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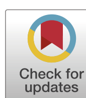
3D TECA hydrogel reduces cellular senescence and enhances fibroblasts migration in wound healing ☆



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ABSTRACT

This study was designed to investigate the effect of 3D TECA hydrogel on the inflammatory-induced senescence marker, and to assess the influence of the gel on the periodontal ligament fibroblasts (PDLFs) migration in wound healing *in vitro*. PDLFs were cultured with 20 ng/ml TNF-α to induce inflammation in the presence and absence of 50 µM 3D TECA gel for 14 d. The gel effect on the senescence maker secretory associated-β-galactosidase (SA-β-gal) activity was measured by a histochemical staining. Chromatin condensation and DNA synthesis of the cells were assessed by 4′,6-diamidino-2-phenylindole and 5-ethynyl-2′-deoxyuridine fluorescent staining respectively. For evaluating fibroblasts migration, scratch wound healing assay and Pro-Plus Imaging software were used. The activity of senescence marker, SA-β-gal, was positive in the samples with TNF-α-induced inflammation. SA-β-gal percentage is suppressed (>65%, $P < 0.05$) in the treated cells with TECA gel as compared to the non-treated cells. Chromatin foci were obvious in the non-treated samples. DNA synthesis was markedly recognized by the fluorescent staining in the treated compared to non-treated cultures. Scratch wound test indicated that the cells migration rate was significantly higher (14.9 µm^2/h, $P < 0.05$) in the treated versus (11 µm^2/h) for control PDLFs. The new formula of 3D TECA suppresses the inflammatory-mediated cellular senescence and enhanced fibroblasts proliferation and migration. Therefore, 3D TECA may be used as an adjunct to accelerate repair and healing of periodontal tissues.

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1. Introduction

Periodontal disease is an inflammatory condition which results in the destruction of tooth supporting tissues (alveolar bone, periodontal ligament and cementum) and triggered by the bacterial infection from dental biofilm [1]. Chronic periodontitis affects the quality of life negatively as it may eventually lead to the loss of teeth involved [2,3]. Treatment of the affected teeth requires not only combating the bacterial infection, but also restoring the lost structure supporting the tooth.

Cellular senescence is a state of irreversible cell growth arrest (irreversible cell cycle arrest) which occurs due to aging [4] or due to other stimuli such as ionizing radiation, chemical exposure, or bacterial infection and inflammation [5–8]. Inflammation can be provoked by a variety of inducers such as tumor necrosis factor-α (TNFα), interleukin-1β, IL-6, reactive oxygen species, ultraviolet irradiation or bacterial lipopolysaccharides [9–11].

The typical senescence phenotype consists of an enlarged or swollen cell, and an increases in the activity of senescence biomarker called "senescence-associated β galactosidase" (SA-β-gal). In the senescent cells, the accumulation of membrane-impermeant proteins increases the osmotic pressure within the cell [12]. Senescent cells are unable to release their proteins to be part of the connective stroma. Protein accumulation in a cell creates inflows of water and/or ions across the water-permeable plasma membrane, and results in cell distention and swelling [13–16].

During senescence, the cells are not able to proliferate so they release mediators that escalate and spread the inflammation. A specific feature of senescent cells is condensed heterochromatic regions, known as senescence-associated heterochromatic foci (SAHF) [17,18]. SAHF plays a role in limiting proliferation-promoting genes 19]. Disturbance of SAHF formation causes mutagenic changes 20], which assumes that SAHF provides a barrier to malignant transformation and contributes to the tumor suppressive function of senescence. SAHF may also prolong the viability of the senescent cells by limiting the DNA damage signaling 21]. However, during chronic inflammation, apoptosis takes place due to the extensive DNA damage [8].

Senescent cells undergo apoptosis and then clearance by phagocytosis [22–24]. In a case of chronic periodontal infection, it is evident that the inflamed senescent supporting structure cells are cleared and definite replacement of the lost structure is not materialized. This is very obviously manifested by the gingival recession, pocketing, and loss of attachment [25,26].

Migration of the healing cells to the wound or inflamed site is crucial to achieve accelerated healing with minimal loss or changes in the tissues architecture [27,28]. During periodontal healing, the proliferation and remodeling stages are essential phases in proper wound repair and regeneration. Periodontal ligament fibroblasts play an important role in these phases. PDLFs are recruited to damaged site to reinstitute tissue repair and matrix remodeling [29–31].

2D gel environment is not the real representation of the physiological cell environment as cell movement; cell signaling and the exposure to the extracellular matrix (ECM) are limited in 2D environment [32]. 3D gel environment is therefore, considered to be a more reflective picture of the morphological and physiological characters of the *in vivo* environment that facilitate cell–cell and cell–ECM interactions which ultimately influence gene expression and cellular behavior [33,34]. Growth of cells in 3D is steadier than that in 2D environment which require the cells to undergo trypsinization in order to provide sufficient nutrients for normal cell growth [35,36]. *In vitro* 3D system can provide analogous ECM properties and simulate the mechanical environment of the *in vivo* cell microenvironment. 3D scaffolds can be made from natural or synthetic materials and can be engineered to allow cells to migrate and grow within the network of the scaffold [37–39]. A hydrogel is a biologically compatible (biocompatible) polymer network with high water content and physical properties that closely mimic the natural extracellular matrix (ECM) and the living cells can be cultured either in or on a hydrogel [38]. Biodegradable polymers are mainly used where the transient existence of materials is required and they find applications as sutures, scaffolds for tissue regeneration, tissue adhesives, hemostats, and transient barriers for tissue adhesion, as well as drug delivery systems [39].

Centella asiatica herb has been used for hundreds of years as a traditional medicine of many Asian countries for healing of skin cuts and ulcers. These properties are ascribed to the active ingredients, asiatic acid, asiaticoside, and madecassic acid. In a previous study, titrated extract of *C. asiatica* (TECA) was used to improve connective tissue formation, epithelization, and angiogenesis when applied locally on wounds [40].

Although many adjunctive remedies are currently available for the non-surgical or surgical treatment of periodontal disease, almost all of them are made by a complicated manufacturing process, require training and skill for handling, and are mostly not cost-effective [41]. Although several gel preparations are used for gum treatment, the statistics for these conventional gum treatments is entirely disappointing [42–45]. In this study, the effect of 3D TECA hydrogel on the cellular senescence of the periodontal fibroblasts has been tested by assessing the senescence marker. The effect of gel on the cells migration during healing was evaluated by *in vitro* scratch wound healing assay.

2. Materials and methods

2.1. 3D gel preparation

In this study, silanized hydroxyl propyl methyl cellulose gel (Si-HPMC) was prepared. The natural water-soluble cellulose ethers HPMC 2%–4% w/w (Methocels E4M, Colorcon, Kent, UK) was grafted with 3-glycidoxypropyltrimethoxysilane (3-GPTMS) (Sigma-Aldrich, Germany). Then, the silanized Si-HPMC was dissolved in NaOH (pH 12.9). The basic Si-HPMC solution was made more viscous by adding 0.26M HEPES, buffer (Sigma-Aldrich, Germany) and 10% (v/v) of culture medium (Fibroblast medium, Sigma-Aldrich, Germany) supplemented with 10% FCS, 1% penicillin/streptomycin and 1% L-glutamine. The pH of hydrogel was adjusted with a Mettler Toledo pH meter (Mettler Toledo, Greifensee, Switzerland), and ranged from 7 to 8 [46]. The final composition of the hydrogel includes

50 µM titrated extract of *C. asiatica* (TECA). The percentages of the bioactive components of TECA were previously tested and customized for enhancing the proliferation of PDLFs in our previous study (unpublished data).

2.2. Cell culture

Human periodontal ligament fibroblast (HPDLF) was obtained from the ScienCell Research Laboratories, (Carlsbad, CA, USA). HPDLF cells were cultured in a fibroblast medium containing antibiotics (10% Fetal bovine serum (FBS), 1% of penicillin/streptomycin and 1% of Gentamycin). The Fetal bovine serum was deactivated by immersing the container in water bath at 50 °C for 5 min. The cell culture was incubated in 5% CO_2 at 37 °C and 95% air and the medium was changed every three days. Seven days later, each confluent culture dish was trypsinized and split onto 5 new 10 cm-dishes in 10% DMEM. The growing cells were kept in vials with 10% dimethyl sulfoxide/20% DMEM and stored in a liquid nitrogen tank. The cell cultures were examined by using phase contrast microscopy (Olympus, Tokyo, Japan).

Cell cultures after 25 passages were also prepared to check the naturally expressed senescence marker in aged cells. Three groups of samples were made and analyzed; two of them were treated with TNF-α. The third group of samples used was serially sub-cultured cells or naturally senescent cells.

2.3. Senescence-associated β-galactosidase (SA-β-gal) activity

PDLFs were cultured with 20 ng/ml TNF-α [47,48] for 14 days in a 3D gel medium in the presence or absence of 50 µM TECA. The SA-β-gal-positive cell ratios were determined using a senescence detection kit, according to manufacturer's protocol (BioVision, CA, USA). In brief, sparse cultures at a density of 4×10^3 cells were washed once with 1 ml of 1X PBS. Then, the cells were fixed with the kit Fixative Solution for 10 min at room temperature. Staining of the samples was done by adding 470 µl of kit Staining Solution, 5 µl of Staining Supplement 25 µl of 20 mg/ml X-gal in DMF for each plate well. The samples were washed twice with 1 ml of 1X PBS. Then, another 0.5 ml of the kit Staining Solution Mix was added to each well. The plates were incubated overnight at 37 °C. Observation of the cells was done under a phase contrast microscope for development of blue color (200× total magnification).

2.4. DNA replication assay, EdU and DAPI analysis

Analysis of DNA replication or cell proliferation was achieved by cells treatment with 10 mM 5-ethynyl-2′-deoxyuridine (EdU) (ThermoFisher) followed by incubation of the samples for 24 h, then fixation with 3.7% formaldehyde in 0.1 M PBS for 10 min. Permeabilization was done by a 0.5% Triton® X-100 for 10 min. All steps of the assay were performed in the dark. The Click-iT® reaction cocktail was prepared following the protocol from Click-iT EdU Alexa Fluor 488 Imaging Kit (ThermoFisher). 0.5 ml of Click-iT® reaction cocktail was added to each well containing a coverslip followed by plate incubation for 30 min at room temperature. The reaction cocktail was then removed and each well was washed with 3% BSA in PBS for 2 min. Before microscopic observation, samples were washed with ddH_2O and stained with 2.5 µg/ml 4′,6-diamidino-2-phenylindole (DAPI) (Sigma-Aldrich, MO, USA) in PBS for 20 min. Afterwards, the samples were carefully placed face down on glass slides and mounted with Vectashield antifade (Vector Laboratories, CA, USA). The samples were observed on a Nikon Eclipse 80i fluorescence microscope.

2.5. Cell migration/scratch wound healing assay

6-well Costar® culture plates (Corning Inc., NY, USA) were layered with 2 ml of the 3D hydrogel with or without TECA. Each well was seeded with 500,000 cells in 10% FCS/DMEM and placed at 37 °C and 5% CO_2 incubator for 48 h. Wounds were induced by scratching the samples with a sterile pipette tip to leave a scratch of approximately 1 mm width. Suspended cells were removed by aspiration. Digital images were captured with an inverted microscope (Nikon Eclipse Ti-U, Nikon, Japan) and QImaging Retiga 2000R CCD digital camera (QImaging, BC, Canada). The selected fields were captured every 2 min during 24 h using phase microscopy. The distance between the selected cells and wound edge was measured. Migration rate was calculated as cell migration distance/time (µm^2/h). The digitized images were analyzed using Image-J software and Pro-Plus Imaging software [49,50].

2.6. Statistical analysis

Data presented in this study are means ± standard error of the mean. Kruskal–Wallis test was run followed by a pairwise Wilcoxon rank sum test. For scratch wound assay, statistical significance was obtained by Student's t-test. Variances with a value of $P < 0.05$ were considered significant.

3. Results and discussion

3.1. Detection of senescence-associated β-galactosidase (SA-β-gal) activity

Cellular senescence was confirmed by measurements of SA-β-gal positive cells. The activities of SA-ß-gal activity, chromatin condensation (DAPI), and detecting DNA proliferation marker were analyzed 14 days after treatment with TECA gel (Fig. 1). Late passage cell samples were used as a control.

Detection of biomarker SA-β-gal activity permits the identification of senescent cells in culture and mammalian tissues [51–54]. In this study, senescence marker SA-β-gal percentage (Fig. 1; A1 & B1) is suppressed (>65%, $P < 0.05$) in the treated cells (˜32% positive cells) as compared to the non-treated cells (˜81% positive cells) (Fig. 2). The histochemical analysis has showed that inflammatory senescence was induced in the fibroblasts cultured with TNF-α only. Detection of senescence was manifested by the blue staining of the enlarged senescent cells in the non-treated samples compared to the test samples treated with TECA. The samples which consisted of late passages cells were also exhibited with senescence marker stain. Treatment of the cells with TECA encountered the TNF-α

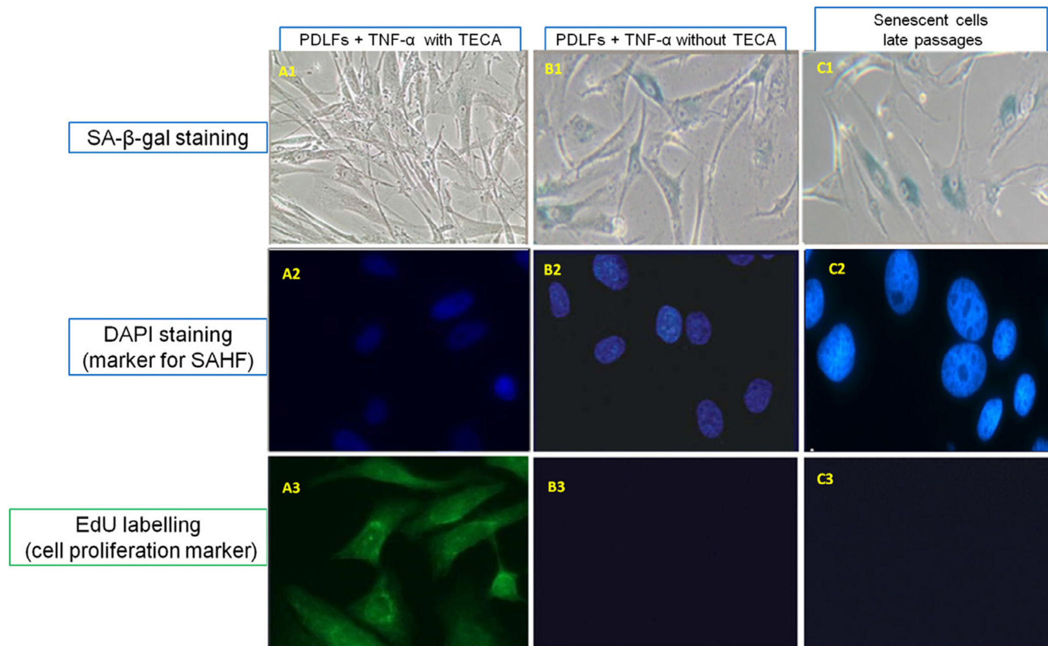

Fig. 1 – Senescence marker histochemical blue staining (A1, B1 & C1), fluorescent staining by DAPI for SAHF (A2, B2 & C2) and by EdU for DNA synthesis (A3, B3 & C3).

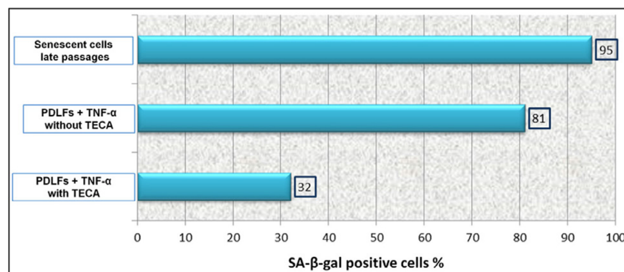

Fig. 2 – Senescence marker SA-β-gal percentages.

mediated inflammation and subsequently limited the senescence process in these test samples (Fig. 1; A1). This result is consistent with previous studies that revealed blockade of TNF-α would be beneficial in suppressing inflammation and senescence [55–57]. The results also indicate that TECA hydrogel was having a remarkable action in sustaining the cell vitality and protecting the cells from senescence. Cellular senescence is characterized by permanent cell cycle arrest [58–60]. Senescent cells secrete bioactive peptides [61,62], generate and release ROS [60,63] which provoke further inflammatory reaction within the affected tissues [64]. Senescent cells generate DNA damage response and induce a bystander effect, spreading senescence toward their neighbors *in vitro* and, possibly, *in vivo* [65,66]. Thus, senescent cells may contribute to loss of tissue homeostasis.

Previous studies on osteogenic activity of fibroblast reported that osteocalcin production is impaired in senescent periodontal ligament fibroblasts which exhibit short telomere and loss of the production potential of bone-associated proteins during periodontal disease [67]. The presence of the senescent cells in the tissues, by itself, has a damaging effect as senescent cells release pro-inflammatory cytokines which, in turn, provoke the inflammation and increase the intensity of destruction and apoptosis [68–77]. By suppression of inflammatory senescence, the new formulated TECA hydrogel may be able to maintain tissues homeostasis, reduce the DNA damage and limit the periodontal tissue loss.

3.2. *Detection of SAHF*

SAHF were observed by the blue fluorescence DAPI stain. The heterochromatic condensations were evident in the non-treated samples (Fig. 1; B2) compared to the test samples. The non-treated cells presented with SAHF (similar to that in aging cells sample) are probably undergoing cell growth arrest and no more proliferation as SAHF plays a role in sequestering proliferation-promoting genes during inflammation [18,19,78]. Condensation of chromatin was not materialized in the treated samples with TECA because these samples were almost free of inflammatory senescence (Fig. 1; A2). The late passage cells were clearly showing the chromatin foci as one of the features of senescence (Fig. 1; C2) [79]. The results are consistent with previous study [72] which concluded that pro-inflammatory cytokines released in chronic inflammation conditions are similar to that released due to aging. Indeed, heterochromatin foci (which are formed due to inflammation) are associated with repression of E2F target genes and have been shown to be a hallmark of senescent cells, thus, these foci were thought to harbor epigenetic modifications that govern maintenance of senescence [19,68,80].

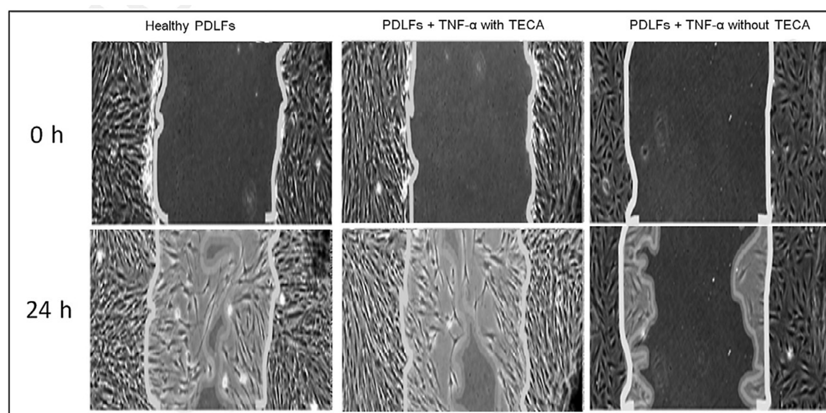

Fig. 3 – Cell migration assay at 0 h and after 24 h, closure of the scratch was remarkable in the TECA treated samples. Normally cultured fibroblasts were used as a control.

3.3. *DNA replication assay (EdU)*

Proliferation assay was performed by EdU (5-ethynyl-2′-deoxyuridine) fluorescence staining. EdU provided in the kit is a nucleoside analog of thymidine and is incorporated into cell DNA during active DNA synthesis [81]. The EdU contains the alkyne which can be reacted with an azide-containing detection reagent, to form a stable, triazole ring. In our study, the treated samples were shown to be proliferating and actively synthesizing their DNA (Fig. 1; A3). However, the non-treated as well as the late passage samples (Fig. 1; B3 & C3) showed no signs of clear green fluorescence stain, probably because these samples were not proliferating, nor replicating their DNA and undergoing irreversible growth arrest [82,83].

These results indicated that the cells treated with TECA gel were able to survive the inflammation and produced a new DNA (or their DNA is replicating) which was stained by the fluorescent stain EdU. Previous studies found that collagen synthesis was suppressed in fibroblast during chronic inflammation [84,85]. In our study, TECA hydrogel protected the cells which were consequently proliferating and positive to the DNA synthesis marker EdU.

Regarding TECA, it elicits a strong and consistent change in the levels of gene expression in fibroblast culture [86,87], induces cell-cycle progression and collagen type-1 synthesis in human dermal fibroblasts [88,89]. Of 1053 genes analyzed, 82 were found to have statistically recognizable changes in expression [86]; considering the direction of this change along with the role of these genes play in cellular function provides an insight into the biological activity of TECA.

Non-TECA hydrogel treated samples and also samples of the late passages senescent cells (control) showed very low percentages of cells positive for EdU. DNA synthesis is an indication of cell growth or cell cycle progression and proliferation [90–93] where DNA must be duplicated precisely once before cell division occurs [94,95].

3.4. *Cell migration/scratch wound healing assay*

The effect of TECA hydrogel on the cell migration during inflammatory senescence was assessed by scratch wound healing assay (Fig. 3). The *in vitro* scratch assay is an easy, low-cost and well-developed method to measure cell migration in cell culture [96]. The basic steps involve creating a “scratch” in a cell monolayer [37,97,98], capturing the images at the beginning and at regular intervals during cell migration to close the scratch, and comparing the images to quantify the migration rate of the cells [37,99,100]. Stimulated by the availability of empty space (scratch), the cells at the edges of the newly created gap proliferate and move toward the center of the scratch area [101,102].

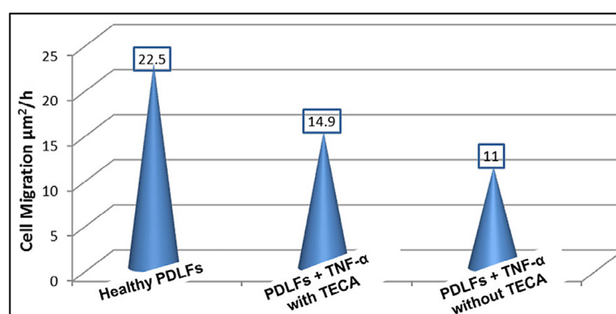

Fig. 4 – Cell migration rates. Non-treated cells migration was remarkably lower than the normal healthy samples.

In this study, scratch wound assay showed that the cells migration rate (Fig. 3 & 4) was higher (14.9 µm^2/h, $P < 0.05$) in the TECA treated samples versus (11 µm^2/h) for non-treated PDLFs samples. Healthy PDLFs without TNF-α or TECA treatment were used as a control.

Application of the 3D TECA gel was significantly enhancing the migration of cells to the scratch field. The explanation for the higher migration rate in the treated sample would be due to the suppression of senescence activity and limiting the condensation of chromatin foci. As the treated cells samples were almost free of inflammatory senescence and SAHF, they were able to move faster than the enlarged senescent cells [103–105]. The reduced ability to migrate may be related to alterations in the cytoskeleton during cellular senescence [106]. Cellular migration requires actin which is an important

component of the cytoskeleton. However, in senescent fibroblasts, actin is down-regulated and instead, vimentin is produced [106]. This migration discrepancy has negative implications during wound healing since cells are stimulated to migrate into the wound, proliferate and build a new extracellular matrix. Senescent cells secrete pro-inflammatory cytokines, chemokines, and proteases which provoke the inflammation and degrade the matrix [107]; hence, wound repair would be further impaired [108].

4. Conclusion

In the current study, inflammatory senescence was induced in PDLF cultured with TNF-α. Elimination of the senescence was possible with the use of TECA gel as samples treated with TECA gel showed a reduced percentage of cells with senescence-associated-β-galactosidase (SA-β-gal) staining and suppressed SAHF formation.

Senescence (irreversible cell growth arrest) of periodontal cells occurs during chronic inflammation of these cells which means that aging factor is not required for the periodontal fibroblast to be senescent cells. The formation of senescent cells during chronic periodontitis would not be beneficial as the apoptotic senescent cells (as in the case of periodontitis) are not replaced by new cells; consequently, this leads to a very evident loss of tooth supporting tissues. As the formula of 3D TECA has suppressed the inflammatory mediated cellular senescence, preserved cell proliferation and enhanced the migration of fibroblasts, it may be used as an adjunct to accelerate tissues repair and healing of periodontal tissues.

Conflicts of interest

The authors declare that there are no conflicts of interest.

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