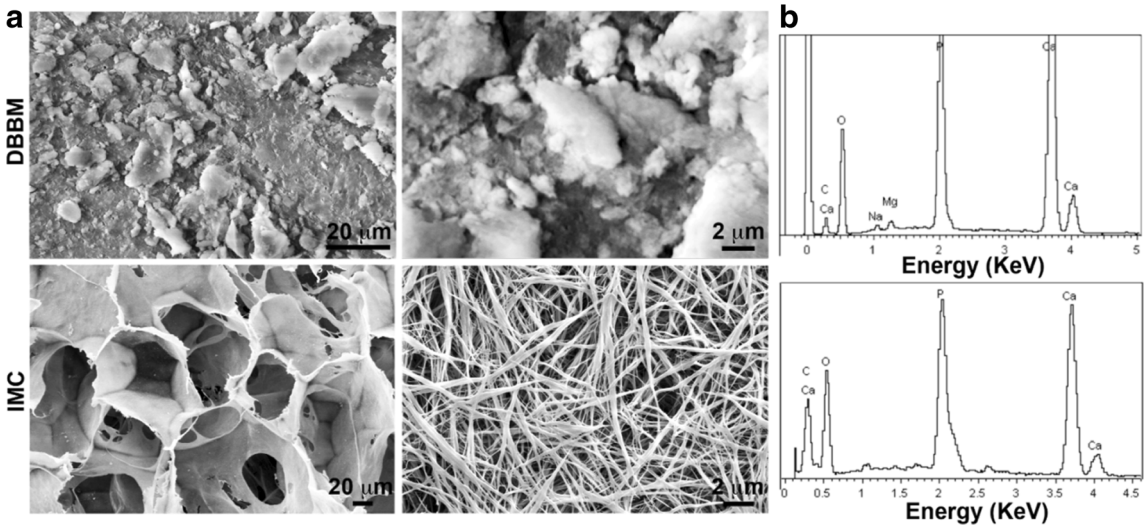

Fig. 2 **a** Representative (left) low- and (right) high-magnification SEM images of DBBM and IMC. **b** EDS of DBBM and IMC

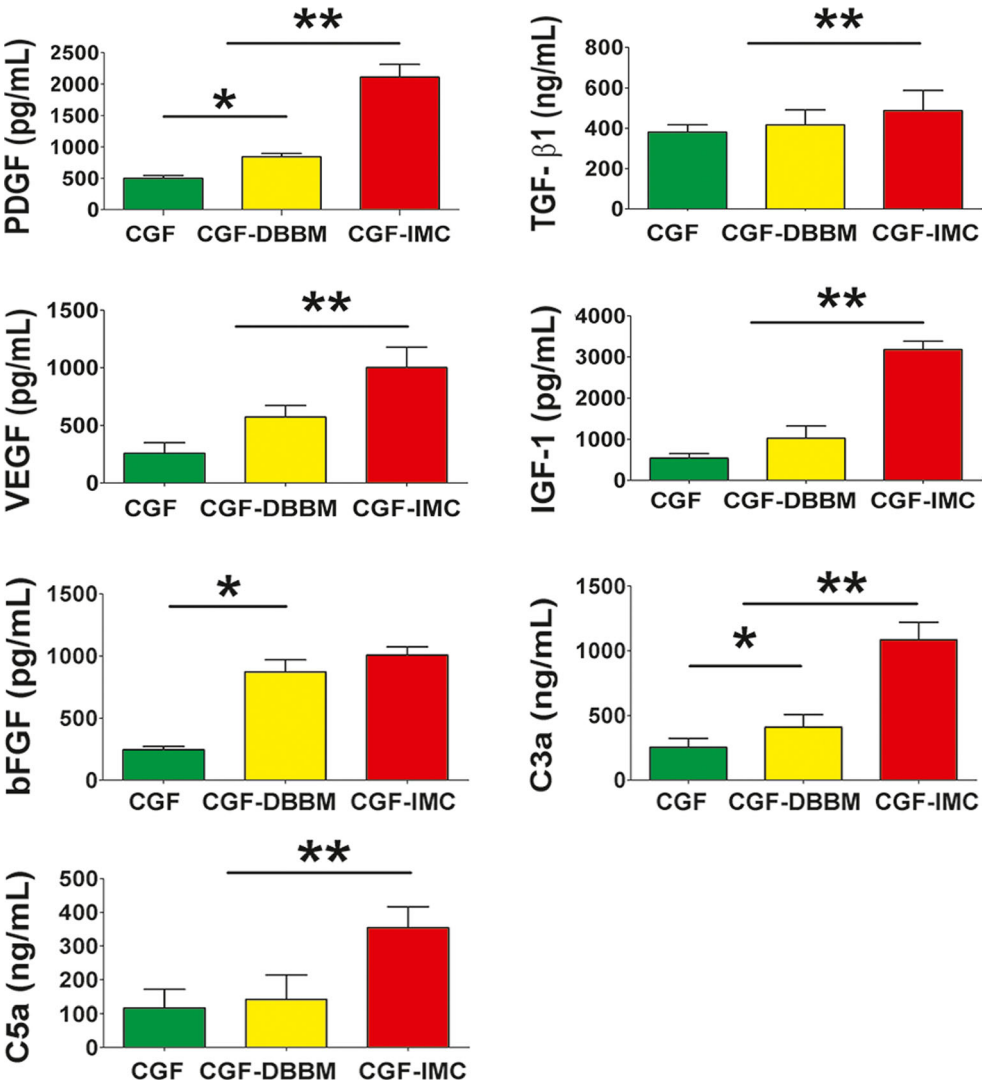

Fig. 3 The total accumulated protein released over 28 days. Data were presented as mean ± SD from triplicate measurements of nine samples. Statistical evaluation was done using one-way ANOVA. Significant differences among CGF, CGF-DBBM, and CGF-IMC for each factor were indicated: $*p < 0.05$, $**p < 0.01$

and a high peak value at 14 days (PDGF-BB in three groups, IGF-1 in CGF-DBBM), 21 days (VEGF in CGF and CGF-DBBM, IGF-1 in CGF, C3a in CGF-IMC, C5a in 21 days), or 28 days (VEGF in CGF-IMC, IGF-1 in CGF-IMC, bFGF in all three groups, C3a in CGF). The release of TGF-β1 also showed a bimodal pattern but with a high peak value at 24 h and a low peak value at 14 days (CGF-IMC) or 21 days (CGF and CGF-DBBM). The release of C3a showed a similar bimodal pattern with a high peak value at 24 h and a low peak value at 21 days. The release of C5a in CGF-DBBM and CGF-IMC showed a multimodal pattern: a low peak at 24 h, and two high peaks at 14 and 28 days.

Discussion

Platelet derivatives have been widely used in wound healing, tissue regeneration, and tissue engineering [26–28]. As a novel generation of platelet concentrates product, CGF, usually combined with graft materials, has been applied in soft and hard tissue healing and regenerative medicine, such as periodontal regenerative therapy [12] and bone regeneration [14, 29, 30]. Although CGF has been demonstrated to contain high concentrations of certain growth factors, the release kinetics of different growth factors and cytokines in CGF was still unclear. This study provides an important evidence for the release kinetics of CGF and CGF combined with IMC and DBBM.

Our data showed that the highest release occurred in CGF-IMC for all growth factors and cytokines investigated except bFGF. Then, CGF-DBBM released more quantities of PDGF-BB, bFGF, and C3a than CGF alone. Several factors can influence total release as well as the dynamics of growth factor release. A possible explanation is the architecture of different scaffolds. Compared to structured fiber network in CGF, DBBM and IMC provided more scaffolds for sustained release of growth factors. The spongy morphology and subfibrillar nanostructures in the IMC provide a much

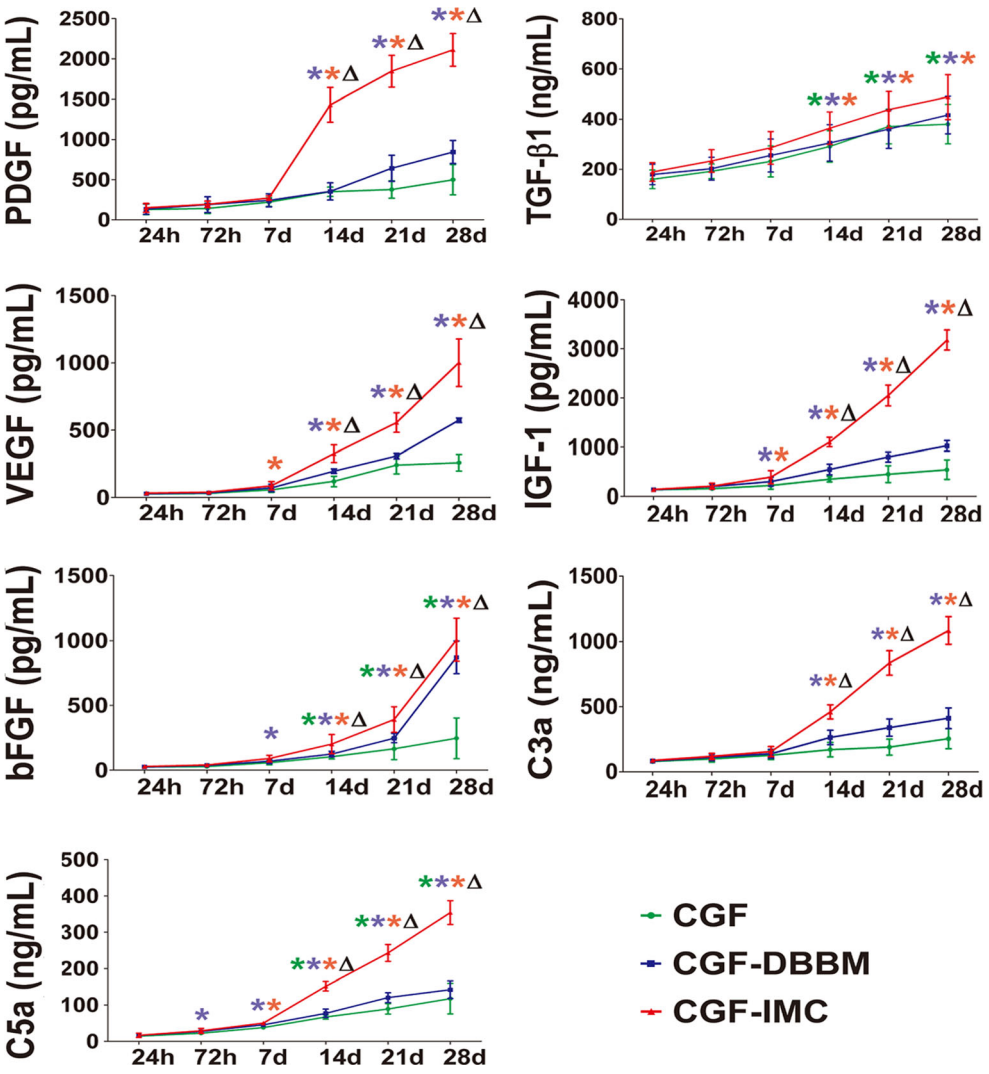

Fig. 4 Kinetics of growth factor accumulated release from CGF, CGF-DBBM, and CGF-IMC. Release of each factor was determined at each time point (24 h, 72 h, 7, 14, 21, and 28 days). Asterisk: compared to other time points in the same group, $p < 0.05$; triangle: compared to the same time point in CGF and CGF-DBBM group, $p < 0.05$

larger interface area compared to DBBM. The second reason may be the components of different scaffolds. DBBM is mainly composed of hydroxyapatites, while IMC was a composite of nanohydroxyapatites and collagen fibrils. Fufa et al. found that type I collagen was a safe and effective alternative to bovine thrombin in activating PRP and stimulating growth factors release from the platelets [31]. In another study, Zhang et al. fabricated a novel scaffold that integrates PRP activated by type I collagen, and found that collagen/PRP scaffolds provided a long period release of growth factors compared to thrombin-activated PRP [32]. Collagen is much more efficient at loading growth factors when compared to DBBM [33, 34]. Furthermore, it is well known that cell adhesion is greatly affected by collagen-containing scaffolds and collagen supports better cell attachment [35, 36]. Therefore, we speculated that the collagen component in IMC may be an activator for CGF and provided a carrier for the controllable release of growth factors. Another possible explanation is "nano." In the present study, a 3D IMC was fabricated using a modified bottom-up biomimetic approach, enabling the formation of subfibrillar nanostructures at the molecular and nanoscale levels. Biological activity of nanoparticles can be dictated by their composition, size, and charge [37]. This hypothesis is also supported by the differential degrees of platelet aggregation induced by multiwalled and single-walled nanotubes, C60 fullerenes, mixed carbon nanoparticles, or collagen-coated latex nanoparticles compared with inhibition of platelet aggregation by positively charged nanoparticles or those formed from PEGylated and nonPEGylatedcetyl alcohol/polysorbate nanoparticles [38–42]. The IMC used in the present study might participate in modulating platelets secretion. Further studies are needed to investigate the possible mechanism of "nano" on CGF or platelets functions.

The release pattern of the growth factors analyzed in our study showed sustained release from CGF, CGF-DBBM, and CGF-IMC till 28 days. The release curves of most growth factors and cytokines included two peaks, one occurring at 24 h, and the other occurring during 14 to 28 days. C5a

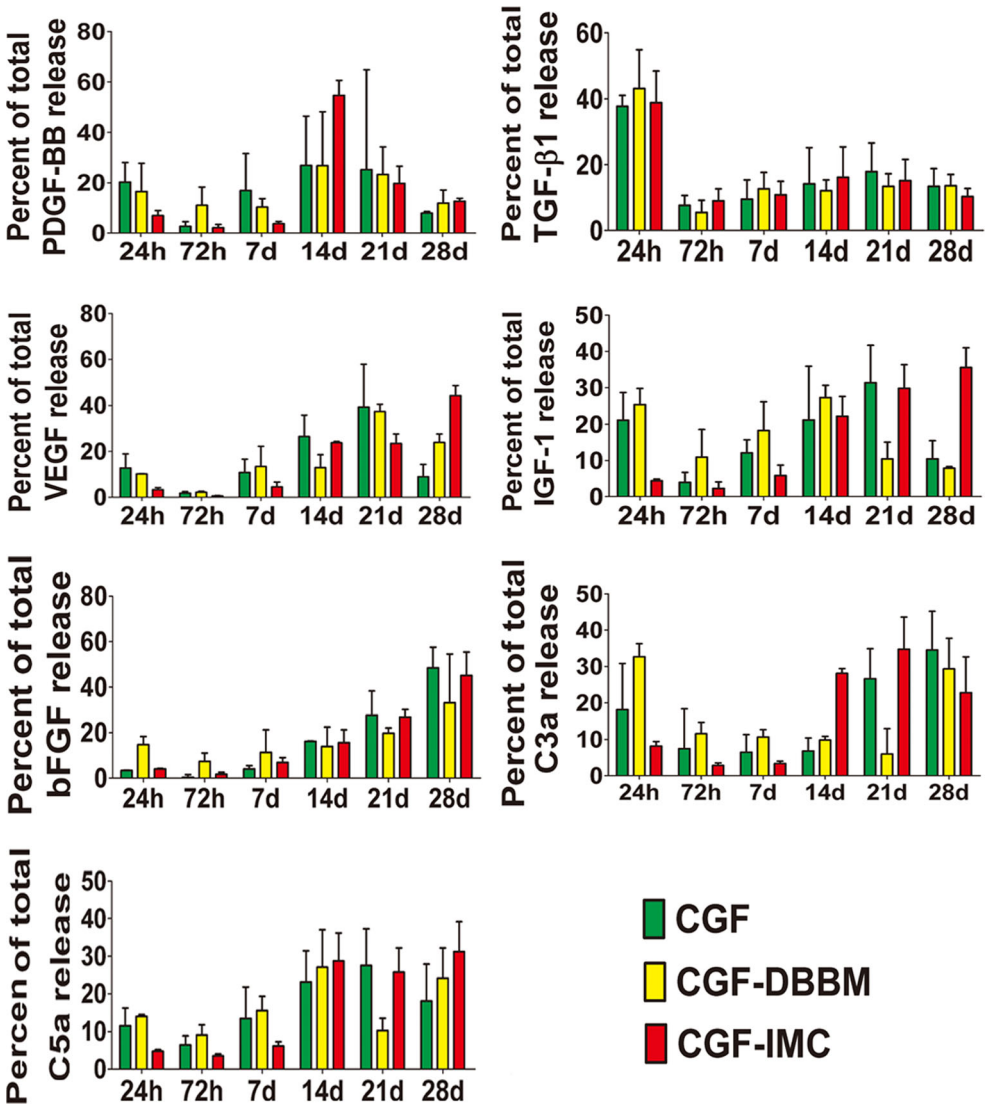

Fig. 5 Kinetics of growth factor release from CGF, CGF-DBBM, and CGF-IMC. The amounts released were expressed as a percentage of total release at each time point

released in CGF-IMC showed a triple humped releasing curve, peaking at 24 h, 14 days, and 28 days. Furthermore, CGF-IMC released much more growth factors compared to CGF and CGF-DBBM during 14 to 28 days. The immediate release peak at 24 h could be attributed to instant release from activated platelets during centrifuge or simple diffusion from plasma. The late release peak during 14 to 28 days could be explained by release of growth factors after degradation of fibrin structure and production of growth factors by the leukocytes present in CGF. In the data obtained from previous studies, fibrin clots can be maintained without substantial degradation under similar protease-free conditions for longer than 1 week [43, 44]. CGF have shown a much larger, denser, solider, and more structured fibrin network consistency compared to natural fibrin clot. Therefore, the dissolving time of fibrin structure in CGF might be longer than usual fibrin clot. This could partly explain the time when the late peak occurred.

The centrifugation speeds we used for CGF preparation were between 323×*g* and 503×*g*. More recently, lower centrifugation speeds and time have been proposed to further optimize the number of leukocytes and subsequent release of growth factors from PRF formulations. Fujioka-Kobayashi et al. have demonstrated that modifications to centrifugation speed and time with the low-speed concept could favor an increase in growth factor release from PRF clots, which might in turn directly influence tissue regeneration by increasing fibroblast migration, proliferation, and collagen mRNA levels [45]. Utilizing CGF with lower centrifugation speeds will be a tendency in the future.

The results presented in this study have important biomedical indications that IMC scaffolds could be used as a carrier for sustained release of growth factors from autogenous CGF in tissue regeneration therapy. In the future work, we will continue to investigate the release dynamics of growth factors in CGF in vivo.

Conclusions

In summary, the release pattern of growth factors from CGF, CGF-DBBM, and CGF-IMC were different. CGF-IMC offers advantages over CGF-DBBM and CGF. CGF-IMC can release higher levels of growth factors and provide a sustained, long-time release. IMC could be used as a new carrier for sustained release of growth factors in CGF. We need more studies to investigate the effect of sustained release of growth factors on different types of cells, and the in vivo effect in the field of regenerative medicine.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

Informed consent Informed consent was obtained from all individual participants included in the study.

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