# Carbodiimide Cross-Linked Nanocomposite Materials Designed for Bone Tissue Regeneration

**OANA CRACIUNESCU[1]*, LUCIA MOLDOVAN[1], CHRISTU TARDEI[2], GABRIELA SBARCEA[2]**

[1]National Institute of Research and Development for Biological Sciences, Department of Cellular and Molecular Biology, 296 Splaiul Independentei, 060031, Bucharest, Romania

[2]National Institute for Research and Development in Electrical Engineering, ICPE-CA, 313 Splaiul Unirii, 030138, Bucharest, Romania

*The paper presents the preparation of two new 3-D nanocomposite porous materials, based on collagen type I (COL) and β-tricalcium phosphate nanopowder (n-β-TCP) and the selection of the best method for their chemical cross-linking using 1-ethyl-3-(3-dimethyl aminopropyl)carbodiimide hydrochloride (EDC). To our knowledge, no EDC cross-linked COL/n-β-TCP composite materials for orthopedic use were reported. The physico-chemical and biological properties of the cross-linked composite materials were investigated by porosity, dissolution, enzymatic degradation and cytotoxicity analyses.*

*Key words: carbodiimide, nano-beta-tricalcium phosphate, collagen, cross-linking, biocompatibility*

Bone is a highly specialized connective tissue that provides internal support and confers marked rigidity, strength and elasticity. Its repair and regeneration can be enhanced through implantation of biocompatible and biodegradable polymeric substitutes with similar mechanical properties to bone. The bone matrix is a natural composite material which consists of calcium phosphate in the form of hydroxyapatite crystals, collagen (COL) (mainly type I), proteoglycans and small amounts of lipids and peptides [1]. Calcium hydroxyapatite (HA), ($Ca_{10}(PO_4)_6(OH)_2$) and β-tricalcium phosphate (β-TCP), ($Ca_3(PO_4)_2$) are the most commonly investigated ceramics for biomedical application [2]. Experimental and clinic investigations showed that they possess the ability to improve new bone formation, due to their osteoconductive properties [3]. Also, their crystal size should be similar to the nanometer size of the apatite from the natural bone, in order to increase the protein adsorption and the osteoblast adhesion [4]. Several studies have demonstrated a better conductivity, osteocompatibility and resorption rate for β-TCP than for HA [5]. COL type I, the major organic component of the bone matrix, has excellent biocompatible properties. It is easily degraded and resorbed by the body and allows a good attachment to bone cells, but its mechanical properties are relatively low in comparison to bone [6].

A series of COL-calcium phosphate composite matrices have been previously developed and used as temporary bone substitutes [7, 8]. The addition of COL to a ceramic material was shown to provide many advantages for medical applications: shape control, spatial adaptation and ability for clot formation [9]. Conversely, the addition of calcium phosphates to COL scaffolds is supposed to improve the osteoconductive properties of the material [10]. COL-based scaffolds conditioned as sponges, sheets or gels do not possess mechanical strength after hydration. Scaffold cross-linking could be used in order to control their degradation rate and biomechanical characteristics, but it might compromise their biocompatibility. The commonly used cross-linking agents, including glutaraldehyde, formaldehyde and epoxy compounds were showed to be cytotoxic owing to reactive moieties, covalently coupled between neighbour COL fibrils [11]. Recently, it were reported efficient cross-linking methods using biocompatible compounds, such as polyethylene glycol [12], proanthocyanidins [13], mTGase [14].

Another cross-linking agent that fulfill both requirements -efficiency and biocompatibility- is the heterobifunctional carbodiimide, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) that does not incorporate itself into the polymer macromolecules. EDC has been used to cross-link COL-based skin substitutes [15], vascular scaffolds [16] and gelatin-HA composites for bone repair [17]. To our knowledge, there are no reports of COL/β-TCP composite scaffolds cross-linked with EDC.

The aim of the present paper was to select the best method for the chemical cross-linking of 3-D porous nanocomposite materials, prepared from mixtures of COL and β-TCP nanopowder (n-β-TCP). For this, their physico-chemical and biological characteristics were investigated and discussed.

## Experimental part

*Preparation of β-TCP nanopowder and collagen*

β-TCP nanopowder was synthesized by chemical precipitation technique from salt solutions, in the ICPE-CA Ceramic lab. The reaction mixture was prepared by mixing stoichiometric proportions of high-purity $(NH_4)_2HPO_4$ and $Ca(NO_3)_2 \cdot 4\,H_2O$ (Merck), at 60 °C. The *p*H of the reaction solution was maintained constant at 8.5 by adding a diluted $NH_4OH$ solution (25%, w/w), with continuous stirring, for 1-3 h. This reaction ensures the formation of a precursor product, the apatitic tricalcium phosphate, $Ca_9(HPO_4)(PO_4)_5OH$. The product was then washed with distilled water and dried at 60°C, for 6 h, followed by calcination in air atmosphere, at 800 °C, for 2 h. COL type I was extracted in NIRDBS lab from bovine tendons by pepsin treatment, purified by precipitation at a salt concentration of 2.4 M NaCl and dialysed against distilled water. The COL solution was analyzed for the hydroxyproline and the hexosamine content and its molecular weight was determined [18].

*Preparation of composite materials*

A solution of COL type I (0.8 %, w/w) was mixed with n-β-TCP powder, in two different dry weight ratios of 1:1 (variant I) and 1:2 (variant II). The mixtures were freeze-

* *e-mail: oana_craciunescu2009@yahoo.com; Tel.: 0770333296*

dried using freezing temperatures of - 35°C and drying temperature of + 30°C. The obtained porous scaffolds were cut in small pieces (0.5 x 0.5 cm), sealed in plastic bags and exposed to UV-radiation, for 8 h, in a sterilization cabinet (Scie-Plas, UK).

*Cross-linking of composite materials*

Cross-linking of COL/n-β-TCP scaffolds (20 mg) was performed by incubation in 30 mM EDC (Sigma-Aldrich) ethanolic solution, with shaking, for 18 h or by treatment with EDC and N-hydroxysuccinimide (NHS), in a molar ratio of 5: 1, for 4 h [19]. Then, samples were washed in 0.1 M sodium phosphate, 1 M NaCl, 2 M NaCl, distilled water and, finally, lyophilized again. COL scaffolds were also cross-linked using both methods, in order to be used as controls.

*Cross-linking assessment*

The quantity of free amine groups in cross-linked materials was spectrophotometrically assayed using 2,4,6-trinitrobenzenesulfonic acid (TNBS) [20]. The degree of cross-linking was expressed as percentage loss in free amino groups after cross-linking and was calculated as follows:

$$\% \text{ Cross-linked} = 1 - \frac{ABSc/MASSc}{ABSnc/MASSnc} \quad (1)$$

where *ABS* is absorbance at 346 nm, *MASS* is sample weight, c is the cross-linked sample and *nc* is the non-cross-linked sample.

*Density and porosity measurement*.

The density (*d*) and porosity (ε) of COL and composite materials were measured by water displacement method [21]. Briefly, a sample with a known weight (*w*) was immersed in a graded test tube holding a known volume of water (*v1*). The sample was kept in water for 3 h and pressed to force air from the scaffold and allow the water to penetrate and fill the pores. The total volume of water plus the water-impregnated sponge was recorded as *v2*. The water-impregnated scaffold was removed from the test tube and the residual water volume was recorded as *v3*. The following equations were used:

$$d = w/(v2-v3) \quad (2)$$

and

$$\varepsilon = (v1- v3)/( v2- v3) \times 100\ \% \quad (3)$$

Three measurements were taken for each average value.

*In vitro calcium release*

Samples of material (5 mg) were incubated in phosphate buffer saline (PBS), *p*H 7.4, for 5 days. At each 24 h, 5 μL of supernatant were harvested after centrifugation and their calcium content was determined using the QuantiChrom Calcium Assay kit (BioAssay Systems, USA).

In vitro degradation test of the materials was performed in the presence of bacterial collagenase. A sample of each type of composite material (10 mg) was incubated in 5 mL of TES buffer, *p*H 7.4, containing 50 mM $CaCl_2$, at 37°C, for 30 min. Subsequently, 100 μL collagenase type IA solution (Sigma Chemical Co.) was added. After 24 h at 37°C, reaction was stopped by the addition of 0.2 mL 0.25 M EDTA and cooling on ice. After centrifugation at 3000 rpm for 10 min, 200 μL of supernatant were analyzed using the colorimetric ninhydrin method. The percentage of degradation was calculated from the degradation of samples in buffer without collagenase. Experiments were performed in triplicate.

*Fibroblast cell culture*

A cell culture line of human fibroblasts (MRC-5) purchased from ECACC was used in this experiment. Cells ($1.2 \times 10^4$ cells/well) were grown in Dulbecco's modified Eagle medium (DMEM) containing 15 % (v/v) foetal bovine serum and antibiotics on 24-well culture plates. After 24 h, scaffolds (Φ5 mm) were added onto the wells and cells were grown in their presence. Plates were incubated for 48h and the mitochondrial dehydrogenase activity of cultured cells was assayed by MTT test [22]. Experiments were performed in triplicate and a control (cells cultivated in the presence of COL scaffold) was considered as 100 % viable cells.

*Statistics*

Data were expressed as mean values ± standard deviation of the mean (S.D.). Statistical analysis was performed using Student's test. Differences were considered significant at $p<0.05$.

**Results and discussion**

*Characterization of n- β-TCP and COL*

It is well known that the phase purity of β-TCP is an important factor for successful bone regeneration. The synthesis of pure β-TCP powders can be performed using either high-temperature solid state reactions or wet precipitation methods. A chemical precipitation method at low temperature is recommended in order to obtain nanosized β-TCP powders [23]. Nanosized β-TCP has an advantage over micro sized powders, leading to increased mechanical properties and sinterability; in addition, its degradability can be regulated [24]. However, it is important to know and to control the phase evolution during this process in order to obtain the β–TCP compound. The precipitation process has a high number of variables that influence chemical equilibrium and physical nature of precipitates: stoichiometry, temperature, stirring time, *p*H and thermal treatment.

In this study, intermediary precursors were obtained using the chemical precipitation method; characteristic peaks for calcium-deficient apatite -$Ca_9(HPO_4)(PO_4)_5OH$- were observed, in accordance with ASTM 46-0905 files (fig. 1a). X-ray diffraction analysis of samples calcined at 800°C indicated the transformation of apatitic tricalcium phosphate to β-TCP, as unique phase. Differential thermal analysis (DTA) data indicated an endothermic peak at 790°C, corresponding to the transformation of precursor compound to β-TCP (fig. 1b), in correlation with X-ray diffraction measurements.

The unit cell parameters and average crystallite size values for two sorts of β-TCP powder are calculated from XRD data using Sherrer formula and are listed in table 1. The microstructure of sintered ceramic and its crystallite morphology was observed by scanning electron microscopy (SEM). The micrographs showed the presence of clusters made of fine particles (fig. 2). Their chemical composition and physico-chemical characteristics were analyzed by XRD and thermal analysis (TG/DTG/DTA) [25].

The atelocollagen solution obtained by pepsin-treatment of mature bovine tendons consisted of approx. 97% non-immunogenic COL type I. It had a content of 11.2 % hydroxyproline and 0.8% hexosamine. Its molecular weight of 320 kDa is comparable to that of tropocollagen (300 kDa) indicating that the enzymatic extraction, used in this

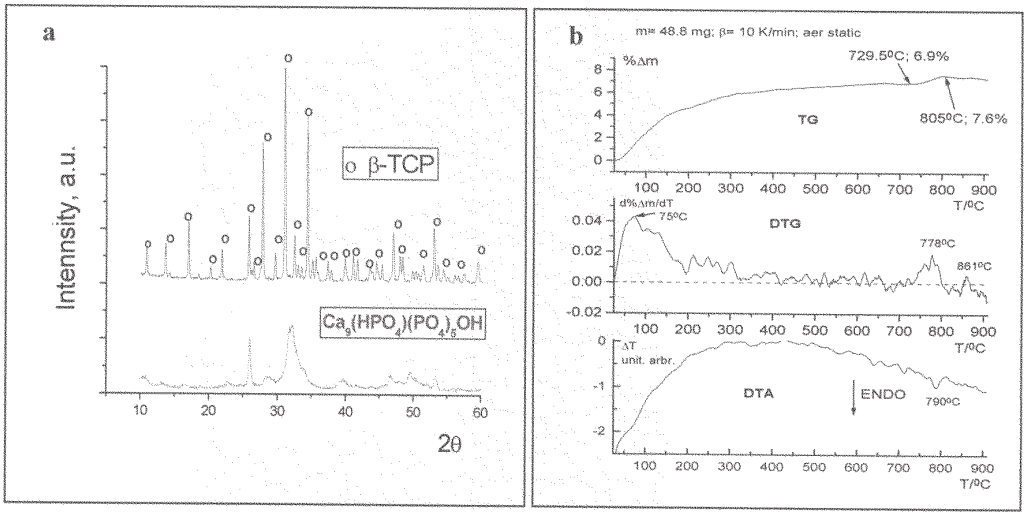


Fig. 1. (a) XRD pattern of β-TCP precursor (apatitic tricalcium phosphate) and β-TCP after calcination of the precursor at 800°C for 3h; (b) The curves of TG/DTG/DTA indicating the formation of β-TCP compound

| Compound | a (Å) | c (Å) | V(Å) | D(nm) |
|---|---|---|---|---|
| $Ca_3(PO_4)_2$ *cf. ASTM 09-169* | *10.429* | *37.380* | *3,476.08* | |
| Conventional β-TCP* | 10.436 | 37.379 | 3,525.55 | ≤ 5μm |
| Low temperature β-TCP | 10.419 | 37.307 | 3,483.55 | ≤ 84nm |

**Table 1**
THE UNIT CELL PARAMETERS AND CRYSTALLITE AVERAGE SIZE VALUES FOR β-TCP COMPOUNDS

* *β-TCP obtained in INCDIE ICPE-CA laboratory, by solid-state sintering reaction*

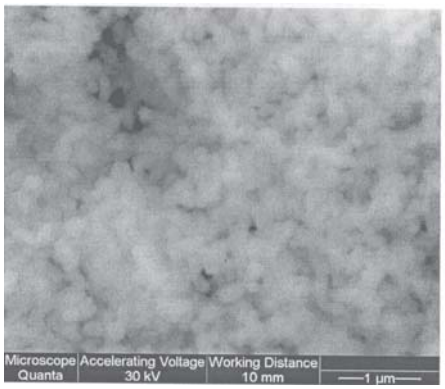


Fig. 2. SEM micrograph of n-β-TCP powder prepared by a precipitation technique

study, is a non-denaturing method, which preserves the native triple helix structure of COL.

*Characterization of the composite materials*

In order to prepare two variants of biomimetic and biodegradable composite materials, a nano-sized tricalcium phosphate powder (n-β-TCP) and the natural polymer COL type I were mixed in dry weight ratios of 1:1 and 1:2, respectively. Composite variants were then cross-linked by chemical treatment with 30 mM EDC or EDC/NHS in a molar ratio of 5:1. It was shown that, adding NHS to EDC reaction, a more efficient cross-linking is recorded through minimizing any intermediate reaction compounds [19]. Also, using ethanol as solvent in the cross-linking process is important in prevention of morphological changes in COL porous structure. Ethanol has a lower dielectric constant of 35, compared with 81 for water, which reduces dipolar forces and allows bond changes; the salt bridges are coverted to hydrogen bonds when immersed in alcohol solutions which may be advantageous in maintaining COL biomaterial structure [20].

The values calculated after physico-chemical evaluation of the COL/n-β-TCP scaffold variants are shown in table 2. The porosity varied between 70-97 % depending on the quantity of n-β-TCP and the cross-linking degree. The porosity of non-cross-linked materials was higher than that of cross-linked materials. These values may be explained by sample rehydration that occur during the cross-linking treatment and the additional freeze-drying process that can induce a slightly collapse of scaffold pores [26]. When comparing composites with the same cross-linking type, the porosity decreased with increasing n-β-TCP quantity, so that variant I had higher porosity than variant II. Differences were not significant ($p>0.05$). The small variation in porosity values for composites with increasing quantities of n-β-TCP could prove a tightly bonding of ceramic nanoparticles to COL fibers. For the same variant of COL/n-β-TCP composite, the porosity was higher for EDC cross-linked scaffolds than for EDC/NHS cross-linked ones. Still, the porosity values are high enough (> 70 %) to allow a good infiltration of cells. In turn, their density varied in an inverse proportion to porosity.

The cross-linking process results in a decreased content of free amine groups, relative to non-treated scaffolds. Cross-linking efficiency was highest for EDC/NHS method, in all scaffold variants. COL scaffold was cross-linked to a higher extent than COL/n-β-TCP scaffolds, because more amine groups were present. Also, increasing n-β-TCP quantity resulted in an increase in the amount of free amino groups. The cross-linking degree was smallest for COL/n-β-TCP variant II scaffold.

*Dissolution behavior*

*In vivo* dissolution of ceramic particles takes place by a decrease in crystal size and increase in macroporosity and microporosity [27]. When ceramics are soaked in buffer solution, a dissolution reaction takes place which leads to increasing calcium and phosphate ion concentrations in

| Sample | Cross-linking agent | Porosity (%) | Density (g/cm$^3$) | Cross-linking degree (%) |
|---|---|---|---|---|
| COL | Non-cross-linked | 97.60 ± 8.40 | 1.72 ± 0.14 | 0 |
| | EDC | 88.42 ± 2.54 | 2.55 ± 0.06 | 56.83 ± 5.27 |
| | EDC/NHS | 80.85 ± 5.28 | 2.95 ± 0.15 | 58.92 ± 6.39 |
| COL: n-β-TCP 1:1 (variant I) | Non-cross-linked | 94.83 ± 9.63 | 2.01 ± 0.19 | 0 |
| | EDC | 83.76 ± 4.23 | 2.79 ± 0.12 | 25.64 ± 4.46 |
| | EDC/NHS | 78.25 ± 8.15 | 3.34 ± 0.22 | 28.01 ± 2.98 |
| COL: n-β-TCP 1:2 (variant II) | Non-cross-linked | 91.37 ± 3.41 | 2.38 ± 0.08 | 0 |
| | EDC | 79.85 ± 8.10 | 3.18 ± 0.25 | 20.76 ± 3.85 |
| | EDC/NHS | 70.18 ± 10.24 | 3.98 ± 0.31 | 24.34 ± 4.14 |

**Table 2**
PHYSICO-CHEMICAL CHARACTERISTICS OF THE CROSS-LINKED COMPOSITES

the solution. A decrease in calcium concentration is registered when the reprecipitation reaction occurs.

In this study, the dissolution of ceramic nanoparticles attached to COL fibrils in composite materials, was analyzed by assessment of calcium release in solution, in physiological conditions, over a 5 days period. The dynamic of the dissolution behavior of n-β-TCP from non-cross-linked and cross-linked material variants is shown in figure 3. The calcium quantity released from the non-cross-linked materials was higher than from the cross-linked ones after 5 days. The cross-linked scaffolds had a similar pattern for calcium release regardless of the cross-linking method, having a maximum value after 24 h of soaking. The value was smaller than that for non-cross-linked scaffolds at 48 h. COL/n-β-TCP 1:2 material (variant II) released a higher quantity of calcium than variant I. It was concluded that the cross-linking process is beneficial for COL/n-β-TCP materials because the calcium release is more controlled in comparison to the non-treated composite matrices.

*Sensitivity to collagenase degradation*

Bone regeneration takes place over a duration of several months. That is why it is important for scaffolds to degrade in a controlled fashion. Figure 4 compares the biodegradation degree of pure COL scaffold and COL/n-β-TCP composite variants I and II before and after the cross-linking treatment. The non-cross-linked materials had been thoroughly degraded after incubation in collagenase solution for only 2 h. The addition of n-β-TCP had no effect on COL scaffold biostability. After cross-linking treatment, the biostability of the matrices was enhanced according to the cross-linking degree and their composition. The EDC-cross-linked materials were only 42-60 % degraded in 24 h and the EDC/NHS-cross-linked materials had a better ability to resist collagenase degradation (max. 53 %) due to their larger cross-linking degree. Also, variant I was more stable in the presence of collagenase than variant II. These results reveal that both cross-linking methods can improve COL/n-β-TCP material biostability, but EDC/NHS treatment is faster and more efficient.

*Biocompatibility of the composite materials*

Cell adhesion and proliferation are important for a polymeric bone substitute in order to support and guide tissue regeneration. *In vitro* measuring of mitochondrial dehydrogenase activity by MTT test gives information on cell viability and proliferation in the presence of composite

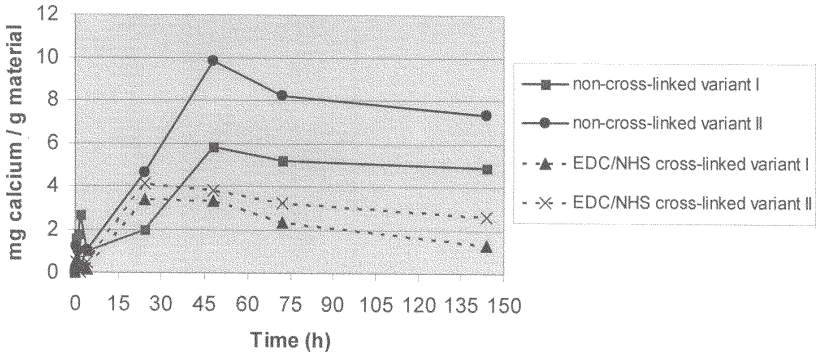


Fig. 3. Variation of calcium concentration assessed in 0.1 M PBS, *p*H 7.4

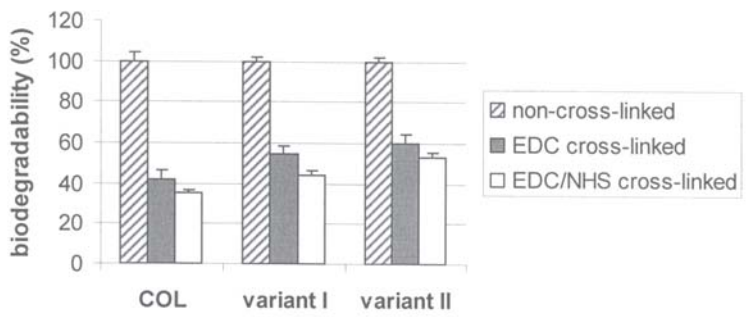


Fig. 4. The degree of biodegradation for pure COL material and COL/n-β-TCP composite variants, non-cross-linked and cross-linked, after incubation in 20 μg/mL collagenase, for 24 h. Values are mean ± S.D. (n=3). (variant I – COL:n-β-TCP 1:1; variant II – COL:n-β-TCP 1:2)

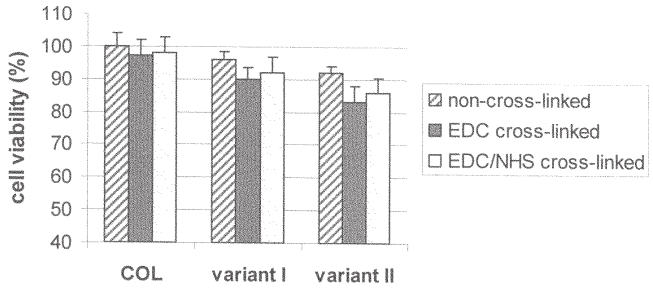


Fig. 5. Cell viability diagram for human fibroblasts cultivated in the presence of COL/n-β-TCP composite variants. Measurements represent mean ± S.D. (n=3) for control (cells cultivated with non-cross-linked COL scaffold, 100 %) and samples (cells cultivated with cross-linked scaffolds). (variant I – COL:n-β-TCP 1:1; variant II – COL:n-β-TCP 1:2)

materials [28]. When cross-linked samples were added in the culture medium of human fibroblasts, cell viability was higher for variant I than for variant II (fig. 5). For the same variant, EDC/NHS cross-linked scaffolds induced a slightly higher proliferation than the EDC scaffolds. Also, cell viability for cross-linked scaffolds was similar to that of non-cross-linked ones.

These results showed that all variants were biocompatible. The biocompatibility of the non-cross-linked composite scaffolds was diminished with increasing n-β-TCP quantity. After cross-linking, cell viability values decreased in the same manner, but were higher than 80 %.

## Conclusions

Two new bone-mimetic materials were designed as 3-D scaffolds by integrating n-β-TCP powder in a solution of type I COL. These composites had a porosity higher than 70%. We reported a fast and efficient chemical cross-linking method using EDC/NHS. Cross-linked materials were 50 % more stable in the presence of collagenase and released calcium ions in a controlled manner. Their biocompatibility was good in the presence of fibroblasts from a cell culture line, after 48 h of cultivation. These properties are essential for composite materials with applications in bone tissue engineering, used as scaffolds for cell infiltration and tissue regeneration.

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