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

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28

29 **Abstract**

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

45 **Keywords:** hydroxyapatite; macrophage; fibrous capsule; EGCG

46

47 **1 Introduction**

48 One of the key principles of guided bone regeneration (GBR) is to
49 avoid the damaged area being filled with soft tissue, leaving no space for
50 deposition of bone tissue; this can be attained by removing
51 non-osteogenic tissues from interfering bone regeneration while
52 implanting biomaterials. The design of implanted bone substitutes should
53 base on the certain characteristic of the defect, restoring the
54 microenvironment of the tissue before being impaired and keeping from
55 severe fibrosis to promote integration and recovery the function ^{1,2}. As a
56 primary component of mineral bone, hydroxyapatite (HA) comprised in
57 60–70 % bone and 98 % dental enamel has been known to possess good
58 bio-compatibility, high osteoconductivity and osteoinductivity ³,
59 remaining in the implant site with slow absorption rate ⁴. A 20 years
60 follow-up in maxillary sinus floor elevation by means of commercial
61 bovine- derived bone mineral, which is mainly composed of HA, revealed
62 that new mineralized bone volume remained stable and increased from
63 16.96% to 22.05% in 20 years, while the volume of the implanted graft
64 has fallen from 35.87% to about 4% ⁵. However, in some cases, HA
65 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-inflammatory⁶, anti- carcinogenic⁷, anti-microbial and

anti-oxidative ability⁸. 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 achieved⁹ to prevent inflammatory bone loss

caused by inhibiting prostaglandin E synthesis¹⁰. 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

macrophage¹¹. 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

85 further scrutiny; the process was confirmed to be associated with the
 86 participation of certain phenotype of macrophage and various immune
 87 cells. This fibrous response usually began with severe foreign body
 88 reaction as cells being damaged, necrotic and stressed ¹²⁻¹⁴. Nevertheless,
 89 principal cells that prompt tissue repair are no different from those
 90 leading to fibrosis capsule, except the long persistence and drastic
 91 communication of the inflammatory cells and myo-fibroblasts ¹⁵. In fact,
 92 with different degree of fibrosis, the outcome of implantation could be
 93 either advantageous to repair and prognosis (deposited provisional
 94 extracellular matrix providing frame for angiogenesis) or harmful to the
 95 host (impair the integration resulting in failure) ¹⁶. Hence, the ideal
 96 situation is to avoid serious foreign body reaction, and direct healing in
 97 the meanwhile through controlling the infiltrated cells and their behavior
 98 to modulate immune response. To sum up, immunoreaction plays a vital
 99 role in the healing process and determines the final result; considering the
 100 broad clinical application of HA material, it is of interest to understand
 101 the immune response after the involvement of EGCG which is capable to
 102 reduce the amount of M1 macrophage. The purpose of the presented
 103 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 °C 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% CO₂ and
scanned by ultraviolet-visible spectrophotometry to obtain the release

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

133 **2.3 Surface morphology observation**

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

138 **2.4 Cell viability**

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

142 treated HA at 10^4 cells/well cultured in 1640 medium supplemented with
143 10% fetal bovine serum (FBS). Immediately and after culture for 1, 3, 5
144 and 7 d, cell viability were evaluated using Cell Counting Kit-8 (CCK-8,
145 Dojindo Laboratories, Kumamoto, Japan) according to the
146 manufacturer's instructions. OD value at 450 nm was measured using
147 micro-plate reader (Multiskan, Thermo, USA).

148 **2.5 Establishment of *in vivo* Model**

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

156 **2.6 Surgical procedures**

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

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

168 **2.7 Hematoxylin-eosin (HE) staining**

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

178 **2.8 Immunofluorescent staining**

179 The paraffin-embedded tissue was simply cut into 8 μ m thick

180 sections. After dewaxing and hydrating, immunostaining was conducted.

181 Sections were pre-treated with 0.1% Triton X-100 in PBS with 1% bovine

182 serum albumin for 1 h, 1% t20 for 20 min, and PBS was washed for 20

183 minutes. Briefly, the sections were incubated for 30 min in the dark. The

184 excess dye was washed away with PBS. Sections were incubated isotype

185 with antibodies to exclude false positive staining. At least three parallel

186 sections from different implantation sites were observed under a

187 fluorescence microscope (Zeiss stereoscopic finding, V20, Germany).

188 Five fields were randomly selected for immunofluorescence

189 determination. Macrophages were magnified 400 times to exclude false

190 positive staining. Semi-quantitative analysis was then performed at a

191 magnification of 40 times. CaseViewer 2.1 and Image Pro Plus 7.0 (n = 5)

192 were used to measure the fluorescence intensity of 5 random spots.

193 Hoechst staining analysis was performed at the same time.

194 **2.9 Statistical analysis**

195 All quantitative data are presented as mean $\pm$ standard deviation.

196 Statistical calculations were performed using GraphPad Prism 5.0 (San

197 Diego, CA, USA). The semi-quantitative data of immunofluorescent

198 staining that did not conform to the normal distribution were further

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

205 **3 Results**

206 **3.1 *In vitro* release profile of E-HA**

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

218 7.8-500 $\mu\text{g/ml}$ for EGCG, presenting in **Fig. 3B**. Cumulative release
219 profile of E-HA was obtained by detecting the amount of EGCG released
220 to the buffer with time. The release profile demonstrated a minimal initial
221 release, with only approximately 10 μg EGCG being released in the first 3
222 days, followed by a continuous release up to about 100 μg the next 3 days.
223 Extra release about 75 μg was measured at 216 h (**Fig. 3C**).

224 **3.2 Surface morphology**

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

231 **3.3 Cell viability**

232 RAW 264.7 were seeded on the material for 7 days; during all period
233 of time, cell viability of group HA stayed relatively constant, whereas the
234 effect of EGCG towards which increased when the co-culture time was
235 prolonged. On day 0 and day 1, there was no statistical significance
236 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 biotoxicity¹⁷, effects on osteoblasts¹⁸, chemotaxis of macrophages¹⁹, phenotypic regulation¹¹, 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

275 a part of it, such as BMP-2 immobilized PLGA/hydroxyapatite fibrous
 276 scaffold²¹; or modified, such as loading Nerve Growth Factor²².
 277 Therefore, in order to better stimulate bone ECM and provide suitable
 278 microenvironment for the regeneration of the hard tissue, we used a
 279 bovine-derived decellularized bone graft which composed of HA and
 280 collagen type I, which is similar to the composition of bone and the
 281 current commercial dental bone substitute.

282 As a biomaterial is implanted, not only osteoblasts are involved in the
 283 repair, but the interaction between the material and immune cells is the
 284 key in the healing of tissue. The adsorption of proteins is the beginning
 285 of the acute inflammatory phase of FBR, and the inflammation will
 286 gradually transit to tissue repair. Right after the surgery was performed,
 287 macrophages quickly identified the implant, and were recruited to
 288 biomaterial the forming specific immune microenvironment. Macrophage
 289 is involved in the starting of inflammation and the following tissue
 290 reconstruction with the secretion of cytokines. Macrophage phenotype
 291 transformation for tissue from M1 to M2 is necessary in both hard and
 292 soft tissue repair; long-term or severe inflammation usually contribute to
 293 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-1 β 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

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

316 Apart from absented M1 macrophage, we also found that EGCG
 317 made the difference to the surface morphology, shown under scanning
 318 electron microscope, which has close relationship with FBR. Researchers
 319 had speculated that the mechanism by which HA coating promotes
 320 osteogenesis may take place on the basis of its surface morphology rather
 321 than on stimulating macrophages to express BMP2, since HA could
 322 significantly up-regulate proinflammatory factors, TNF- α , by M1
 323 macrophages, further inhibited M2 macrophages from expressing BMP2
 324 that featured in osteoconductive ²⁷. In other words, HA has a
 325 proinflammatory tendency and is not conducive to the expression of
 326 BMP2, both of which may be improved by the addition of EGCG ^{6,9}.

327 However, whether EGCG can promote the polarization of macrophages
 328 toward M2 and stimulate the secretion of desired cytokines, as our
 329 previous studies on collagen membranes have shown, it needs further
 330 investigation.

331 Cytotoxicity is another essential aspect to evaluate biocompatibility.

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

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

348 **5 Conclusion:**

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

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

361 **Grant**

362 National Natural Science Foundation of China: No. 81870801

363 National Natural Science Foundation of China: No. 81902976

364 **Statement Conflict of Interest**

365 There are no conflicts of interest related to this manuscript

366 **Reference**

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484 **Figure 1. Schematic illustration.**

485 Diagram of in vivo experiments. (A) Showing the period to harvest and

486 the subsequently executed testing. (B) Cell viability, macrophage

487 polarization, and fibrosis outcome of EGCG modified bone graft,

488 compared with bone graft alone.

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492 **Figure 2. Representative image of HA and E-HA. (A) Surface**

493 morphology of materials is clearly presented under digital camera and

494 (B) scanning electron microscope.

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498 **Figure 3. The establishment and release of EGCG on the bone graft.**

499 **(A)** E-HA fabrication assessment by detecting the concentration of EGCG

500 solution before and after immersing HA. **(B)** The standard curve of

501 EGCG solution at the concentration between 7.8 and 500 $\mu g/ml$, **(C)**

502 obtained under OD value of 272. *In vitro* cumulative release profile of

503 E-HA.

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507 **Figure 4. Cell viability assay.** Culturing RAW 264.7 on deproteinized
508 bone material with or without EGCG treatment, then the cell viability was
509 detected on day 0, 1, 3, 5 and 7. NS= no significance; *= $P < 0.05$, **=
510 $P < 0.01$, ***= $p < 0.001$.

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.