 doi:10.20944/preprints202208.0298.v1

Article

Morphological and Quantitative Evidence for the Altered Mesenchymal Stem Cells Remodeling of Collagen in Oxidative Environment – Peculiar Effect of Epigalocatechin Gallate

Regina Komsa-Penkova[1], Svetoslava Stoycheva[1], Pencho Tonchev[2], Galya Stavreva[3], Svetla Todinova[4], Galya Georgieva[1], Adelina Jordanova[5], Stanimir Kyurkchiev[5] and George Altankov[4#,6] *

[1] Department of Biochemistry, Medical University Pleven, Pleven,5800, Bulgaria.
[2] Department of Surgery, Medical University Pleven, Pleven, 5800 Bulgaria
[3] Department of Experimental and Clinical Pharmacology,Medical University Pleven, Pleven, Bulgaria.
[4] Institute for Biophysics and Biomedical Engineering, Bulgarian Academy of Sciences, Sofia, Bulgaria
[4#] Associate Member Institute for Biophysics and Biomedical Engineering, Bulgarian Academy of Sciences, Sofia, Bulgaria
[5]Tissue Bank BulGen, Str. Hristo Blagoev 25, 1330 Sofia, Bulgaria, adi.7@abv.bg
[6] Medical University Pleven, Research Institute, Pleven, Bulgaria
*Correspondance: altankov@abv.bg

Abstract: Mesenchymal stem cells (MSCs) are involved in the process of extracellular matrix (ECM) remodeling where collagens play a pivotal role. We recently demonstrated that the remodeling of adsorbed collagen type I might be disordered upon oxidation following its fate in the presence of human adipose derived MSC (ADMSCs). With the present study we intended to learn more about the effect of polyphenolic antioxidant Epigallocatechin gallate (EGCG) attempting to mimic the conditions of oxidative stress in vivo and its putative prevention by antioxidants. Collagen Type I was isolated from mouse tail tendon (MTC) and labeled with FITC before oxidized according to Fe2+/H2O2 protocol. FITC-collagen remodeling by ADMSC was assessed morphologically before and after EGCG pretreatment and confirmed via detailed morphometry analysis measuring the anisotropy index (AI) and fluorescence intensity (FI) in selected regions of interest (ROI), namely: outside the cells; over the cells and central (nuclear perinuclear) region, whereas the pericellular proteolytic activity was measured by de-quenching of fluorescent collagen probes (FRET effect). Here we provide morphological evidence that MTC undergoes significant reorganization by the adhering ADMSC along with the substantial activation of pericellular proteolysis, and further confirm that both processes are suppressed upon collagen oxidation. An important observation was that this abrogated remodeling cannot be prevented by the EGCG pretreatment. Conversely, the detailed morphometry analysis showed that oxidized FITC-collagen rather tends to accumulate beneath the cells and around cell's nuclei suggesting the activation of alternative routes for its removal, such as internalization and/or transcytosis. Morphometry analysis also revealed that both processes are supported by EGCG pretreatment.

Keywords: Adipose tissue-derived mesenchymal stem cell; collagen type I; EGCG; oxidation; remodeling

1. Introduction

Collagens are the most abundant proteins of the ECM important for the mechanical properties of connective tissues, regulation of cell functions, and communication [1]. Recently become clear that they are tightly involved also in the regulation of adult stem cell functioning such as renewal and differentiation [2]. Mesenchymal stem cells (MSCs) isolated from different sources can differentiate into other types of cells [3,4], providing

unique properties for various cellular therapies. In humans, these sources include bone marrow, adipose, and umbilical tissues, or amniotic fluid [2,5,6] An important unresolved issue however, is how to guide MSCs behavior in engineered environments.

Under physiological conditions, MSCs are capable of self-renewal or differentiation induced by environmental stimuli, either soluble growth factors [7] or distinct structural cues related to the tissue specific extracellular matrix (ECM) organization. Though it is well known that ECM has important roles in regulating the development and homeostasis of eukaryotic cells [8,9], relatively little is known about how stem cells behave within the specific ECM environment, often approximated as stem cells niche [10,11]. The molecules that are usually associated with ECM include collagens, laminins, fibronectin, proteoglycans, and other specific glycoproteins [12], which associates with the rough ECM [13], or highly specialized basement membranes (BM) [14,15]. The way they assembly determines the structural organization of the stem cells niche and regulates the balance between stem cell differentiation and self-renewal [16]. Particularly adipose tissue-derived MSCs (ADMSCs) draw notable attention in tissue engineering combining relatively easy availability, less donor site morbidity, and multi-potency [2,17]. In addition like most MSCs, they lack expression of major *histocompatibility complex* (MHC) *class II* surface molecules (important for immune rejection) and further provide regulatory function influencing the phenotype of immune cells and their cytokine secretions upon stimulation of their toll-like receptor (TLR)[18]. Thus, ADMSCs provide unique properties for various regenerative therapies.

Though our knowledge of the composition and function of the ECM molecules is continuously growing [19], the structural aspects of the ECM, including the intimate organization of collagen molecules, are still not well understood, particularly in pathological environments [20]. Oxidative stress is one such a condition that strongly affects the collagen structure and turnover [21], including synthesis, post-translational processing [22], and remodeling [23], but the effect on stem cell functioning is rather sparsely studied. In this respect our recent study showing that the post translational oxidation of collagen type 1 (obtained from rat tails) significantly alters its remodeling by stem cells, including mechanical reorganization and proteolytic degradation [24], opens the scores for further investigations.

The collagen superfamily in vertebrates comprises 28 members (I–XXVIII) with a common structural feature - the presence of a triple helix that can range between different types of collagens. For type I collagen, the most abundant type of collagen, it is about 96% [25], but in a tendon its content can rich about 100% of the total dry mass [26]. Thus, the typical structural “brand” of collagen type 1 is the tight right-handed triple helix composed of three left-handed polypeptide chains. The anisotropic character of collagen molecule and its sequence variability is widely discussed. For example, it is well known that sequence variability along the length of collagen results in local differences in helix pitch, dynamics, and thermal stability [27], which suggests that distinct regions of the triple helix may be sensitive to the oxidative environment. Despite the extensive investigations on the role of oxidative stress in collagen turnover, particularly related to vari-

 doi:10.20944/preprints202208.0298.v1

ous pathological events, the studies in vitro utilizing direct cellular models are rather sparse [28]. Our recent study showed that the oxidative environment inhibits the ability of ADMSCs to remodel adsorbed collagen type 1 because of minute structural changes in the collagen molecules, but not because is harmful to the cells. To go more inside this phenomenon, here we describe the potential preventing role of bio-antioxidants. One of the most intensively studied antioxidants is the epigallocatechin-3-gallate (EGCG) the major polyphenolic compound of green tea (occupying about 30-40 % of the dry weight), which is presumably the most frequently drunk beverage after water [29,30]. The phenol rings of EGCG act as electron scavengers of free radicals [31,32] thus inhibiting the formation of reactive oxygen species and reducing the harm caused by oxidative stress [33]. It has been shown, however, that EGCG effects are not only anti-oxidative, but may affect also other biochemical routes, acting for example as anti-inflammatory agent, with additional an antiproteolytic, antiapoptotic, and antifibrotic functions [34,35], apart from its suggested protective effect in various cardiovascular and neurodegenerative diseases, diabetes and certain cancers [34]. These diverse effects vary on cell type environment, and a load of oxidants [36,37]. However, considering our recently observed effect of oxidation on stem cells remodeling of collagen type I [24] here we focus on the effect of EGCG on the ADMSCs remodeling in an oxidative environment applying the same in vitro system.

2. Material and Methods

2.1 Collagen preparation

Mouse tail tendon collagen (MTC) was produced by acetic acid extraction and salting out with NaCl, as described elsewhere [38]. After centrifugation at 4 000 rpm at 4°C, the pellets were redissolved in 0.05 M acetic acid. The excess NaCl was removed by dialysis against 0.05M acetic acid. All procedures were performed at 4°C. The nearly monomolecular composition of a tail tendon, in which the collagen type I content approaches 100% of the total dry mass was reasonably expected. The collagen concentration in the solutions was measured by modified Lowry assay [39] and from the optical absorbance at 220-230 nm [40].

2.2 Fluorescent labeling of collagen

The modified protocol of Doyle [41] was used for the FITC labelling of collagen obtained from murine tendons. MTC (2 mg/ml) was dissolved in 0.05 M borate buffer (pH 8) and 20 μg of FITC (from 1 mg/ml stock in DMSO) per 1 mg of protein were added and incubated at room temperature in dark for 90 min. 0.05M Tris buffer (pH 7.4) was used to stop the reaction, followed by extensive dialysis versus 0.05 M acetic acid aiming to remove the excess FITC.

2.3 Collagen oxidation procedure

 doi:10.20944/preprints202208.0298.v1

The MTC solution (2 mg/ml) was incubated in 0.05M acetic acid, pH 4.3, with 50 μM $FeCl_2$ and 5 mM H_2O_2 for 18 hours at room temperature as previously described [42]. The oxidant solutions were freshly prepared. 10 mM EDTA was used to stop the reaction before intensive dialysis versus 0.05 M acetic acid was aimed to remove the excess oxidants. Pretreatment with EGCG was performed via 30 min incubation of native collagen with 10 μM EGCG at room temperature before the oxidation procedure. The oxidized collagen (MTC-OXI) and EGCG pretreated (MTC-OXI/EGCG) were freshly prepared before all experiments.

2.4 Cells

Human ADMSCs of passage 1 was received from Tissue Bank BulGen using healthy volunteers undergoing liposuction with sophisticated written consent. The cells were maintained in DMEM/F12 medium containing 1% GlutaMAX™, 1% Antibiotic-Antimycotic solution and 10% Fetal Bovine Serum (FBS) all purchased from (Thermo Fisher Scientific, USA). In every two days the medium was replaced until the cells reach approximately 90% confluency to be used for the experiments up to 7th passage.

2.5 Morphological studies

FITC labelled MTC (100 μg/ml), was coated (60 min) on (12x12) glass coverslips (ISOLAB Laborgerate GmbH) as dissolved in 0.05M acetic acid. For the morphological studies the collagen-coated slides were placed in 6-well TC plates (Nunc, Denmark). Then the cells were seeded with 5 x 104 cells/well ADMSCs at final volume of 3 mL in serum-free medium and incubated for 2h before the initial cell adhesion and overall cell morphology were monitored at phase contrast using inverted microscope Leica DM 2900. Then 10% serum was added to each sample and the cells were cultivated up to 24 h, then washed 3x with PBS, fixed with 4% paraformaldehyde and permeabilized with 0.5% Triton X-1000 for 5 min before fluorescence staining. Red fluorescent Rhodamine-Phalloidin (Invitrogen) was used (dilution 1:100) to visualize actin cytoskeleton while cell nuclei were stained simultaneously by Hoechst 33342 (Sigma-Aldrich) in dilution 1:2000 for 30 min in PBS containing 10% albumin. Finally, the samples were mounted upside down on glass slides with Mowiol and viewed using the blue (for nuclei), red (for actin cytoskeleton) and green (for FITC-collagen) channels of an inverted fluorescent microscope (Olympus BX53, Upright Microscope) at magnification 400x with objective (40x/0.50) UPlan FLN. Minimum three representative images were obtained for each sample. The different colors were merged using the image processing software.

2.6 Measurement of collagen degradation by ADMSC

Glass bottomed 24 well TC plates (Greiner, Sensoplate black, Germany) were pre-coated as above with 100 μg/ml native or oxidized FITC-Col I and washed 3 times with PBS before ADMSCs ($1x10^4$ per well) were added in a final volume of 1 ml serum-free medium (assuring single protein adhesion of cells to Col I). After 2 h of incubation, 10% serum was added and the cells were further cultured up to 24 h in a humidified CO_2 incubator. Then the supernatants were collected for the fluorescence measure-

 doi:10.20944/preprints202208.0298.v1

ment of released FITC-Col, while the adsorbed (substratum associated) FITC-Col I was measured directly from the bottom of the plate (in 1ml PBS) using Multimode Microplate Reader (Mithras LB 943, Berthold Technologies GmbH & Co. KG, Germany) set at 485/525 nm. To estimate the particular effect of ADMSCs control samples without cells (-cells) were processed in the same way. All experiments were quadruplicated.

2.7 Quantitative analysis of raw format images by ImageJ

Qualitative data were gathered via the measurement tool of Java build image post-processing ImageJ and additional plug-in FibrilTool developed for ImageJ measuring the anisotropy of the putative fibrillary structures [43]. The fluorescence intensity and the anisotropy of the fibrillary arrays were measured based on raw format images of cells in the same and a separate experiment performed under the same conditions. Several sequential steps were done: optimization, segmentation, analysis, and measurement steps. In the optimization module, pixel-based treatments are performed to highlight the regions of interest (ROIs) and allow the removal of artefacts. A default black and white threshold was used in the segmentation module. Images of equal size (W: 1600 px/H: 1200 px) were examined. The regions of interest (ROI) for the images of native MTC, MTC-OXI, and MTC-OXI/EGCG were delineated (a minimum of four ROIs for each collagen sample were examined). All measurements were performed at the green channel of the three colored images. The Mean fluorescence intensity (MFI) and the Anisotropy index (AI) were then calculated as the average of each ROI for the region of interest specified as: Outside the cell, Cellular region, and Central (nuclear/perinuclear) part. The MEI ratio inside/outside the cell was further calculated.

2.8 DSC measurements

DSC measurements were performed using DASM4's (Privalov, BioPribor) built-in high-sensitivity calorimeter with a cell volume of 0.47 ml. The samples were prepared in 0.05 M acetic acid. The protein concentration was adjusted to 2 mg/ml. To prevent any degassing of the solution under study, constant pressure of 2 atm was applied to the cells. The samples were heated at a scanning rate of 1.0°C/min from 20°C to 65°C and were preceded by a baseline run with a buffer-filled cell. Each collagen solution was reheated after cooling from the first scan to evaluate the reversibility of the thermally induced transitions. The calorimetric curve corresponding to the second (reheating) scan was used as an instrumental baseline and was subtracted from the first scans, as collagen thermal denaturation is irreversible. The obtained excess heat capacity profiles were normalized to the protein concentration. The calorimetric data were analysed using the Origin Pro 2018 software package.

2.9 Statistical analysis

Data were analyzed using SPSS Statistics for Windows, version 23.0 (Armonk, NY: IBM Corp). The quantitative results were obtained from at least four samples. Descriptive data were compared using Chi-square and Mann-Whitney U-tests. Nonparametric dif-

 doi:10.20944/preprints202208.0298.v1

ferences between groups were compared using the Friedman test; pairwise comparisons were achieved using the Dunn-Bonferroni post-hoc analysis. Data were expressed as mean ± standard deviation (SD). Differences with $p < 0.05$ were considered statistically significant.

3. Results

In a preliminary study, we found morphologically that ADMSCs can reorganize the adsorbed FITC-labelled murine collagen (FITC-MTC) forming bright streaks of mechanically reorganized protein in a fibril-like pattern within 24 h of incubation. Signs for proteolytic remodeling of the adsorbed collagen were also observed (dark zones around some of the cells), presumably caused by peri-cellular proteolysis.

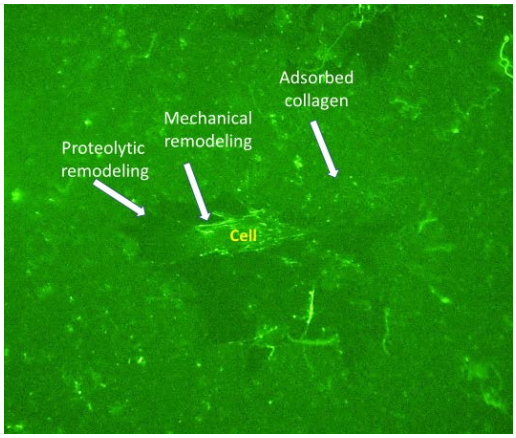

Figure 1. FITC-MTC collagen remodeling by stem cells. ADSCS were cultured for 24 h on the fluorescent FITC-MTC substratum (green). The white arrows point to the places of adsorbed collagen with fibril-like reorganization and places of collagen removal caused by cellular proteolytic activity.

3.1 Overall design of the experiments

Further experiments were designed to establish the particular effects of oxidation with and without the antioxidant pretreatment. More specifically the ADMSC remodeling of adsorbed fluorescent collagen was studied by comparing the native FITC-MTC and the oxidized collagen before (FITC-MTC-OXI) and after EGCG pretreatment (FITC-MTC-OXI/EGCG).

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 17 August 2022 doi:10.20944/preprints202208.0298.v1

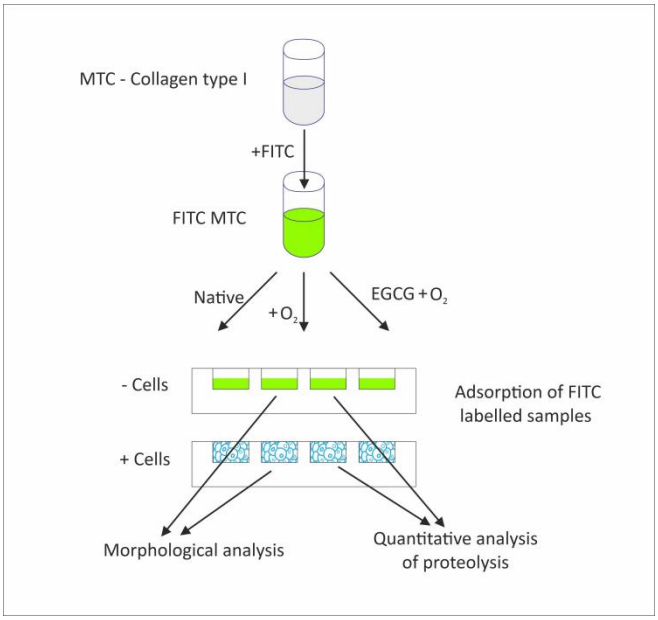

Figure 2. Overall experimental design

According to the diagram in Figure 2, the MTC was first labelled with FITC (FITC-MTC) to easily follow its fate upon adsorption. After the application of the protocols for oxidative modification (before or after EGCG pretreatment), the remodeling of collagen samples was investigated employing of two approaches: morphological and enzymatic.

3.2 Morphological study

For the morphological investigations, ADMSCs were cultured for 24 h on fluorescent FITC-MTC substrata (pre-coated glass coverslips) and then stained to view simultaneously the substratum adsorbed protein (green) and the cells stained for actin cytoskeleton (red) and nuclei (blue). The results are presented in Figure 3. In conjunction with the previous experiments, the adhering ADMSCs on regular collagen (left panels A, D, and G) tend to rearrange mechanically the underlining fluorescent layer forming typical fibrillary assemblies mostly at the cells' periphery. Note, characteristic dark zones of FITC-collagen removal in regions adjacent to the cells suggesting the activation of peri-cellular proteolytic activity, were also observed in native MTC samples (A, D, and G).

 doi:10.20944/preprints202208.0298.v1

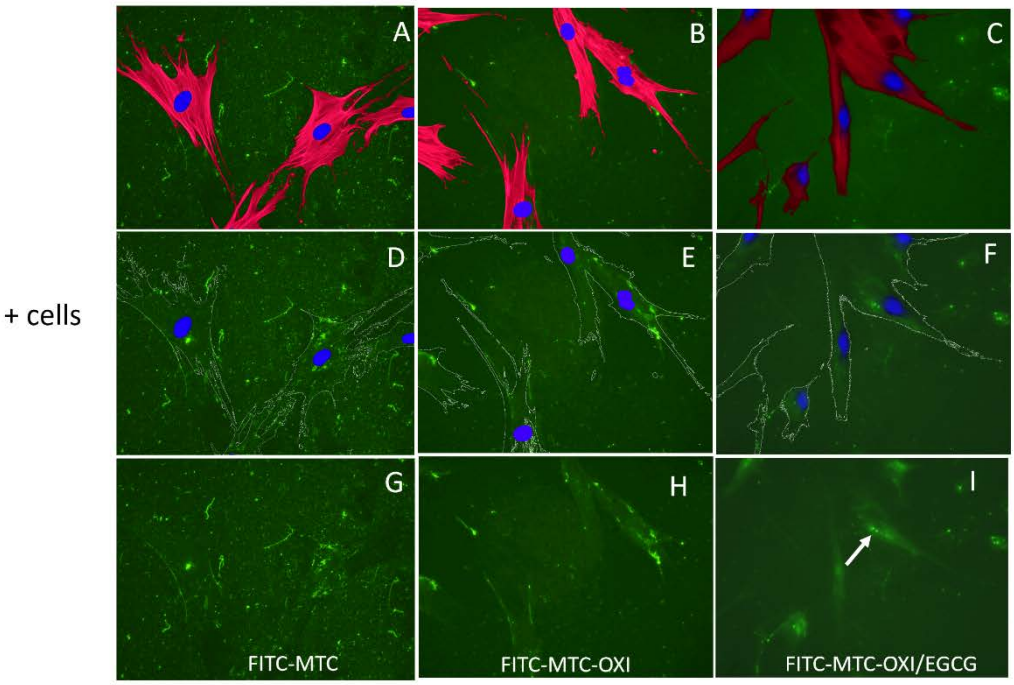

Figure 3. The overall morphology of ADSCS adhering to regular collagen (A, D and G), oxidized collagen (C, E and H) and oxidized collagen pretreated with EGCG (C, F, and I). The top images (A, B and C) present the overall cell morphology viewed by the actin cytoskeleton (red) and cell nuclei (blue) on the green, fluorescent background of FITC collagen. The middle row (D, E, and F) presents the substratum (green) with artificially superimposed cell contours and nuclei, while the bottom row (G, H, and I) presents the collagen substratum only.

Conversely, on oxidized collagen, both mechanical and proteolytic activities tend to abrogate (images D, E, and F), suggesting that oxidized collagen is hardly remodeled by ADMSCs, though the cells spread equally well on both substrata showing similar polarized morphology (compare A, B, and C). No morphological changes were observed also for cells adhering to oxidized samples pretreated with EGCG (C, F, and I). However, here the reorganization occurs in differently, the cells tended to accumulate more collagen beneath, and the formation of some clusters located along the cell nuclei (Golgi localization) might be recognized, suggesting a partial internalization of the adsorbed collagen. Signs for adsorbed collagen degradation were missing.

No significant morphological difference was observed also for the bare substrata, without cells. Though some spontaneous fibrillary assembly of FITC-MTC might be observed, but it is rather independent of the oxidation (as shown in Figure 4) and presumably represent artefacts appearing from the spontaneous fibrillation of collagen in the coating solution.

 doi:10.20944/preprints202208.0298.v1

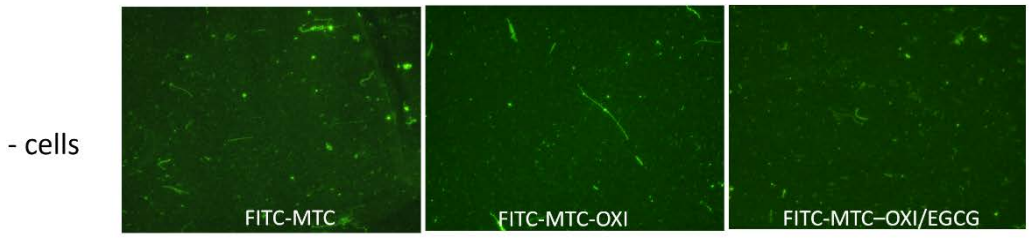

Figure 4. Spontaneous self-assembly of native FITC-MTC, oxidized (FITC-MTC-OXI), and oxidized after EGCG pre-treatment (FITC-MTC-OXI/EGCG) samples

3.4 Quantitative morphometric analysis

ImageJ software was further applied to better characterize the morphological observations. More specifically Fibril Tool was used to quantifying the overall fibrils organization [43] via measuring an anisotropy index (AI) and a Java build-in tool was further explored for measuring the fluorescence intensity (FI) in a given region of interest (ROI). For that purpose, ROIs of similar size (±0.7 %) were selected, namely: "Outside the cells", the "Cellular region" and the "Central (nuclear/perinuclear) part", where both the AI and FI (in pixels) measured for the green, FITC-collagen channel only. The results are presented in Tables 1 and 2:

Table 1. Anisotropy index of FITC labelled collagen samples, consisting of native (MTC), oxidized (MTC-OXI) and MTC-OXI pretreated with EGCG (MTC OXI/EGCG) measured for bare samples (- cells) and for samples (+ cells). For later the measurements were performed in selected regions of interest (ROI) including Outside the cells, the Cellular region, and the Central part including (the nuclear/perinuclear) region.

Collagen Sample	Total anisotropy (- cells)	Total anisotropy (+ cells)	Change in the anisotropy (ΔAI)	Region of interest (ROI)	Anisotropy of the region
MTC	0.069	0.212	0.143	Outside Cell	0.009
				Cell region	0.383
				Central part	0.119
MTC OXI	0.028	0.116	0.088	Outside Cell	0.020
				Cell region	0.448
				Central part	0.553
MTC OXI/EGCG	0.053	0.083	0.031	Outside Cell	0.060
				Cell region	0.374
				Central part	0.381

 doi:10.20944/preprints202208.0298.v1

Table 2. Fluorescence intensity (FI) of FITC collagen studied as native (MTC), oxidized (MTC-OXI), and EGCG pretreated (MTC-OXI/EGCG) measured for samples containing cells. FI is presented in pixels for different regions of interest (ROI) as specified in Table 1.

Sample	Total area (pixels)	Type ROI	FI mean (pixels)	SD	Ratio Inside/Outside the cells
MTC	1904820	Outside Cell	4.88	1.85	
		Cellular region	5.41	2.56	1.11
		Central part	5.73	1.94	1.17
MTC-OXI	1878228	Outside Cell	3.03	0.80	
		Cellular region	3.58	1.47	1.18
		Central part	4.01	1.48	1.32
MTC-OXI/EGCG	1893672	Outside Cell	4.48	0.96	
		Cellular region	5.62	1.44	1.26
		Central part	7.02	1.14	1.57

Total anisotropy (AI) was measured for the bare collagen samples (-cells) and for samples with cells (+cells) as shown in the first two rows of Table 1. As expected, the bare MTC substrata showed very low AI, of about 0.07, suggesting a rather random collagen distribution. It was about twice lower (0.03) for the oxidized samples, while EGCG pretreatment partly "restore" the anisotropy index to about 0.05, though in the same low range. In the samples (+cells) however, the AI substantially increase, ranging between 0.21 for the native MTC collagen, again lower for the oxidized sample (0.12), and lowest (0.08) for the EGCG pretreated one. Nevertheless, apart from the value for the bare EGCG pretreated substrata, the anisotropy was substantially higher here. All this suggests that stem cells tend to reorganize all three substrata. To better characterize this increase of anisotropy in the presence of cells, a specific parameter ΔAI was introduced. For the native collagen samples, ΔAI was 0.143, while for oxidized and EGCG treated samples it drops to 0.088 and 0.023, respectively.

A similar trend was observed for the selected ROIs in the samples (+cells). ROI Outside cells again showed comparable with the bare substrata low anisotropy varying as: 0.09, 0.020, and 0.060 for the native MTC, MTC-OXI and MTC-OXI/EGCG, respectively. It sharply increased however in ROIs Cell region: to 0.383, 0.488, and 0.374, respectively, confirming the significant reorganization of collagen by ADMSCs. It was surprisingly high for the oxidized MTC-OXI and MTC-OXI/EGCG, thus didn't match well the morphological observations, presumably due to the added values of ROIs from the Central region (nuclear/perinuclear zone), showing about 5 times higher signal: 0.374 and 0.381 for the MTC-OXI and MTC-OXI/EGCG, respectively, and only 0.060 for the native MTC

 doi:10.20944/preprints202208.0298.v1

sample, suggesting a trend for different structuring the collagen within the perinuclear region.

For some accumulation of collagen in the ROI (Central region) suggest also the data presented in Table 2 compares the FI in different ROIs. Apart from the inherently higher signal for ROI (Inside cells) and ROI (Central region), compared to ROI (Outside cells), presented in the 3rd row of Table 2, the FI was substantially higher for the nuclear regions of MTC-OXI, and particularly higher for the MTC-OXI/EGCG, reflecting well in the parameter "Ratio inside/outside cells" (5th row) amounting to 1.32 and 1.57 respectively, on the background of 1.17 for the native MTC samples.

3.5 Proteolytic activity of ADMSCs

As described elsewhere, the measuring of proteolytic activity may rely on the de-quenching of fluorescently labelled protein, known as the FRET effect [44], which is based on the assumption that in the conditions of excess dye during the labeling procedure some of the labels remain quenched (due to the high density of FITC molecules) and may de-quench (i.e. increase its fluorescence) upon proteolytic degradation of protein sample. Actually, this concept was proven for adsorbed proteins, including type 1 collagen, in our previous studies [45,46].

As shown in Fig 5 A in the presence of ADMSCs a substantial increase of the fluorescent signal (de-quenching) was observed suggesting a significant proteolytic activity of ADMSs toward adsorbed FITC-MTC collagen.

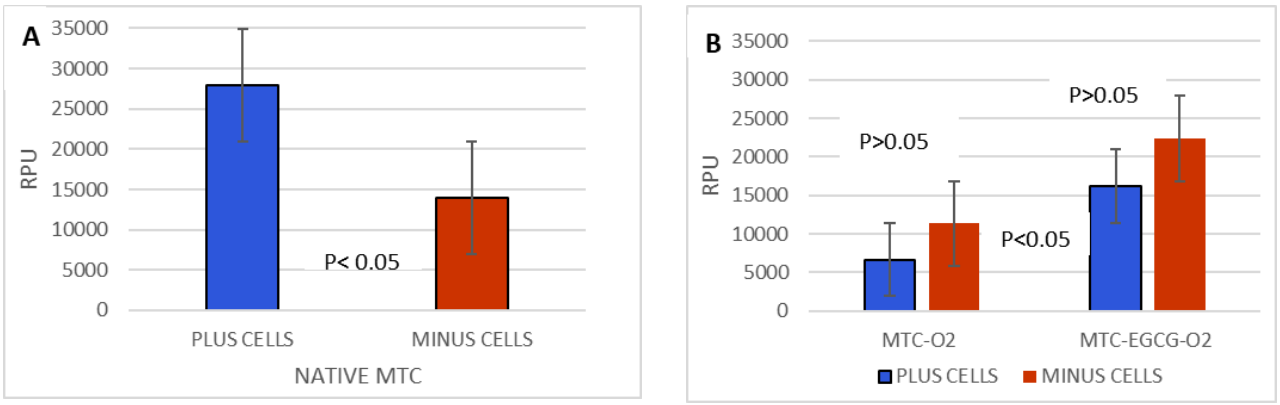

Figure 5. De-quenching activity of ADMSCs toward native (A) and oxidized (B) FITC-collagen samples, studied either without (left) or with EGCG pretreatment (right).

Data in Figure 5 B, however, shows that this is not valid for the oxidized FITC-MTC-OXI and EGCG pretreated samples. Interestingly, in both cases in Fig. 5 B the basic fluorescent signal (red right columns) showed higher fluorescence versus the signal in the presence of cells (blue bars). Though this difference was not significant (p>0.05) we are prone to explain this trend with the "shadowing" effect of adhering cells (see be-

 doi:10.20944/preprints202208.0298.v1

low). Significantly higher however was the fluorescence of EGCG pretreated samples, moreover for both "+cells" and "–cells" samples.

3.6 Thermal denaturation profiles of collagen. Effect of EGCG pretreatment.

This experiment was performed to evaluate the putative impact of EGCG on the previously established change in the thermal stability of oxidized collagen observed by DSC [43] giving rise to an additional low-temperature pre-pick at 34.1°C. More specifically the aim here was to compare the thermal stability of MTC-OXI and MTC/OXI/EGCG. The heat capacity was measured as a function of temperature and presented as the transitional curves shown in Fig. 6, further used to calculate the melting temperature (TM), the total transition enthalpy (ΔH total), and the half-widths of transition (Table 3).

As expected, the maximum heat absorption of native FITC-Col I was observed in the region of 39.6°C (TM2) (Figure 5, Table 3). As a result of the oxidation, the thermogram splits into two well-resolved transitions with melting temperatures at 34.1 °C (TM1) and 39.6°C (TM2), respectively (Table 3), which confirms our previous investigation with calf skin and rat tail collagen type I, showing similar changes in collagen structure upon oxidation [43].

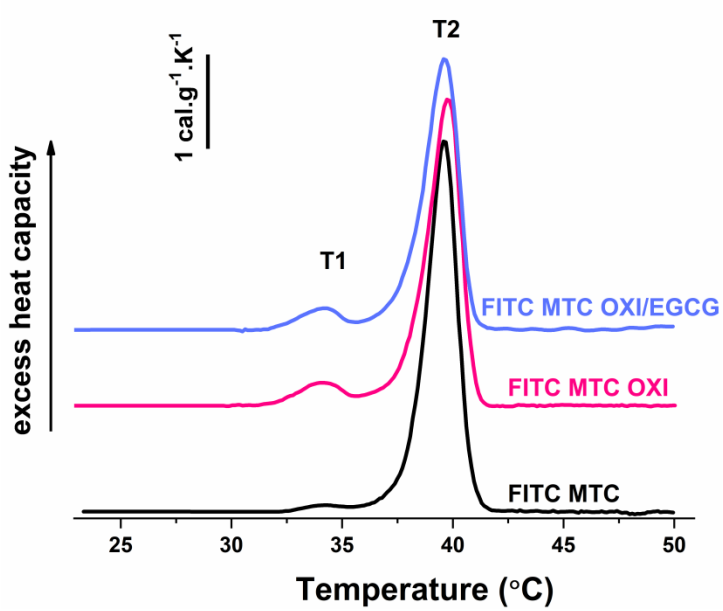

Figure 6. DSC thermograms of native FITC-MTC (black line), oxidized FITC-MTC-OXI (red line), and EGCG pretreated FITC-MTC-OXI/EGCG (blue line) samples.

Table 3. Thermodynamic parameters: transition temperature (TM), total calorimetric enthalpy (ΔH total), and transition half-widths (Tm ½) obtained from DSC profiles of FITC-MTC, FITC-MTC-OXI, and FITC-MTC-OXI/EGCG.

 doi:10.20944/preprints202208.0298.v1

Sample	T_{m1} (°C)	T_{m2} (°C)	ΔH_{total} (cal g^{-1})	T_{m2} ½ (°C)
FITC-MTC	33.8	39.6	7. 2	1.61
FITC-MTC-OXI	34.1	39.7	7.00	1.80
FITC-MTC-OXI/EGCG	34.1	39.6	6.47	1.74

As described elsewhere [43] the pre-transition of the MTC-OXI sample results from the destabilization of the oxidized domains of collagen. However, no specific effect of EGCG pretreatment on the slope and the shape of thermograms were observed. According to the data in Table 3, the effect of EGCG pretreatment was also rather negligible. The presented results demonstrate a small reduction in the total enthalpy of the transition (7.5%) and a slight change in the half-width of transition, when compared to the oxidized collagen without pretreatment. Collectively, these data show that EGCG did not change the pretransition, which presumes that the oxidation is not prevented.

4. Discussion

Abnormal ECM remodeling upon oxidation has an important bio-medical aspect as might be a sign of abrogated balance between the formation and degradation of ECM components leading to pathological consequences [47]. The collagens turnover plays a significant role encompassing the balance between its synthesis, organization, and degradation. [48]. Though the common belief that the physiological role of its remodeling is to remove the excess or the altered collagen in the body [49], this structural protein undergoes also significant organization and reorganization by the adjacent cells, a process, playing a pivotal role in the structuring of ECM in tissues and organs [48]. Indeed, this often goes in concert with the action of proteases [49], which actually assure its successful turnover. Proteolytic remodeling is mediated by several proteinases, among which the matrix metalloproteinases (MMPs) are prominent, comprising a family of more than 23 zinc-dependent enzymes, which act not only degrading the ECM components but also processing the signaling molecules such as cytokines, chemokines, cell receptors and growth factors [50].

Our recent study revealed that the adsorbed collagen Type I undergoes significant remodeling by stem cells, involving its mechanical reorganization in a fibril-like pattern, combined with the activation of cellular proteolytic machinery [38]. Likewise, we showed that both processes were abrogated in an oxidative environment due to the distinct structural alterations in collagen molecules [24]. The implication of stem cells in these studies, particularly ADMSCs, was an important issue considering their pivotal role in various regenerative routes. The effects of oxidative stress caused by ROS [or similar oxidative processes) on the repair of injured tissues are extensively studied, though still not well understood [51]. An important query here is whether this altered

 doi:10.20944/preprints202208.0298.v1

remodeling of collagen depends on the abrogated functioning of stem cells or is caused by intrinsic changes in the collagen molecules in an oxidative environment. In fact, we demonstrated that the abolished remodeling depends rather on the minute changes in the collagen structure, resulting in less susceptibility for proteases, than to the altered cell functioning. The present study confirms this trend, but with collagen from another source, murine tendon, apart from the previously used rat tail collagen [38].

The real novelty in this study, comes from the implication of antioxidant EGCG (a well-known major constituent of the green tee), as we anticipate that it can block the oxidation, in this case via pre-treatment of the collagen solution. However, the DSC analysis shows that after pretreatment of collagen with EGCG (e.g., before oxidation) the denaturation curves were nearly identical to the oxidized sample, with the same slop and shape and a very small reduction in the enthalpy (roughly 7.5%), confirming still discrete structural changes in the collagen molecule, but no "restoring" effect of the antioxidant. Thus, it turned out that the effect of EGCG is not so simple. The morphological investigations and the related quantifications (Tables 1 and 2) confirmed that MTC underwent significant reorganization by ADMSCs, evident from the sharp increase of anisotropy index (AI) in the whole sample in the presence of cells, as well as in the selected ROIs matching the cell regions. The AI however was surprisingly high for the oxidized MTC-OXI and MTC-OXI/EGCG, thus it notably did not confirm the morphological observations. We anticipate that the reason for this high anisotropy at the cell regions comes from the added values of ROIs (Central region) reflecting the nuclear and perinuclear zone of cells, showing about five times higher anisotropy signal, thus suggesting a trend for different structuring of collagen within the perinuclear region. In fact, distinct accumulation of FITC-collagen in this region was already observed morphologically and further confirmed with ImageJ morphometry analysis (Table 2), revealing that the fluorescence intensity (FI) was substantially higher for the nuclear regions of MTC-OXI, particularly in EGCG pretreated sample. This implies the activation of other routes for intracellular processing of oxidized collagen by ADMSCs, presumably involving internalization and subsequent transcytosis. How it is supported by antioxidant EGCG, however, remains unclear. We hypothesise that the EGCG cannot prevent the oxidation of collagen (or do it only slightly) but may affect its subsequent processing by the cells. The morphological signs for the formation of clusters along the cell nuclei (Golgi localization) partly confirm this, suggesting that MSC may possess alternative routes for the removal of oxidized collagen, but that might be further supported by EGCG, for example, via endocytosis and vesicular trafficking ending with intracellular degradation of collagen. In the past few years, a great deal in the understanding of endosome functioning was achieved. Proteins following internalization at the cell surface arrive at endosomes [52] for "sorting," and afterwards some of them are being delivered back to the cell surface, via endosome-to-plasma membrane recycling, and other are sent to the trans-Golgi network via retrograde transport before reaching the degradative lysosomes [53]. We speculate that the oxidation may trigger the first mechanism, assuring transcytosis of the oxidized collagen, while EGCG further activates the second one. The literature analysis

 doi:10.20944/preprints202208.0298.v1

shows that the effects of EGCG may involve direct interactions with plasma membrane components including distinct proteins and phospholipids, unlocking intracellular signaling pathways. Thus, it may stimulate or inhibit various cellular processes ranging from direct metabolic changes like lipogenesis and gluconeogenesis to apoptosis, autophagy, and mitochondrial and nuclear changes [33,54]. In addition, its chemical reactivity makes it susceptible even to generating reactive oxygen species (ROS) via auto oxidation, or to binding and crosslinking with other membrane proteins [34]. Therefore, EGCG could play a dual role, being both beneficial and harmful for the cells depending on concentration and the way of administration. In this case, we suppose a rather positive effect of EGCG, supporting the removal of inactive collagen molecules via its intracellular trafficking.

The enzymatic degradation is another rote for the remodeling of collagen known to be critical for its *in vivo* turnover. Based on previous studies the degradation of adsorbed FITC- collagen might be easily quantified by measuring its de-quenching in the presence of ADMSCs proteases. As expected, the de-quenching worked also very well here, accompanied by the above-mentioned trend of mechanical remodeling in the presence of adhering ADMSCs. While the de-quenching was strongly expressed in the native collagen samples (Fig. 5A) it was significantly inhibited in oxidized ones (Fig 5B). A bit surprising was again the effect of the EGCG. Though the basic fluorescence background increased when compared to the oxidized samples no de-quenching in the presence of ADMSCs was detected. On the contrary, even some nonsignificant trend for diminished fluorescence in the presence of cells was observed, which we are prone to explain by optical shadowing of fluorescent signal from the adhering cells on the bottom of wells. Nevertheless, ADMSC-induced de-quenching in oxidized samples was absent apart from the native collagen ones.

5. CONCLUSION

Collectively this study confirms that due to the distinct structural and thermodynamic changes in the collagen molecule in an oxidative environment ADMSCS hardly remodel oxidized collagen via the “classical” modes of mechanical reorganization and proteolytic degradation constraining them to activate alternative routes for its removals like internalization and transcytosis. EGCG cannot block the oxidation of collagen and thus restore its normal remodeling, but rather potentiate its removal via alternative routes.

Author Contributions: AG and RK-P – Conceptualization of the study; RK-P, ST, GS, GG, SS, AJ and GA – Methodology and Investigation; RK-P, GA, SK, PT and ST – Analysis and interpretation; PT-formal analysis, GA and RK-P – Writing, Original Draft Preparation; GA RK-P Writing – Review & Editing, RK-P –Project Administration, funding acquisitionGS,GA, SK, and RK-P , GA and RK-P – Primary responsibility for the final content. All authors have read and agreed to the published version of the manuscript.

Funding: This work was conducted with the financial support of multidisciplinary project BG05M2OP001-1.002-0010 financed by the operative program "Science and education for intelligence growth" within the European Regional Development Fund and further supported by the Medical University, Pleven, Bulgaria (MU-Project 16/2020 and MU-Project 14/2021).

Acknowledgments: We acknowledge the support and donations of materials used for experiments, given by Tissue Bank BulGen, Bulgaria.

Institutional Ethics Committee Statement: The study was conducted according to the guidelines of the Declaration of Helsinki and approved by the Institutional Ethics Committee of Medical University-Pleven.

Conflicts of Interest: The authors declare no conflict of interest.

References

1. Mouw JK, Ou G, Weaver VM. Extracellular matrix assembly: a multiscale deconstruction. Nat Rev Mol Cell Biol. 2014;15(12):771-785. doi:10.1038/nrm3902
2. Ahmed M, Ffrench-Constant C. Extracellular Matrix Regulation of Stem Cell Behavior. Curr Stem Cell Rep. 2016;2(3):197-206. doi:10.1007/s40778-016-0056-2
3. Zuk, P. A.; Zhu, M.; Mizuno, H.; Huang, J.; Futrell, J. W.; Katz, A. J.; Benhaim, P.;Lorenz, H. P.; Hedrick, M. H., Multilineage cells from human adipose tissue: Implications for cell-based therapies. Tissue Eng. 2001, 7 (2), 211-228. DOI: 10.1089/107632701300062859
4. Mehrabani D, Mehrabani G, Zare S, Manafi A. Adipose-Derived Stem Cells (ADSC) and Aesthetic Surgery: A Mini Review. World J Plast Surg. 2013;2:65–70. [PMC free article] [PubMed] [Google Scholar]
5. Aghamir SMR, Mehrabani D, Amini M, Mosleh Shirazi MA, Nematolahi S, Shekoohi-Shooli F, Mortazavi SMJ, Razeghian Jahromi I. The regenerative effect of bone marrow-derived stem cells on cell count and survival in acute radiation syndrome. World J Plast Surg. 2016;5:1–4. [Google Scholar]
6. S Eskandarlou M, Azimi M, Rabiee S, Seif Rabiee MA. The healing effect of amniotic membrane in burn patients. World J Plast Surg. 2016;5:39–44. [PMC free article] [PubMed] [Google Scholar]
7. Justin D Glenn and Katharine A Whartenby. Mesenchymal stem cells: Emerging mechanisms of immunomodulation and therapy World J Stem Cells. 2014 Nov 26; 6(5): 526–539. doi: 10.4252/wjsc.v6.i5.526, PMCID: PMC4178253, PMID: 25426250
8. Kloareg B, Badis Y, Cock JM, Michel G. Role and Evolution of the Extracellular Matrix in the Acquisition of Complex Multicellularity in Eukaryotes: A Macroalgal Perspective. Genes (Basel). 2021;12(7):1059. Published 2021 Jul 10. doi:10.3390/genes12071059
9. Karsdal, M. A.; Nielsen, M. J.; Sand, J. M.; Henriksen, K.; Genovese, F.; Bay-Jensen, A. C.; Smith, V.; Adamkewicz, J. I.; Christiansen, C.; Leeming, D. J., Extracellular Matrix Remodeling: The Common Denominator in Connective Tissue Diseases Possibilities for Evaluation and Current Understanding of the Matrix as More Than a Passive Architecture, but a Key Player in Tissue Failure. Assay Drug Dev. Technol. 2013, 11 (2), 70-92. DOI: 10.1089/adt.2012.474
10. Kolf, C.M., Cho, E. & Tuan, R.S. Mesenchymal stromal cells: Biology of adult mesenchymal stem cells: regulation of niche, self-renewal and differentiation . Arthritis Res Ther 9, 204 (2007). https://doi.org/10.1186/ar2116
11. F. Gattazzo, A. Urciuolo, P. Bonaldo, Extracellular matrix: A dynamic microenvironment for stem cell niche, Biochimica et Biophysica Acta (BBA) - General Subjects, Volume 1840, Issue 8, 2014, Pages 2506-2519, ISSN 0304-4165, https://doi.org/10.1016/j.bbagen.2014.01.010.
12. Bosman FT, Stamenkovic I. Functional structure and composition of the extracellular matrix. J Pathol. 2003 Jul;200(4):423-8. doi: 10.1002/path.1437. PMID: 12845610.
13. Karamanos, N.K., Theocharis, A.D., Piperigkou, Z., Manou, D., Passi, A., Skandalis, S.S., Vynios, D.H., Orian-Rousseau, V., Ricard-Blum, S., Schmelzer, C.E., Duca, L., Durbeej, M., Afratis, N.A., Troeberg, L., Franchi, M., Masola, V. and Onisto, M. (2021), A guide to the composition and functions of the extracellular matrix. FEBS J, 288: 6850-6912. https://doi.org/10.1111/febs.15776
14. Halfter, W.; Candiello, J.; Hu, H.; Zhang, P.; Schreiber, E.; Balasubramani, M. Protein composition and biomechanical properties of in vivo-derived basement membranes. Cell Adhes. Migr. 2013, 7, 64–71. [Google Scholar] [CrossRef]]
15. Pozzi, A.; Yurchenco, P.D.; Iozzo, R.V. The nature and biology of basement membranes. Matrix Biol. 2017, 57–58, 1–11. [Google Scholar] [CrossRef
16. Liu S, Ginestier C, Ou SJ, Clouthier SG, Patel SH, Monville F, Korkaya H, Heath A, Dutcher J, Kleer C, et al. Breast cancer stem cells are regulated by mesenchymal stem cells through cytokine networks. Cancer Res. 2011; 71:614–24. [PubMed: 21224357]
17. Razmkhah F, Soleimani M, Mehrabani D, Karimi MH, Kafil-Abad SA. Leukemia cell microvesicles promote survival in human umbilical cord blood hematopoietic stem cells. EXCLI J. 2015;14:423–9. [PMC free article] [PubMed] [Google Scholar]
18 . Bernardo ME, Fibbe WE. Mesenchymal stromal cells: sensors and switchers of inflammation. Cell Stem Cell. 2013;13:392-402.
19. Stretching the boundaries of extracellular matrix research.Hynes RO.Nat Rev Mol Cell Biol. 2014 Dec;15(12):761-3. doi: 10.1038/nrm3908.PMID: 25574535
20. Kennett EC, Chuang CY, Degendorfer G, Whitelock JM, Davies MJ. Mechanisms and consequences of oxidative damage to extracellular matrix. Biochem Soc Trans. 2011 Oct;39(5):1279-87. doi: 10.1042/BST0391279. PMID: 21936802.
21. Boin, F., Erre, G.L., Posadino, A.M. et al. Oxidative stress-dependent activation of collagen synthesis is induced in human pulmonary smooth muscle cells by sera from patients with scleroderma-associated pulmonary hypertension. Orphanet J Rare Dis 9, 123 (2014). https://doi.org/10.1186/s13023-014-0123-7
22. Myllyharju, J. Intracellular Post-Translational Modifications of Collagens. In: Brinckmann, J., Notbohm, H., Müller, P.K. (eds) Collagen. Topics in Current Chemistry, vol 247. Springer, Berlin, Heidelberg. https://doi.org/10.1007/b103821
23. Lu P, Takai K, Weaver VM, Werb Z. Extracellular matrix degradation and remodeling in development and disease. Cold Spring Harb Perspect Biol. 2011;3(12):a005058. Published 2011 Dec 1. doi:10.1101/cshperspect.a005058

24. Komsa-Penkova R, Stavreva G, Belemezova K, Kyurkchiev S, Todinova S, Altankov G. Mesenchymal Stem-Cell Remodeling of Adsorbed Type-I Collagen-The Effect of Collagen Oxidation. Int J Mol Sci. 2022 Mar 11;23(6):3058. doi: 10.3390/ijms23063058. PMID: 35328478; PMCID: PMC8953637.
25. Ricard-Blum S. The collagen family. Cold Spring Harb Perspect Biol. 2011;3(1):a004978. Published 2011 Jan 1. doi:10.1101/cshperspect.a004978
26. Fullerton GD, Rahal A. Collagen structure: the molecular source of the tendon magic angle effect. J Magn Reson Imaging. 2007;25(2):345-361. doi:10.1002/jmri.20808
27. Michael WH Kirkness, Kathrin Lehmann, Nancy R Forde, Mechanics and structural stability of the collagen triple helix, Current Opinion in Chemical Biology, 2019, 53,98-105, ISSN 1367-5931,
28. Liu, C.; Yang, Q.; Fang, G.; Li, B. S.; Wu, D. B.; Guo, W. J.; Hong, S. S.; Hong, L., Collagen metabolic disorder induced by oxidative stress in human uterosacral ligament-derived fibroblasts: A possible pathophysiological mechanism in pelvic organ prolapse. Mol. Med. Report. 2016, 13 (4), 2999-3008. DOI: 10.3892/mmr.2016.4919
29. Chowdhury, A.; Sarkar, J.; Chakraborti, T.; Pramanik, P.K.; Chakraborti, S. Protective role of epigallocatechin-3-gallate in health and disease: A perspective. Biomed. Pharmacother. 2016, 78, 50–59. [CrossRef] [PubMed]
30. Chacko,S. M., P. T. Thambi, R. Kuttan, and I. Nishigaki, "Beneficial effects of green tea: a literature review," Chinese Medicine, vol. 5, article 13, 2010
31. Rice-Evans C. A. , N. J. Miller, and G. Paganga, "Structure-antioxidant activity relationships of flavonoids and phenolic acids," Free Radical Biology and Medicine, vol. 20, no. 7, pp. 933–956, 1996.
32. Chung,J. E., M. Kurisawa, Y.-J. Kim, H. Uyama, and S. Kobayashi, "Amplification of antioxidant activity of catechin by polycondensation with acetaldehyde," Biomacromolecules, vol. 5, no. 1, pp. 113–118, 2004.
33. Tipoe,G. L., T.-M. Leung, M.-W. Hung, and M.-L. Fung, "Green tea polyphenols as an anti-oxidant and anti-inflammatory agent for cardiovascular protection," Cardiovascular and Hematologi cal Disorders—Drug Targets, vol. 7, no. 2, pp. 135–144, 2007.
34. Chu C., DengJ., Man Y., and QuY. Green Tea Extracts Epigallocatechin-3-gallate for Different Treatments Hindawi BioMed Research International Volume 2017, Article ID 5615647, 9 pages, https://doi.org/10.1155/2017/5615647
35. Payne A, Nahashon S, Taka E, Adinew GM, Soliman KFA. Epigallocatechin-3-Gallate (EGCG): New Therapeutic Perspectives for Neuroprotection, Aging, and Neuroinflammation for the Modern Age. Biomolecules. 2022 Feb 25;12(3):371. doi: 10.3390/biom12030371. PMID: 35327563; PMCID: PMC8945730.
36. Kumar R, Sharma A, Kumari A, Gulati A, Padwad Y, Sharma R. Epigallocatechin gallate suppresses premature senescence of preadipocytes by inhibition of PI3K/Akt/mTOR pathway and induces senescent cell death by regulation of Bax/Bcl-2 pathway. Biogerontology. 2019 Apr;20(2):171-189. doi: 10.1007/s10522-018-9785-1. Epub 2018 Nov 19. PMID: 30456590.
37. Rice-Evans, C. A, N. J. Miller, and G. Paganga, "Structure-antioxidant activity relationships of flavonoids and phenolic acids," Free Radical Biology and Medicine, vol. 20, no. 7, pp. 933–956, 1996.
38. Miller, E. J.; Rhodes, R. K., Preparation and characterization of the different types of collagen. Methods Enzymol. 1982, 82, 33-64.
39. KomsaPenkova, R.; Spirova, R.; Bechev, B., Modification of Lowry's method for collagen concentration measurement. J. Biochem. Biophys. Methods 1996, 32 (1), 33-43. DOI: 10.1016/0165-022x(95)00046-t
40. Kuznetsova, N.; Leikin, S., Does the triple helical domain of type I collagen encode molecular recognition and fiber assembly while telopeptides serve as catalytic domains? Effect of proteolytic cleavage on fibrillogenesis and on collagen-collagen interaction in fibers. J. Biol. Chem. 1999, 274 (51), 36083-36088. DOI: 10.1074/jbc.274.51.36083
41. Doyle, A. D., Fluorescent Labeling of Rat-tail Collagen for 3D Fluorescence Imaging. Bio-Protocol 2018, 8 (13). DOI: 10.21769/BioProtoc.2919
42. Komsa-Penkova, R.; Koynova, R.; Kostov, G.; Tenchov, B., Discrete reduction of type I collagen thermal stability upon oxidation. Biophys. Chem. 2000, 83 (3), 185-195. DOI: 10.1016/S0301-4622(99)00135-0 2 32
43. Arezki Boudaoud, Agata Burian, Dorota Borowska-Wykręt, Magalie Uyttewaal, Roman Wrzalik, Dorota Kwiatkowska & Olivier Hamant. FibrilTool, an ImageJ plug-in to quantify fibrillar structures in raw microscopy images Nature Protocols 753volume 9, pages457–463 (2014) 75
44. Jedeszko, C.; Sameni, M.; Olive, M. B.; Moin, K.; Sloane, B. F., Visualizing Protease Activity in Living Cells: From Two Dimensions to Four Dimensions. Curr. Protoc. Cell Biol. 2008, 39 (1), 4.20.1-4.20.15. DOI: 10.1002/0471143030.cb0420s39
45. Llopis-Hernandez, V.; Rico, P.; Moratal, D.; Altankov, G.; Salmeron-Sanchez, M., Role of Material-Driven Fibronectin Fibrillogenesis in Protein Remodeling. Bioresearch Open Access 2013, 2 (5), 364-373. DOI: 10.1089/biores.2013.0017
46. Toromanov, G.; Gugutkov, D.; Gustaysson, J.; Planell, J.; Salmeron-Sanchez, M.; Altankov, G., Dynamic Behavior of Vitronectin at the Cell-Material Interface. Acs Biomaterials Science & Engineering 2015, 1 (10), 927-934. DOI: 656 10.1021/acsbiomaterials.5b00147
47. Bonnans, C., Chou, J. & Werb, Z. Remodelling the extracellular matrix in development and disease. Nat Rev Mol Cell Biol 15, 786–801 (2014). https://doi.org/10.1038/nrm3904
48. Topol,H., Demirkoparan,H., PenceT. J. Fibrillar Collagen: A Review of the Mechanical Modeling of Strain-Mediated Enzymatic Turnover Appl. Mech. Rev. 2021, 73(5): 050802 (29 pages).

49. Daley WP, Peters SB, Larsen M. Extracellular matrix dynamics in development and regenerative medicine. J Cell Sci. 2008 Feb 1;121(Pt 3):255-64. doi: 10.1242/jcs.006064. PMID: 18216330.
50. Rodríguez, D., Morrison, C.J., Overall, C.M., Matrix metalloproteinases: what do they not do? New substrates and biological roles identified by murine models and proteomics. Biochim. Biophys. Acta 2010, 1803 (1), 39–54.
51. Gonçalves, R. V. E.; Costa, A. M. A. E.; Grzeskowiak, L. E., Oxidative Stress and Tissue Repair: Mechanism, Biomarkers, and Therapeutics (Special Issue in: Oxidative Medicine and Cellular Longevity). Hindawi: 2019.
52. Burd, C., and Cullen, P. J. (2014). Retromer: a master conductor of endosome sorting. Cold Spring Harb. Perspect. Biol. 6:a016774. doi: 10.1101/cshperspect. a016774
53. Y. Tu, L. Zhao, D.D. Billadeau, D.Jia, Endosome-to-TGN trafficking: Organellevesicle and organelle-organelle inter-actions, Front. Cell Dev. Biol. 8 (2020), DOI=10.3389/fcell.2020.00163; URL=https://www.frontiersin.org/article/10.3389/fcell.2020.00163
54. Kim HS, Quon MJ, Kim JA. New insights into the mechanisms of polyphenols beyond antioxidant properties; lessons from the green tea polyphenol, epigallocatechin 3-gallate. Redox Biol. 2014;2:187-195. Published 2014 Jan 10. doi:10.1016/j.redox.2013.12.022