Studia Universitatis "Vasile Goldiş", Seria Ştiinţele Vieţii
Vol. 26 Issue 2, 2016, pp.243--249

RIBOFLAVIN CROSS-LINKING OF COLLAGEN POROUS SCAFFOLDS FOR PERIODONTAL REGENERATION

Ana-Maria Seciu[1,2], Alexandra Gaspar[1], Laura Mihaela Stefan[1], Lucia Moldovan[1], Oana Craciunescu[1]*, Otilia Zarnescu[2]
[1]National Institute of R&D for Biological Sciences, Bucharest, Romania
[2]Faculty of Biology, University of Bucharest, Romania

ABSTRACT. Periodontitis treatment using occlusive membranes presented variable efficacy due to their rapid degradation in the complex biologic environment of the damaged periodontium. The aim of this study was to prepare novel composites based on collagen, chondroitin sulfate and fibronectin, and to establish the optimal parameters for their photochemical cross-linking using riboflavin and UV exposure. The degree of cross-linking, biodegradability and density of all scaffold variants were investigated. Their cytotoxicity was evaluated in a culture of gingival fibroblasts by MTT assay and light microscopy. The results indicated that the higher the cross-linking degree, the lower was the scaffold biodegradation. Cell culture studies showed that composite scaffolds were favorable for cellular survival. In conclusion, the cross-linking method using riboflavin and UV exposure resulted in stable and biocompatible collagen-based composite scaffolds that could be used for periodontitis treatment.

Keywords: periodontitis, cross-linking, cytotoxicity, collagen, riboflavin, scaffold.

INTRODUCTION:

Recent data show that 40% of worldwide active adult population suffers from moderate or severe periodontitis and there is a need for improving patient treatment results (Ferraiolo, 2016). In addition, untreated periodontal disease can lead to systemic diseases, such as rheumatoid arthritis, chronic bronchitis and pulmonary fibrosis (Kaur *et al*., 2013). It was reported that the presence of chronic inflammation in association with plaque has led to the degradation of extracellular matrix (ECM) constituents, increasing the concentration of soluble fragments of glycosaminoglycans, C-terminal telopeptides of type I collagen (COL) molecule and the 40 kDa pro-apoptotic fragment of fibronectin (FN) (Jee *et al*., 2004; Giannobile *et al*., 1995). Increased levels of pro-inflammatory cytokines (TNF-α, IL-1β), matrix proteinases (MMP-8, MMP-9) and apoptosis inducing cell detachment from ECM were also reported (Dai *et al*., 2005).

Currently, guided tissue regeneration (GTR) is the most widely used periodontal treatment. Its goal is the regeneration of ECM from affected connective tissues by application of occlusive barriers made of different materials (Aurer *et al*., 2005). These barriers were designed to restrict the migration of the gingival and epithelial cells into periodontal defects and to increase the number of periodontal ligament cells, cementoblasts and osteoblasts, which could synthesize new functional ECM (Scheller *et al*., 2009). There are different types of occlusive membranes on GTR products market, such as non-resorbable membranes made of expanded polytetrafluoroethylene and resorbable membranes like Avitene® made of calf pericardium COL cross-linked with diphenylphosphoryl azide, Collistat® made of bovine dermis COL (pore size 0.004 μm), BioMend® made of type I COL from bovine tendon used for different shape and size defects (Behfarnia *et al*., 2012) and Paroguide® made of COL and chondroitin sulfate (CS), which regenerated the periodontal ligament, cementum and alveolar bone without inflammation (Parodi *et al*., 1997). Although resorbable membranes have multiple benefits, acting as hemostatic, chemotactic, biocompatible biomaterials, their cross-linking degree and the methods used to date do not provide enough stability to support new tissue building.

COL cross-linking by photopolymerization with riboflavin (RF) (vitamin B2) under UV-A action has been used in the field of ophthalmology for keratoconus treatment (Wollensak *et al*., 2003) and proposed in restorative dentistry as a dentin pre-treatment (Daood *et al*., 2015). RF is noncytotoxic and an essential constituent of living cells, being the precursor of flavin mononucleotide and flavin adenine dinucleotide coenzymes. The mechanism of corneal COL cross-linking using RF as photomediator has not yet been elucidated at the molecular level, but it is thought to create additional chemical bonds between histidine, hydroxyproline, hydroxylysine, tyrosine and threonine amino acid residues (Hovakimyan *et al*., 2012). Other cross-linking agents (e.g., glutaraldehyde, glyceraldehydes) were found unsuitable for eye application, because they have yielded in cytotoxicity, corneal opacity, scarring and prolonged treatment times (Hovakimyan *et al*., 2012). Although a previous study has shown that vitamin B2 consumption was correlated with the reducing of periodontal disease occurrence (Lee *et al*., 2013), RF cross-linking of a biomaterial intended for periodontal regeneration has not yet been studied.

The present study aimed to establish the cross-linking method of novel COL-based scaffolds, using photopolymerization with RF and UV exposure. The cross-linking parameters (UV exposure time, UV radiation type) and the physico-chemical and biochemical characteristics (density, cross-linking degree, biodegradability) of the scaffolds were investigated, in comparison to chemically cross-linked scaffolds. Their cytotoxicity was also evaluated in a

Correspondence*: Dr. Oana Craciunescu, National Institute of R&D for Biological Sciences, 296, Splaiul Independentei, sector 6, 060031, Bucharest, Romania, Tel./Fax +40-21-2200882, Email: oana_craciunescu2009@yahoo.com*

culture of gingival fibroblasts by MTT assay and light microscopy.

MATERIALS AND METHODS:

Materials

CS sodium salt from bovine trachea, FN, RF, catechin, glutaraldehyde, 2,4,6-trinitrobenzenesulfonic acid (TNBS), 4,5-dimethylthiazol-2-yl-2,5-diphenyltetrazolium bromide (MTT), Dulbecco's Modified Eagle's Medium (DMEM), fetal bovine serum (FBS), L-glutamine and all other chemicals and solvents of analytical grade were purchased from Sigma-Aldrich Chemicals (Germany).

Preparation of composite scaffolds

Variants of composite scaffolds were prepared from COL type I solution (0.5%, w/w), enzimatically obtained in our lab from bovine tendons (Moldovan *et al*., 2009), CS solution (1%, w/v) and FN solution (200 ng/ml), mixed in a ratio of 20:1:1 (v/v/v) and homogenized with a manual speed-stirrer (Xenox, Germany). The mixture was poured into round wells of a plastic culture plate and lyophilized for 48 h, as previously described (Craciunescu *et al*., 2011a). COL-CS and COL scaffolds were also fabricated, in the same conditions and were used as controls.

Cross-linking of composite scaffolds

Scaffold samples (COL-CS-FN, COL-CS, COL) were incubated with 0.25 mM riboflavin in ethanol and exposed to UV radiation at 254 nm and 365 nm, respectively, for 15 min, 30 min, 1 h and 2 h. Cross-linked scaffolds were washed in 0.1 M sodium phosphate, 1 M sodium chloride and distilled water and re-lyophilized. Similar, scaffold samples were cross-linked by incubation in 0.01 M catechin solution in ethanol and exposure to vapors of 3% GA solution in ethanol, respectively, washed and re-lyophilized.

Determination of the cross-linking degree

The cross-linking degree was assessed as the quantity of free amino groups in composite scaffolds using TNBS, as previously described (Craciunescu *et al*., 2011b). The concentration of free amino groups was expressed as nM/mg scaffold, considering $\varepsilon=1.4x10^4\ M^{-1}cm^{-1}$. The cross-linking degree (% CL) was calculated using the following equation:

$$\% CL = 1 - (OD_{CL}/m_{CL} / OD_{nonCL}/m_{nonCL}) \times 100 \quad (1)$$

where OD_{CL} is the absorbance of cross-linked sample, OD_{nonCL} is the absorbance of non-cross-linked sample, m_{CL} is the weight of cross-linked sample and m_{nonCL} is the weight of non-cross-linked sample.

Density measurements

The density of cross-linked composite scaffolds was calculated as the ratio between their weight (g) and their volume (cm^3), determined after diameter and height measurements of the scaffolds, as previously described (Loh *et al*., 2013).

In vitro biodegradability test

The biodegradation of cross-linked composite scaffolds was performed in the presence of bacterial collagenase, as previously described (Craciunescu *et al*., 2011b). Briefly, samples of composites (5 mg) were incubated in TES buffer, pH 7.4 containing 10 U/ml bacterial collagenase, at 37 °C, for 6 h, with gentle shaking. At predetermined intervals, the scaffolds were removed, dried and weighted. The biodegradability was expressed as percentage of degraded mass.

Cytotoxicity test

A primary cell culture of human gingival fibroblasts was established from healthy gingival tissue discarded during patient (male, 29 years old) surgery, as previously described (Azzimonti *et al*., 2015). The patient consented and was treated according to ethical guidelines. Briefly, the gingival tissue was incubated overnight with 0.5% dispase, at 4 ^{0}C, in order to separate the connective tissues from the epithelium. Then, the dermal layer was minced and digested with a collagenase solution (3 mg/mL) at 37 ^{0}C, for 40 min. Cells were cultivated in DMEM supplemented with 10% FBS and 1% antibiotic/antimycotic mixture and incubated in 5 % CO_2 atmosphere, at 37 ^{0}C.

The cytotoxicity of cross-linked composite scaffolds was evaluated after 48 h of cultivation in the culture medium of gingival fibroblasts seeded in 24-well culture plate, at a density of $4x10^5$ cells/ml. Mitochondrial dehydrogenases activity in viable cells was assessed using MTT assay, as previously described (Craciunescu *et al*., 2014). The cell viability of each group was expressed as percent from the untreated cells (control), considered 100% viable. Cell morphology of fibroblasts cultivated with composite scaffold variants for 48 h was observed after cell processing and hematoxylin-eosin staining, at an Axio Observer D1 microscope (Carl Zeiss, Germany).

RESULTS AND DISCUSSION:

Physico-chemical and biochemical characterization of the cross-linked composite scaffolds

First, we have compared the variation of the cross-linking degree for composites treated with RF and exposed to UV depending on their composition. Thus, variants of COL-CS-FN, COL-CS and COL cross-linked scaffolds were analyzed by TNBS assay, in order to determine the quantity of free amino groups after cross-linking. The results are presented in Fig. 1.

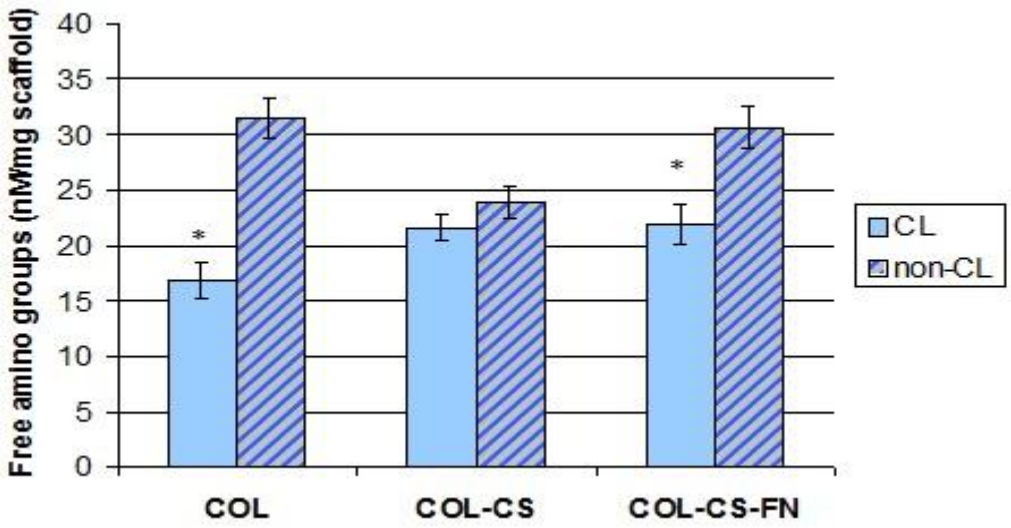

Fig. 1 The variation of free amino groups in composite variants cross-linked (CL) by photopolymerization with riboflavin and UV exposure, depending on scaffold composition. The results were expressed as mean ± SD (n=3). *p<0.05, compared to corresponding non-cross-linked (non-CL) scaffolds

The obtained results showed that the concentration of free amino groups significantly ($p<0.05$) decreased for COL and COL-CS-FN scaffolds, compared to the corresponding non-cross-linked variants. For COL-CS scaffold, there was a non-significantly ($p>0.05$) difference between the cross-linked and non-cross-linked variants. The lower the concentration of free amino groups present in composite scaffolds, the higher was the cross-linking degree. Comparison of these data indicated the advantage of adding CS and FN to COL scaffold composition and their possible role in scaffold cross-linking using riboflavin and UV exposure.

In order to investigate the optimal parameters for UV exposure of COL-CS-FN scaffold incubated in 0.25 mM riboflavin solution in ethanol, we have used a 254 nm source and a 365 nm source for UV irradiation. Also, the time of exposure varied from 15 min, 30 min, 1 h to 2 h. The cross-linking degree was determined and the results are presented in Fig. 2.

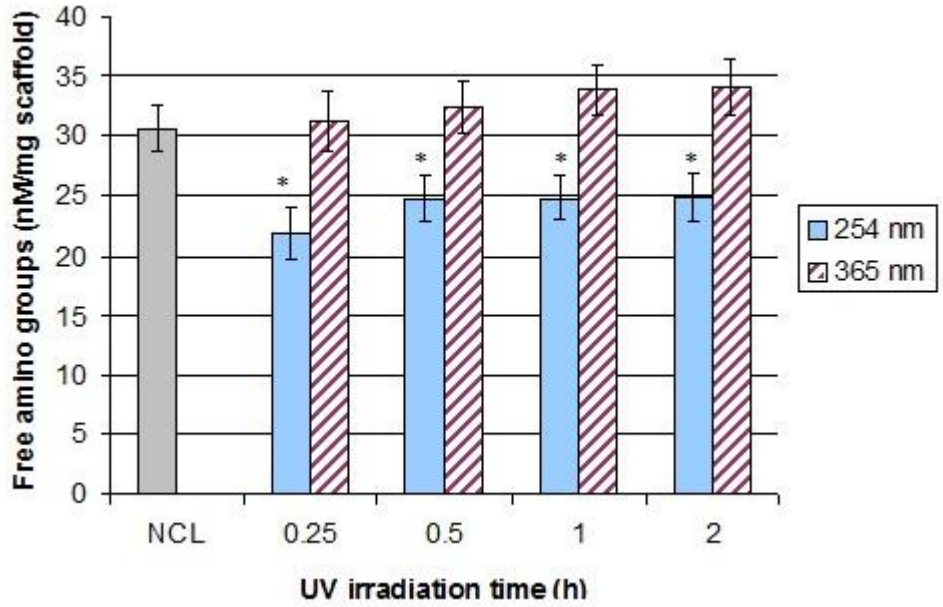

Fig. 2 The variation of free amino groups in composite variants cross-linked by photopolymerization with riboflavin and UV exposure at 254 nm and 365 nm, for different periods of time. The results were expressed as mean ± SD (n= 3). *p<0.05, compared to non-cross-linked (NCL) scaffold

The obtained results have indicated that COL-CS-FN scaffold exposure to UV radiation at 254 nm, for 15 min resulted in the lowest value of free amino groups (21.9 nM/mg scaffold), corresponding to the highest cross-linking degree (31.05%). Exposure of scaffold samples to 365 nm UV source yielded in non-significantly ($p>0.05$) variation of the concentration of free amino groups, for all periods of exposure, compared to the non-cross-linked variant.

The next experiment aimed to investigate the role of riboflavin during the cross-linking reaction. Samples of COL-CS-FN scaffold were incubated in ethanol and 0.25 mM riboflavin in ethanol, respectively and were exposed to UV radiation at 254 nm. Also, a composite sample was incubated in ethanolic riboflavin without exposure to UV. All variants were analyzed for free amino groups and the results are presented in Fig. 3.

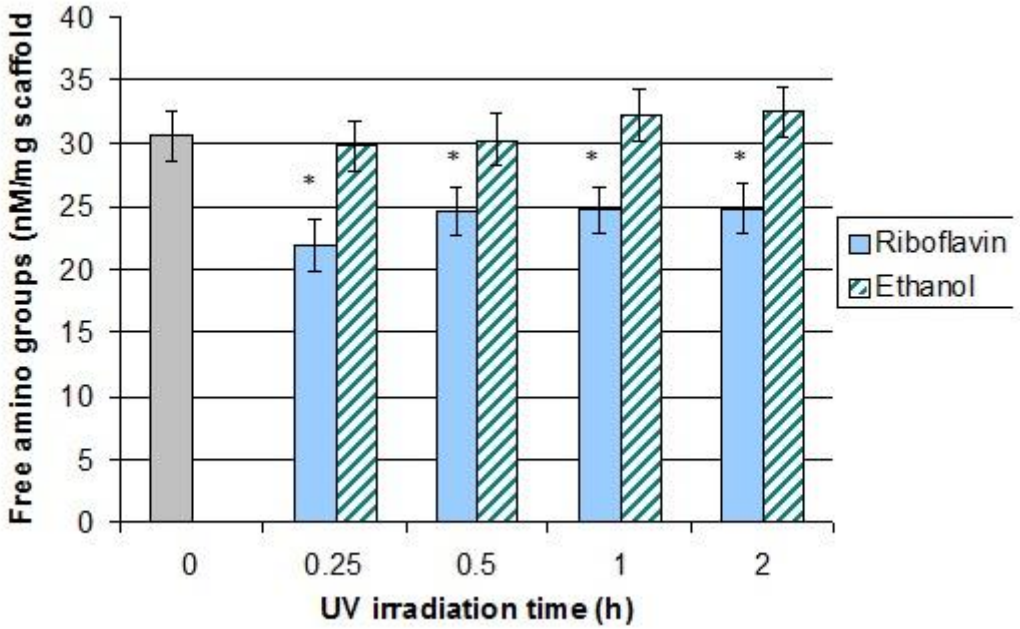

Fig. 3 The variation of free amino groups in composite variants incubated in ethanolic riboflavin or ethanol, with or without UV exposure. The results were expressed as mean ± SD (n= 3). *p<0.05, compared to non-irradiated scaffold (control)

The results showed a significantly ($p<0.05$) decrease of free amino groups for the samples incubated with ethanolic riboflavin and exposed to UV irradiation, compared to the non-irradiated sample. The lowest value (21.9 nM/mg scaffold) and, accordingly, the highest cross-linking degree (31.05%) were calculated for the irradiation period of 15 min. The samples incubated in ethanol and UV irradiated presented non-significantly different ($p>0.05$) values of free amino groups, compared to control, ranging between 29.78-32.50 nM/mg scaffold. Overnight incubation of scaffold samples in riboflavin without UV exposure (non-irradiated sample) resulted in high value of free amino groups (30.60 nM/mg scaffold) and low cross-linking degree (11.43%), respectively. All these data indicated that riboflavin acted as a good cross-linking agent for the novel designed COL-CS-FN scaffolds.

In order to compare the efficacy of photopolymerization and chemical cross-linking methods applied to novel COL-CS-FN scaffolds, we have analyzed their density, cross-linking degree and *in vitro* biodegradation. COL-CS-FN scaffold samples were incubated with riboflavin, catechin and glutaraldehyde, respectively, as described above. The density measurements showed that the scaffolds cross-linked with riboflavin and exposed to 254 nm irradiation were more dense than those incubated with catechin, but similar to glutaraldehyde cross-linked ones (Table 1). Riboflavin incubation of composite scaffolds and exposure to 365 nm irradiation resulted in loose scaffolds, with a density value lower than that of the non-cross-linked scaffolds (Table 1). These results are useful for designing and fabrication of 3D porous scaffolds for tissue regeneration, in order to control their physical microstructure parameters, like density and porosity, which play an important role in cell migration and proliferation.

Table 1.
The density, cross-linking degree and biodegradation of cross-linked composite variants

	Non-CL	RF 254	RF 365	CAT	GA
Density (g/cm^3)	0.053 ± 0.002	0.068 ± 0.002	0.035 ± 0.002	0.057 ± 0.002	0.065 ± 0.002
Cross-linking degree (%)	0	31.75 ± 1.55	22.31 ± 1.33	20.45 ± 1.12	31.23 ± 1.60
Biodegradation (%)	40.34 ± 3.21	20.85 ± 2.59	28.59 ± 2.86	32.78 ± 3.12	16.07 ± 3.95

The calculated cross-linking degree had high values (~31%) for riboflavin photopolymerized and glutaraldehyde treated scaffolds, while catechin incubation of composites resulted in lower values (20.45%) (Table 1). It was previously shown that chemical cross-linking yielded composite scaffolds with better mechanic properties than individual component scaffolds (Craciunescu *et al*., 2011b). COL cross-linking mechanism using chemical agents

Studia Universitatis "Vasile Goldiş", Seria Ştiinţele Vieţii
Vol. 26 Issue 2, 2016, pp.243--249

(glutaraldehyde, carbodiimide, epoxy compounds, isocyanates) or natural substances (catechin, genipin, tannic acid, procyanidins) consists of introducing covalent bonds or other molecular interactions between the residues of its chains, as was demonstrated using bioinformatics (Swamy *et al*., 2011) or lab studies (Ma *et al*., 2014). Glutaraldehyde could significantly improve the tensile strength of COL-based scaffolds, but its toxicity and the induction of calcification resulted in limitations of its use for long-term implants (Ma *et al*., 2014). Catechin, a plant polyphenol, was proposed for the development of a new generation of implantable cross-linked scaffolds and it could stabilize COL through hydrogen bonding and hydrophobic interactions (Madhan *et al*., 2005). Photochemical cross-linking with riboflavin using UV-A or visible blue light was previously proposed as an alternative for producing stable hydrogels by generating free radicals involved in formation of additional bonds between amino acid residues of COL chains (Ibusuki *et al*., 2007).

The results of composite scaffolds biodegradability in the presence of collagenase are presented in Table 1. The values showed that the degraded mass increased from glutaraldehyde-treated (16.07%) to riboflavin-treated (20.85%) and catechin-treated (32.78%) scaffolds. It was observed that the results were in accordance with the variation of the cross-linking degree; thus, the higher the biodegradation, the lower the cross-linking degree. Previous studies have shown that the peptidic sequence -X-Gly-Pro- from COL molecule is attacked by collagenases (Ohan *et al*., 2002). The increase of inter- and intra-molecular covalent bonds after cross-linking could protect the molecule and decrease the enzymatic degradation. Also, we supposed that CS and FN attachment to COL fibrils might play a role in hindering the specific situs.

Evaluation of the cytotoxicity of cross-linked composite scaffolds in gingival fibroblasts culture

COL-CS-FN cross-linked scaffolds were also evaluated for cytotoxicity in a primary culture of gingival fibroblasts, taking into account their applicability in periodontitis treatment. First, we have quantitatively assessed the cell viability of fibroblasts cultivated in the presence of cross-linked composite samples, in standard conditions of incubation, for 48 h. The results are presented in Fig. 4.

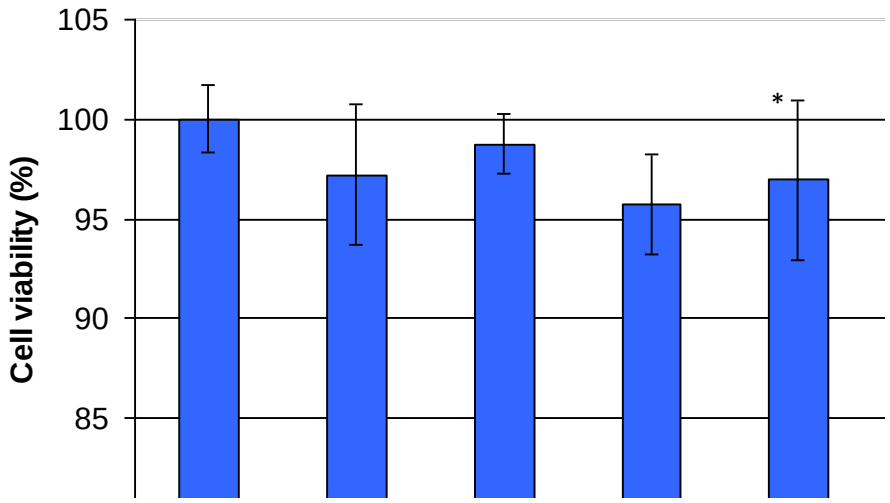

Fig. 4 Cell viability of gingival fibroblasts cultivated in the presence of non-cross-linked (NCL) COL-CS-FN scaffolds and variants cross-linked with riboflavin at 254 nm (RF 254) and 365 nm (RF 365), catechin (CAT) and glutaraldehyde (GA). The results were calculated as mean ± SD (n= 3) and expressed as percent from untreated cells (control). *p<0.05

The cell viability was very high (> 90%) for all cross-linked scaffolds and not significantly different (p>0.05) from that of untreated control cells (100%), except the cells cultivated with glutaraldehyde cross-linked scaffolds. The values ranged from 92.42% for glutaraldehyde-treated variants to 98.73% for variants incubated with riboflavin and exposed to UV radiation at 254 nm (Fig. 4). The results indicated that all cross-linked variants of COL-CS-FN scaffold were biocompatible with gingival fibroblasts cultivated *in vitro*.

Cell morphology observations on the same samples confirmed the quantitative data obtained for cell viability. The cells cultivated with each cross-linked scaffold variant presented a normal phenotype and a similar density to untreated control cells, except the group treated with glutaraldehyde variant that had a slightly decreased density (Fig. 5).

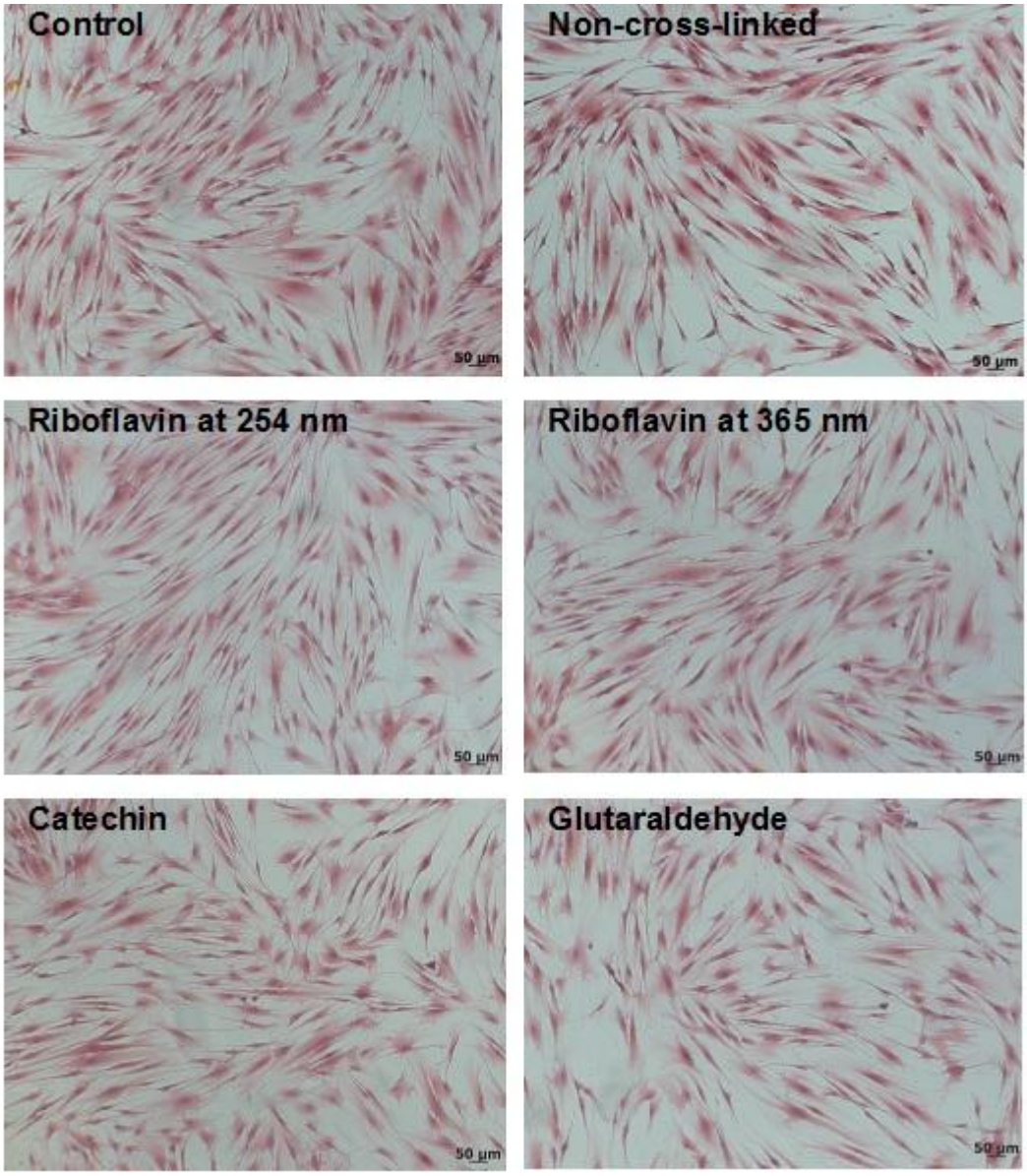

Fig. 5 Light micrographs of gingival fibroblasts cultivated in the presence of non-cross-linked COL-CS-FN scaffold and variants cross-linked with riboflavin at 254 nm and 365 nm, catechin and glutaraldehyde, for 48 h. Untreated cell culture served as control. (Hematoxylin-eosin staining)

Previous studies showed that riboflavin incorporation in COL hydrogels fabricated for tissue engineering did not induce cell toxicity (Tirella *et al*., 2012). Still, UV irradiation, time exposure, radiation type and free radicals generation may induce apoptosis or necrosis of cells encapsulated in hydrogels.

CONCLUSIONS:

In conclusion, we have demonstrated that COL-CS-FN porous scaffolds could be cross-linked using riboflavin and UV exposure method. It was selected the UV radiation wavelength of 254 nm for photo-initiation reaction and an optimal irradiation period of 15 min. The obtained cross-linked scaffolds were stable against collagenase attack and presented no cytotoxicity in a human gingival cell culture. All these data recommend the cross-linked COL-CS-FN composite scaffolds for future testing in periodontitis treatment.

ACKNOWLEDGMENT:

This study was supported by ANCSI, Project BIODIVERS 16-19/2016.

REFERENCES:

Aurer A, Jorgic-Srdjak K, Membranes for periodontal regeneration. Acta Stomatologica Croatica, 39, 107-112, 2005.

Azzimonti B, Cochis A, Beyrouthy ME, Iriti M, Uberti F, Sorrentino R, Landini MM, Rimondini L, Varoni EM, Essential oil from berries of Lebanese *Juniperus excelsa* M. Bieb displays similar antibacterial activity to chlorhexidine but higher cytocompatibility with human oral primary cells. Molecules, 20, 9344-9357, 2015.

Behfarnia P, Khorasani MM, Birang R, Abbas FM, Histological and histomorphometric analysis of animal experimental dehiscence defect treated with three bio absorbable GTR

collagen membrane. Dental Research Journal, 9, 574-581, 2012.

Craciunescu O, Moldovan L, Designing bio-inspired composite materials for medical applications. In: Nanocomposites and Polymers with Analytical Methods. Ed. John Cuppoletti, InTech, Rijeka, Croatia, 309-334, 2011a.

Craciunescu O, Tardei C, Moldovan L, Zarnescu O, Magnesium substitution effect on porous scaffolds for bone repairing. Central European Journal of Biology, 6, 301-311, 2011b.

Craciunescu O, Moldovan L, Moisei M, Utoiu E, Zarnescu O, Roseanu A, Trif M, Selection of an efficient chondroitin sulfate-liposome delivery system as chondroprotective agent for local inflammation treatment. Studia Universitatis Vasile Goldis, Seria Stiintele Vietii (Life Sciences Series), 24, suppl. 1, 93-101, 2014.

Dai R, Iwama A, Wang S, Kapila YL, Disease-associated fibronectin matrix fragments trigger anoikis of human primary ligament cells: p53 and c-myc are suppressed. Apoptosis, 10, 503–512, 2005.

Daood U, Heng CS, Lian JNC, Fawzy AS, *In vitro* analysis of riboflavin-modified, experimental, two-step etch-and-rinse dentin adhesive: Fourier transform infrared spectroscopy and micro-Raman studies. International Journal of Oral Science, 7, 110-124, 2015.

Ferraiolo DM, Predicting periodontitis progression? Evidence-Based Dentistry, 17, 19-20, 2016.

Giannobile WV, Lynch SE, Denmark RG, Paquette DW, Fiorellini JP, Williams RC, Crevicular fluid osteocalcin and pyridinoline crosslinked carboxyterminal telopeptide of type I collagen (ICTP) as markers of rapid bone turnover in periodontitis. A pilot study in beagle dogs. Journal of Clinical Periodontology, 22, 903–910, 1995.

Hovakimyan M, Guthoff RF, Stachs O, Collagen cross-linking: current status and future directions. Journal of Ophthalmology, 2012, Art ID 406850, 12 pages, 2012.

Ibusuki S, Halbesma GJ, Randolph MA, Redmond RW, Kochevar IE, Gill TJ, Photochemically cross-linked collagen gels as three-dimensional scaffolds for tissue engineering. Tissue Engineering, 13, 1995-2001, 2007.

Jee SW, Wang S, Kapila YL, Specific pro-apoptotic fibronectin fragments modulate proteinase expression in periodontal ligament cells. Journal of Periodontology, 75, 523–530, 2004.

Kaur S, White S, Bartold PM, Periodontal disease and rheumatoid arthritis: a systematic review, Journal of Dental Research, 92, 399-408, 2013.

Lee JH, Shin MS, Kim HD, Vitamin B2 and vitamin C intake are associated with the reduction of periodontitis prevalence among Korean representative adults. 141st Annual Meeting and Expo, nov 2-6, Boston, 285275, 2013.

Loh QL, Choong C, Three-dimensional scaffolds for tissue engineering applications: role of porosity and pore size. Tissue Engineering Part B, 19, 485-503, 2013.

Ma B, Wang X, Wu C, Chang J, Crosslinking strategies for preparation of extracellular matrix-derived cardiovascular scaffolds. Regenerative Biomaterials, 9, 81-89, 2014.

Madhan B, Subramanian V, Raghava Rao J, Nair BU, Ramasami T, Stabilization of collagen using plant polyphenol: role of catechin. International Journal of Biological Macromolecules, 37, 47-53, 2005.

Moldovan L, Craciunescu O, Oprita EI, Balan M, Zarnescu O, Collagen-chondroitin sulfate-hydroxyapatite porous composites: preparation, characterization and *in vitro* biocompatibility testing. Roumanian Biotechnological Letters, 14, 4459-4466, 2009.

Ohan MP, Weadock KS, Dunn MG, Synergistic effects of glucose and ultraviolet irradiation on the physical properties of collagen. Journal of Biomedical Materials Research, 60, 384-391, 2002.

Parodi R, Carusi G, Santarelli G, Nanni F, Pingitore R, Brunel G, Guided tissue regeneration employing a collagen membrane in a human periodontal bone defect: a histologic evalaution. International Journal of Periodontics & Restorative Dentistry, 17, 282-291, 1997.

Scheller EL, Krebsbach PH, Kohn DH, Tissue engineering: state of the art in oral rehabilitation. Journal of Oral Rehabilitation, 36, 368-389, 2009.

Swamy RN, Gnanamani A, Shanmugasamy S, Gopal RK, Mandal AB, Bioinformatics in crosslinking chemistry of collagen with selective cross linkers. BMC Research Notes, 4, 399-407, 2011.

Tirella A, Liberto T, Ahluwalia A, Riboflavin and collagen: new crosslinking methods to tailor the stiffness of hydrogels. Materials Letters, 74, 58-61, 2012.

Wollensak G, Spoerl E, Seiler T, Riboflavin/ultraviolet-A-induced collagen crosslinking for the treatment of keratoconus. American Journal of Ophthalmology, 135, 620-627, 2003.