EGCG-modified bone graft to modulate the recruitment of M1 macrophage and alleviate the forming of fibrous capsule

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Abstract

Bone graft with hydroxyapatite, HA, as the main component, is commonly used for bone repair. It will stimulate the bone ECM, providing promising bone regeneration potential. However, due to its pro-fibrosis effect, implant failure of this osteoconductive material is common. To boost the success rate, pure HA is usually modified, or simply being as a part of a biomaterial to improve osteoinductivity. In this paper, we focus on M1 macrophages for its close relationship with fibrosis, acting as the important role during the immune response and the subsequent repairs with its various phenotypes in regulating inflammation. To reduce M1 macrophage recruitment in subcutaneous implantation, we attempt to immerse HA in an extraction of green tea, epigallocatechin-3-gallate (EGCG). The result showed that E-HA considerably compromised the forming of fibrous capsule in HE sections and cell viability was improved in the meanwhile, laying the foundation for future studies on guiding bone regeneration.

Keywords: hydroxyapatite; macrophage; fibrous capsule; EGCG

1 Introduction

One of the key principles of guided bone regeneration (GBR) is to avoid the damaged area being filled with soft tissue, leaving no space for deposition of bone tissue; this can be attained by removing non-osteogenic tissues from interfering bone regeneration while implanting biomaterials. The design of implanted bone substitutes should base on the certain characteristic of the defect, restoring the microenvironment of the tissue before being impaired and keeping from severe fibrosis to promote integration and recovery the function [1,2]. As a primary component of mineral bone, hydroxyapatite (HA) comprised in 60–70 % bone and 98 % dental enamel has been known to possess good bio-compatibility, high osteoconductivity and osteoinductivity [3], remaining in the implant site with slow absorption rate [4]. A 20 years follow-up in maxillary sinus floor elevation by means of commercial bovine- derived bone mineral, which is mainly composed of HA, revealed that new mineralized bone volume remained stable and increased from 16.96% to 22.05% in 20 years, while the volume of the implanted graft has fallen from 35.87% to about 4% [5]. However, in some cases, HA implantation resulted in disappointed healing outcome for thick fibrous

capsule restrained the bone-biomaterial integration.

Epigallocatechin-3-gallate (EGCG), the main catechins of tea, was then regarding as a great substance for modifying HA, with its multiple therapeutic effects against various human diseases through its anti-inflammatory [6], anti- carcinogenic [7], anti-microbial and anti-oxidative ability [8]. Apart from the above biological properties, in the field of bone remodeling, EGCG can promote osteogenesis with the increase the expression of BMP2, Runx2, alkaline phosphatase (ALP), osteonectin and osteocalcin. A Better ALP activity and mineralization of bone defect were then achieved [9] to prevent inflammatory bone loss caused by inhibiting prostaglandin E synthesis [10]. Also, in our previous study, we found that when adding EGCG to the designed material, EGCG seemed to bring about a promising microenvironment for bone regeneration underlining the recruitment and the phenotype of macrophage [11]. Regarding on macrophage, the persistence of inflammatory phenotype of it could contribute to fibrosis, which impacts the bone growth at last. Although the mechanism of fibrosis which features in implant biodegradation and the blocking of the interaction between biomaterial and surrounding tissue is still needed to be studied to

further scrutiny; the process was confirmed to be associated with the participation of certain phenotype of macrophage and various immune cells. This fibrous response usually began with severe foreign body reaction as cells being damaged, necrotic and stressed [12-14]. Nevertheless, principal cells that prompt tissue repair are no different from those leading to fibrosis capsule, except the long persistence and drastic communication of the inflammatory cells and myo-fibroblasts [15]. In fact, with different degree of fibrosis, the outcome of implantation could be either advantageous to repair and prognosis (deposited provisional extracellular matrix providing frame for angiogenesis) or harmful to the host (impair the integration resulting in failure) [16]. Hence, the ideal situation is to avoid serious foreign body reaction, and direct healing in the meanwhile through controlling the infiltrated cells and their behavior to modulate immune response. To sum up, immunoreaction plays a vital role in the healing process and determines the final result; considering the broad clinical application of HA material, it is of interest to understand the immune response after the involvement of EGCG which is capable to reduce the amount of M1 macrophage. The purpose of the presented study was to evaluate whether binding EGCG to HA could mitigate

foreign body reaction with less fibrosis, even further enhance bone regeneration (**Fig. 1**).

2 Materials and Methods

2.1 Materials

We purchased a commercial Heal-All® Bone Repair Material from Zhenghai Biotechnology (Shandong, China), which mainly contains hydroxyapatite and collagen, and retains its three-dimensional porous structure. The EGCG is available from Jiang Xi Lv Kang Natural Products (Jiang Xi, China). The solvents and chemicals used were all of analytical grade which need no further purification. To fabricate the EGCG-modified HA for the follow-up experiment, we dissolved EGCG in aseptic double distilled water at the concentration of 0.64% (w/v) and immersed HA in it for 24 h at 4□ in dark. E-HA is then washed with PBS for three times and preserved in dark at 4°C before use. Optical density (OD) value at 272 of the EGCG solution was obtain before and after immersion to ensure E-HA manufactured successfully.

2.2 *In vitro* release profile of E-HA

E-HA was soaked in PBS in 48-well plate at 37°C, 5% CO_2 and scanned by ultraviolet□visible spectrophotometry to obtain the release

profile. OD value at 272 was used to detect EGCG in solution. At 0 h, the instrument was calibrated to zero, then, measured the OD value of the media at 2, 6, 12, 24, 48, 96, 120, 144, 216 h by removing media (200 μl) and replenishing with fresh buffer (200 μl) each time. The concentration of the solution and weight of ECGC was transferred from OD value to specific concentration by establishing standard curve with different concentrations (7.8, 125, 15.625, 31.25, 62.5, 125, 250 and 500 μg/ml); the vertical axis shows the absorbance value, and the horizontal axis shows the concentration of EGCG solution. Linear regression analysis was performed using the weighted least-square method (W=1/C2).

2.3 Surface morphology observation

Scanning electron microscope (SEM, s-800, HITACHI, Tokyo, Japan) at 15 kV and digital camera (Canon EOS 6D Mark II) were used to characterize the morphology of materials. The typical image of each was captured.

2.4 Cell viability

Murine RAW264.7 cell line (RIKEN BioResource Center, Japan) were used for quantitative and qualitative cell viability assay. The cells were cultured in 48-well plates and seeded on the untreated and EGCG

treated HA at 10^4 cells/well cultured in 1640 medium supplemented with 10% fetal bovine serum (FBS). Immediately and after culture for 1, 3, 5 and 7 d, cell viability were evaluated using Cell Counting Kit-8 (CCK-8, Dojindo Laboratories, Kumamoto, Japan) according to the manufacturer's instructions. OD value at 450 nm was measured using micro-plate reader (Multiskan, Thermo, USA).

2.5 Establishment of *in vivo* Model

This experimental protocol was approved by the Institutional Review Board of West China Dental Hospital (No. WCHSIRB-D-2017-097). *In vivo* experiments were performed with 6-8-week-old C57 mouse (Animal Experimental Center of Sichuan University) weighing about 37.5 g. Two parallel surgical sites were established on each mouse for pure HA and E-HA, respectively ($N \geq 3$). To minimize the bias caused by the surgical site, materials were implanted with different arrangements.

2.6 Surgical procedures

After anesthetized with 0.15ml 10% chloral hydrate, the skin on the back of the mouse above the surgical area was shaved and followed the aseptic process before implantation. Two sagittal incisions of about 10 mm were made on the dorsal skin to create subcutaneous pockets for

bone repair material. Pure bone repair material and the EGCG-modified one were then implanted into the pockets. Subsequently, the incision was sutured with horizontal mattress 3.0 Vicryl suture (Ethicon, CA, USA). The animals were housed in a room specially designed for conducting an animal experiment and fed on a standard laboratory diet. After 7 days of recovery, the animals were sacrificed. Then, bone repair material and whole skin samples around the surgical site were collected.

2.7 Hematoxylin-eosin (HE) staining

For HE staining, 8 μm sections were incubated at 65 ° C for 4 h to dewax. After ethanol dehydration, hematoxylin was stained for 5 min, and 1% hydrochloric acid ethanol was differentiated for 2 h. Sections were then incubated for 2 min in 0.2% ammonia water, followed by staining with eosin for 1 min. After dehydration, the sections were removed and fixed with neutral resin. The slides were viewed with a microscope with 200 × oil immersion objective (Olympus Corporation, Tokyo, Japan); scanned with a digital slide scanning system (PRECICE, Beijing, China).

2.8 Immunofluorescent staining

The paraffin-embedded tissue was simply cut into 8 μm thick

sections. After dewaxing and hydrating, immunostaining was conducted. Sections were pre-treated with 0.1% Triton X-100 in PBS with 1% bovine serum albumin for 1 h, 1% t20 for 20 min, and PBS was washed for 20 minutes. Briefly, the sections were incubated for 30 min in the dark. The excess dye was washed away with PBS. Sections were incubated isotype with antibodies to exclude false positive staining. At least three parallel sections from different implantation sites were observed under a fluorescence microscope (Zeiss stereoscopic finding, V20, Germany). Five fields were randomly selected for immunofluorescence determination. Macrophages were magnified 400 times to exclude false positive staining. Semi-quantitative analysis was then performed at a magnification of 40 times. CaseViewer 2.1 and Image Pro Plus 7.0 (n = 5) were used to measure the fluorescence intensity of 5 random spots. Hoechst staining analysis was performed at the same time.

2.9 Statistical analysis

All quantitative data are presented as mean ± standard deviation. Statistical calculations were performed using GraphPad Prism 5.0 (San Diego, CA, USA). The semi-quantitative data of immunofluorescent staining that did not conform to the normal distribution were further

analyzed by Mann-Whitney U test, as statistical significance between groups was analyzed by analysis of variance (ANOVA) followed by Tukey's multiple comparison tests. $P < 0.05$ was considered statistically significant. The standard curve of EGCG solution was established with linear regression. Pearson's correlation analysis was used to analyze the detected concentration.

3 Results

3.1 *In vitro* release profile of E-HA

After immersing HA into EGCG solution, the OD value of that decreased, which represented that the concentration of which reduced, and E-HA was fabricated successfully (**Fig. 3A**). The concentration was calculated based on the standard curve of EGCG solution. To establish the standard curve, EGCG solutions were detected by ultraviolet□visible spectrophotometry, which revealed that EGCG at different concentrations had a maximum absorption wavelength of 272 nm. Therefore, 272 nm was selected for detection, and the absorbance value was set as the vertical axis with the corresponding concentration setting as horizontal axis. The regression equation was identified to be $y=0.0067x-0.0327$, with $R^2 =0.9998$. The linear concentration range was identified to be

7.8-500 μg/ml for EGCG, presenting in **Fig. 3B**. Cumulative release profile of E-HA was obtained by detecting the amount of EGCG released to the buffer with time. The release profile demonstrated a minimal initial release, with only approximately 10μg EGCG being released in the first 3 days, followed by a continuous release up to about 100μg the next 3 days. Extra release about 75μg was measured at 216 h (**Fig. 3C**).

3.2 Surface morphology

The morphology of bone repair material is shown in **Fig. 2A**. It is inconsistent in shape, different in size, and featuring in the porous structure. After adding EGCG, the surface outlook of bone repair material became directional, dense and uniform, which may be caused by the hydrogen bond between EGCG and collagen fibers. From the SEM image, it can also be seen that the surface roughness decreased (**Fig. 2B**).

3.3 Cell viability

RAW 264.7 were seeded on the material for 7 days; during all period of time, cell viability of group HA stayed relatively constant, whereas the effect of EGCG towards which increased when the co-culture time was prolonged. On day 0 and day 1, there was no statistical significance between group HA and E- HA, whereas on day 3 and day 5, cell viability

slightly improved with the involvement of EGCG, which thrived over time and was highest on day 7 (**Fig. 4**). That is, the modification of EGCG did not harm the cell viability of the RAW 264.7 and was even conducive to the improvement of cell viability.

3.4 The severity of fibrosis after HA and E-HA implantation is related to different phenotypes of infiltrated macrophages

In order to evaluate whether EGCG-modified bone repair materials are able to mitigate fibrosis by modulating the immune microenvironment, the selected materials were implanted subcutaneously (**Fig. 5A-F**), and the reaction of immune cell towards EGCG modification was investigated by HE staining (**Fig. 5G**). On day 7 after the bone repair material was implanted, incisions of both groups healed well. And the nonvascular tubular structure could be observed, whereas more abundant in group E-HA. The fibrous encapsulation effect was much more intense in the group HA, with a large amount of collagen fibers deposited on the surface of the material. And the surrounding immune cells would migrate around the bone repair material, forming an inflammatory cell infiltration zone. The inflammation resulted from the implant of EGCG-load bone repair material is lighter than pure bone repair material. We further explored the

phenotype of macrophage at the same time point. From the immunofluorescence staining images of Hoechst, CD68 and iNOS (**Fig. 5H**), we could find that in group HA and E- HA recruited macrophages of different phenotype. Although the detection of CD68 which serves as the surface marker for macrophage could be seen in both groups, iNOS, a representative for M1 phenotype macrophages, cannot be detected in the E-HA group, whereas existed in group HA, reconfirming macrophage infiltration and the effect of EGCG in anti-inflammation.

4 Discussion:

Serious defects in the hard tissue would cause self-healing to fail. At the present, the application of biomaterial and GBR membrane are often used in clinical practice to block the invasion of soft tissue in the growth space of the hard tissue; this effect has been widely proved. Among them, collagen membrane modified by EGCG had been thorough a deep research, including biotoxicity [17], effects on osteoblasts [18], chemotaxis of macrophages [19], phenotypic regulation [11], and Foreign Body Reaction (FBR) [20].For the implanted biomaterial, the ideal therapeutic effect cannot be achieved due to the absence of organic components and the lack of biological signals in pure HA. When designing a biomaterial, HA is often

a part of it, such as BMP-2 immobilized PLGA/hydroxyapatite fibrous scaffold [21]; or modified, such as loading Nerve Growth Factor [22]. Therefore, in order to better stimulate bone ECM and provide suitable microenvironment for the regeneration of the hard tissue, we used a bovine-derived decellularized bone graft which composed of HA and collagen type I, which is similar to the composition of bone and the current commercial dental bone substitute.

As a biomaterial is implanted, not only osteoblasts are involved in the repair, but the interaction between the material and immune cells is the key in the healing of tissue. The adsorption of proteins i is the beginning of the acute inflammatory phase of FBR, and the inflammation will gradually transit to tissue repair. Right after the surgery was performed, macrophages quickly identified the implant, and were recruited to biomaterial the forming specific immune microenvironment. Macrophage is involved in the starting of inflammation and the following tissue reconstruction with the secretion of cytokines. Macrophage phenotype transformation for tissue from M1 to M2 is necessary in both hard and soft tissue repair; long-term or severe inflammation usually contribute to fibrous capsule formation by M2 macrophages, blocking material with

the surrounding and ending up with bone- biomaterial integration failure [23]. The main cause of implant failure, aseptic loosening, is due to the formation of wear particles from the implant under phagocytosis, which promotes macrophages to secrete cytokines related to bone absorption. HA-induced phagocytosis depended on IL-1β and TNF-α produced by macrophages [24], which was closely related to M1 phenotype. In the meanwhile, the presence of a long-term high M1/M2 phenotypic ratio macrophages often indicated the failure of implantation. Thus, in addition to manufacturing biomaterials close to the natural ECM, the phenotype of macrophages should also be taken into consideration. Biodegradable three-dimensional scaffold which was comprised of polymer matrix (polylactic acid and polyethylene glycol), nano hydroxyapatite, and dexamethasone utilizing 3D printing technology to precisely control properties and shape, promoted macrophage polarization towards M2 phenotype and osteogenesis that downregulated IL-6 and iNOS expression by M1 macrophage [25]. In fact, both M1 and M2 phenotypes are indispensable for tissue healing, and M1 macrophage can even guide the subsequent behavior of the M2. Only the existing time matters, as fortunate repair can never be accompanied by excessive M1 polarization

[26]. CD68, which is widely acknowledged as a marker for macrophage, was stained to identify the presence of it and confirmed the reduction of M1 phenotype by adding EGCG can through iNOS.

Apart from absenting M1 macrophage, we also found that EGCG made the difference to the surface morphology, shown under scanning electron microscope, which has close relationship with FBR. Researchers had speculated that the mechanism by which HA coating promotes osteogenesis may take place on the basis of its surface morphology rather than on stimulating macrophages to express BMP2, since HA could significantly up-regulat proinflammatory factors, TNF-α, by M1 macrophages, further inhibited M2 macrophages from expressing BMP2 that featured in osteoconductive [27]. In other words, HA has a proinflammatory tendency and is not conductive to the expression of BMP2, both of which may be improved by the addition of EGCG [6,9]. However, whether EGCG can promote the polarization of macrophages toward M2 and stimulate the secretion of desired cytokines, as our previous studies on collagen membranes have shown, it needs further investigation.

Cytotoxicity is another essential aspect to evaluate biocompatibility.

Karin H. Müller and Michael Motskin et al. found that HA nanoparticles formed large agglomerates in the medium resulting in extensive particle uptake in macrophage and sequestered in surface-connected compartment [28], triggered dose-dependent cytotoxicity. To reduce cytotoxicity, particles could be citrated or added dispersant Darvan 7 [29]. Our HA particle size is between 1000-2000 μm, and cell viability even improved after adding EGCG. We found that the effect of EGCG on cell viability was related to its released amount from E-HA. The in vitro EGCG release rate assay showed that more EGCG was released over time. The longer the culture time, the more significant the effect EGCG puts on cell viability. Therefore, it is considered that the addition of EGCG will not reduce, but even increase, cell activity, which is beneficial for healing.

This article only mentioned a subcutaneous method to reduce M1 macrophage. As for the specific mechanism and phenotypic transformation, including the ratio of M1/M2 macrophage at different time points, will be discussed in the bone model in the future.

5 Conclusion:

To sum up, we have found a possible strategy to modify bone repair material, which acquired anti-inflammatory effect for lowering the

accumulation of M1 macrophage and further regulated the fibrosis, while the effect of EGCG on the surface morphology cannot be excluded. The specific mechanism of reducing the fibrosis needs to be verified. The results of this study will pave the way for subsequent experiments and explore the osteogenesis effect of E-HA as this is surely the most important effect for bone repair materials. At any circumstances， EGCG do improve cell activity after being added to HA and no $CD68^{+}iNOS^{+}$ cells were detected in the immunofluorescence staining 7D post-surgery, indicating that the addition of EGCG could bring hope for the following regenerative cascade.

Grant

National Natural Science Foundation of China: No. 81870801

National Natural Science Foundation of China: No. 81902976

Statement Conflict of Interest

There are no conflicts of interest related to this manuscript

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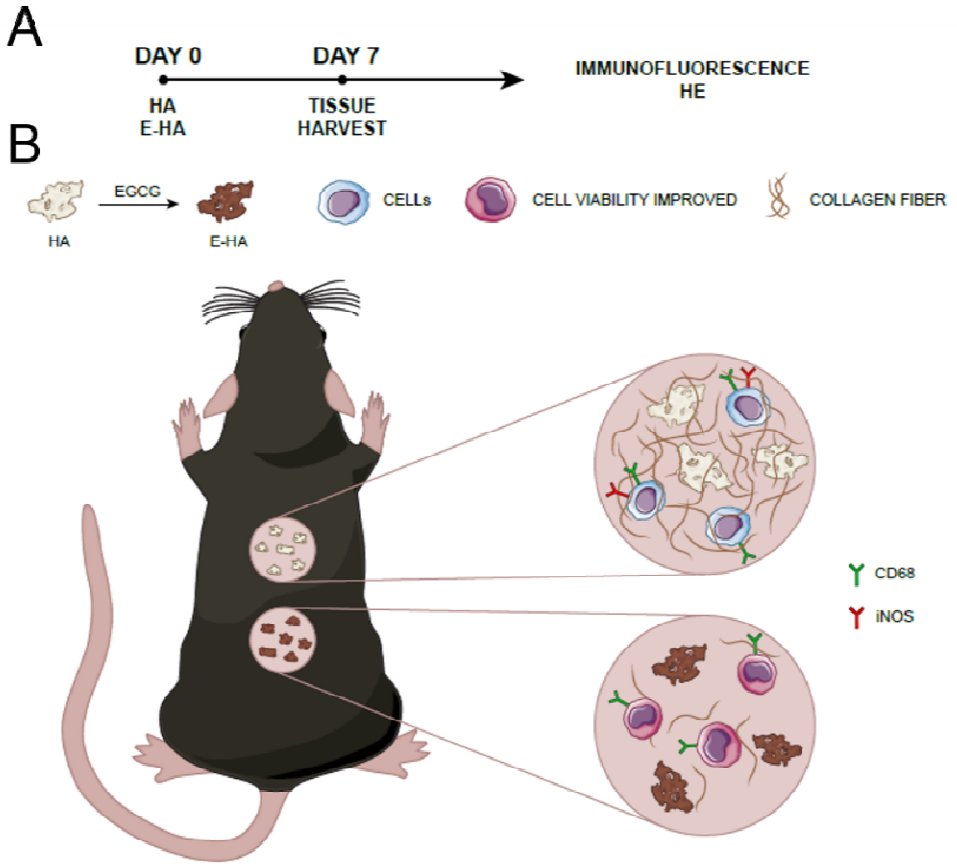

Figure 1. Schematic illustration.

Diagram of in vivo experiments. (A) Showing the period to harvest and the subsequently executed testing. (B) Cell viability, macrophage polarization, and fibrosis outcome of EGCG modified bone graft, compared with bone graft alone.

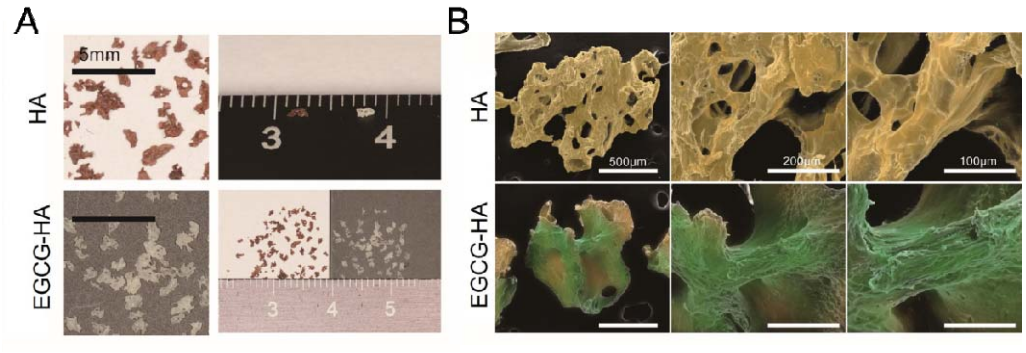

Figure 2. Representative image of HA and E-HA. (A) Surface morphology of materials is clearly presented under digital camera and (B) scanning electron microscope.

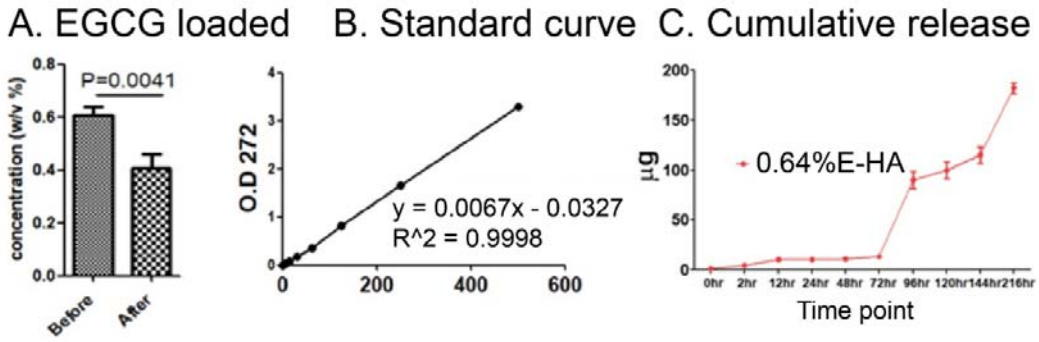

Figure 3. The establishment and release of EGCG on the bone graft. **(A)** E-HA fabrication assessment by detecting the concentration of EGCG solution before and after immersing HA. (B) The standard curve of EGCG solution at the concentration between 7.8 and 500 μg/ml, (C) obtained under OD value of 272. *In vitro* cumulative release profile of E-HA.

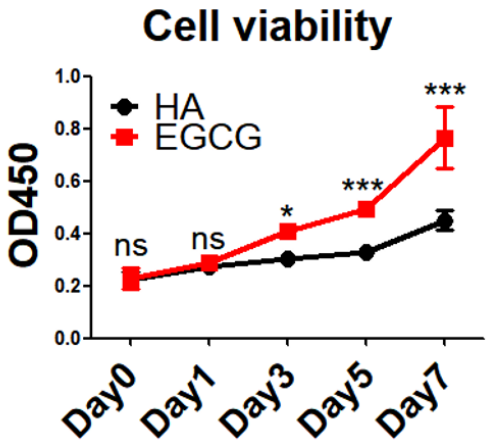

Figure 4. Cell viability assay. Culturing RAW 264.7 on deproteinized bone material with or without EGCG treatment, then the cell viability was detected on day 0, 1, 3, 5 and 7. NS= no significance; *= P<0.05, **= P<0.01, ***= p<0.001.

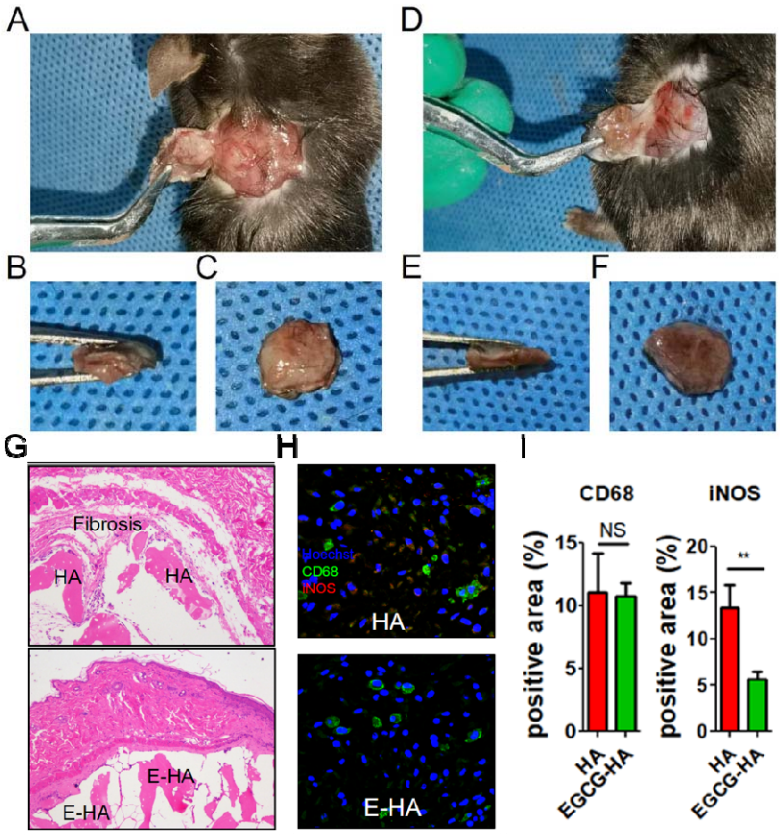

Figure 5. Evaluation of fibrosis and phenotype of infiltrating macrophages after implantation. (A-C) Pictures of harvested tissue encapsulated with (D-E) HA and E-HA. (G) HE staining result on day 7 post-surgery. (H) Immunofluorescent staining result on day 7 post-surgery. Hoechst, blue; CD68, green; iNOS, red. (I) Semi-quantitative analysis of immunofluorescent staining result. NS= no significance; **= P<0.01.