Effect of selected flavonoids on glycosaminoglycans in human skin fibroblasts

Wosek J.[1, A,B,C], Kuźmicz I.[2, B,C], Wiśniewska R.[3,C,E,F], Nazaruk J.[4, C,G], Galicka A,[1, A-F*]

1. Department of Medical Chemistry, Medical University of Bialystok, Poland
2. Laboratory of Cosmetology, Medical University of Bialystok, Poland
3. Department of Pharmacology, Medical University of Bialystok, Poland
4. Department of Pharmacognosy, Medical University of Bialystok, Poland

---

A - Conception and study design, **B** - Data collection, **C** –Data analysis, **D** - Writing the paper, **E** – Review article, **F** - Approval of the final version of the article

---

ABSTRACT

Purpose: Glycosaminoglycans (GAGs) and proteoglycans (PG) in addition to collagen are the main components of extracellular matrix (ECM). They play an important role in intercellular communication and interactions between cells and ECM. The biological changes in ECM that occur during aging are induced by decrease in GAG biosynthesis. The purpose of this study was to evaluate the effect of selected flavonoids isolated from *Cirsium palustre* (L.) Scop. on GAG content in human skin fibroblasts.

Materials and methods: Human skin fibroblasts were treated with eriodictyol 7-O-glucoside (C1), 6-hydroxyluteolin 7-O-glucoside (C2), scutellarein 7-O-glucoside (C3) and pedalitin (C4) at 1, 20 and 40 μM for 24 h. Concentration of GAGs in the medium was assayed using method based on their ability to bind the cationic dye 1,9-dimethylmethylene blue (DMMB).

Results: C1, C2 and C4 at concentration of 20 and 40 μM significantly increased content of sulphated GAGs in the medium. In contrast, treatment of cells with compound C3 did not have a statistically significant impact on GAG level. Ascorbic acid used as a positive control at 50 μM showed no effect on GAG concentration and increased their content at 100 μM but to a much lower extent than flavonoids.

Conclusion: Flavonoids C1, C2 and C4 showed greater than ascorbic acid stimulatory impact on GAGs in healthy human skin fibroblasts, demonstrating their therapeutic potential in the aging.

Key words: Fibroblasts, glycosaminoglycans, flavonoids

DOI: 10.5604/01.3001.0009.5049

***Corresponding author:**
Anna Galicka
Department of Medical Chemistry, Medical University
ul. Mickiewicza 2A, 15-222, Białystok, Poland
Tel.: 857485675; e-mail: angajko@umb.edu.pl

Received: 05.10.2016
Accepted: 22.10.2016
Progress in Health Sciences
Vol. 6(2) 2016 pp 59-63

INTRODUCTION

Glycosaminoglycans (GAGs) are linear polymers of repeated disaccharides, in most cases containing an O-sulphated N-acetylhexosamine and an hexuronic acid, commonly expressed in a variety of tissues. They occur mainly in extracellular matrix (ECM) and associated with the cell membrane [1,2]. GAG family include unsulphated hyaluronic acid (HA), sulphated dermatan sulphate (DS), keratin sulphate (KS), heparin sulphate (HS) and chondroitin sulphates (C4S, C6S). Polysaccharides are very heterogeneous in terms of relative molecular mass, charge density, physicochemical properties as well as biological and pharmacologic activities [3,4]. The specific pattern of their sulphation allow GAGs various electrostatic interactions with many biological molecules and ECM constituents like growth factors and enzymes which facilitate their physiological functions and in some cases prevent their proteolytic degradation [4,5]. Most sulfated GAG chains are components of proteoglycans (PGs) where are they covalently linked to core protein. PGs as major macromolecular components are located at the cell surface and in the extracellular matrix [6,7]. Interactions of these macromolecules with other ECM constituents contribute to the general architecture and permeability properties of the basement membrane. Furthermore, these macromolecules play a role in cellular adhesion, growth, migration and differentiation [6-9].

Flavonoids exhibit multiple biological activities, i.e. antibacterial, antiviral, antioxidant, anti-inflammatory, antiallergic, hepatoprotective, antithrombotic and anti-carcinogenic [10,11].

In the previous study we examined effect of selected flavonoids isolated from *Cirsium palustre* (L.) Scop., marsh thistle (family Asteraceae) on collagen as the main structural component of the ECM [12]. The aim of current research was to evaluate impact of these compounds on other significant components of ECM which are GAGs.

MATERIALS AND METHODS

Plant material

The compounds: eriodictyol 7-O-β-glucoside (C1), 6-hydroxyluteolin 7-O-β-glucoside (C2), scutellarein 7-O-β-glucoside (C3) and pedalitin (C4) were isolated from *Cirsium palustre* (L.) Scop., marsh thistle (family *Asteraceae*) as described previously [12].

Estimation of biological action of flavonoids in skin fibroblasts

Human skin fibroblasts purchased from the American Type Culture Collection, (Manassas, Virginia, USA) were cultured in DMEM supplemented with 10 % FBS, 2 mM glutamine, penicillin (50 U/ml), and streptomycin (50 μg/ml). For experiments, cells were plated at a density of $1x10^6$/well in 6-well culture plates (Costar).

The flavonoids were dissolved in DMSO and added to the medium to a final concentration of 1, 20 and 40 μM. The same concentration of DMSO solution was used as control in order to rule out the possible effect of DMSO on fibroblasts. After 24 h incubation, the exposure medium was used for analysis of GAG content.

GAGs content assay

The assay for GAGs quantification is based on their ability to bind the cationic dye 1,9-dimethylmethylene blue (DMMB) [13,14]. After 24h incubation of fibroblasts with different concentrations of flavonoids the concentrated medium was digested with 50 μg/ml proteinase K in 100 mM solution of K_2HPO_4 pH 8.0 at 56°C overnight. Proteinase K was then inactivated by heating for 10 min at 90°C. Next, 1 ml of DMMB was added to the samples and vigorously vortexed for 30 min to promote complexation of GAGs with DMMB. The GAG/DMMB complex was then separated from solution by centrifugation (12 000 g, 10 min). The pellet was dissolved by adding 1 ml of decomplexation solution and shaking the mixture during 30 min. Absorbance was measured at 656 nm. Sulfated GAGs quantities were determined by comparison with a calibration curve of CS solutions used as standard and normalized to total protein. Protein concentration was measured using BCA Protein Assay Kit (Pierce).

Statistical analysis

The results were subjected to statistical analysis using the one-way analysis of variance (ANOVA) followed by the Duncan's multiple range post hock test. Differences were recognized as statistically significant at $p<0.05$.

RESULTS

Human dermal fibroblasts were exposed to the compounds (C1-C4), which were isolated from methanolic extract of *C. palustre* leaves as described previously [12], at the concentration of 1, 20 and 40 μM each for 24 h. Fig. 1 presents their names and structures. All compounds showed no cytotoxicity to the cells at concentration up to 100 μM (results not shown). GAG quantification is based on their ability to bind the cationic dye DMMB [13,14]. In this assay, we used modified method of Barbosa et al. [14] which has improved specificity, reproducibility and sensitivity as compared to the method of Farndale [13].

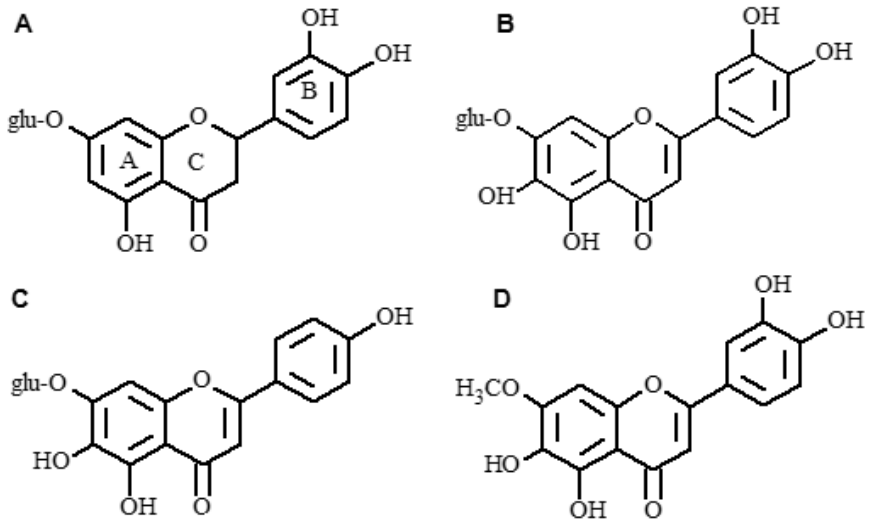

Figure 1. The structure of examined compounds
The following compounds were used in this study: A. eriodictyol 7-O-glucoside (C1), B. 6-hydroxyluteolin 7-O-glucoside (C2), C. scutellarein 7-O-glucoside (C3), and D. pedalitin (C4).

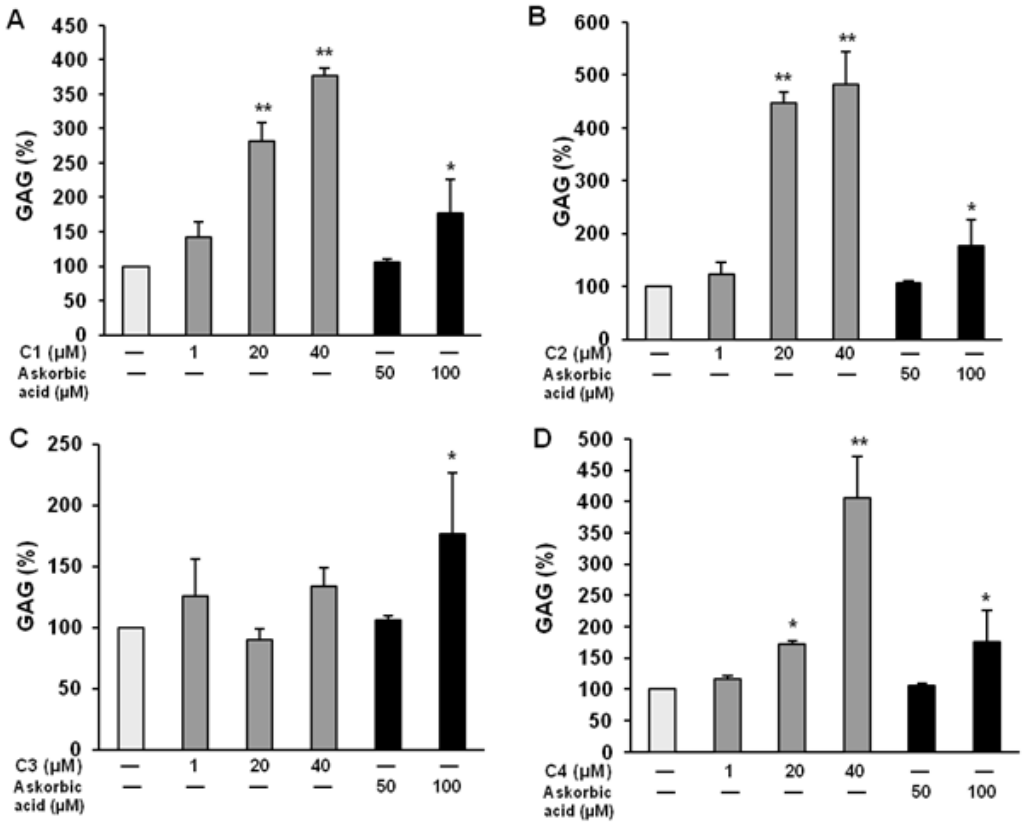

Figure 2. Effect of flavonoids on GAG content in the medium
Skin fibroblasts were treated with compounds C1-C4 at different concentrations (1, 20 and 40 μM) for 24 h. GAG concentration was normalized to total protein and calculated as a percentage of the control (non-treated) medium. Values ± standard deviation (SD) are the mean of three cultures done in triplicates. Statistical significant differences versus respective control were marked with an asterisk (* for $p<0.05$, ** for $p<0.005$).

As shown in Fig. 2 flavonoids C1, C2 and C4 exerted stimulatory effect on GAGs in a dose-dependent pattern. The differences between amount of GAGs in control (DMSO-treated) and compound-treated cells were significant at higher doses of the compounds (20 and 40 μM). Compound C1 increased GAG content 2.8- and 3.7-times at 20 and 40 μM, respectively. Compound C2 showed similar stimulatory impact at 20 and 40 μM increasing GAG content about 4-times. In the case of compound C4 1.7- and 4-times increase of GAG content at 20 and 40 μM, respectively was noted. In contrast, treatment of cells with compound C3 did not have a statistically significant effect on GAG level as compared to control. Ascorbic acid as known antioxidant was simultaneously used in experiments as a positive control. At 50 μM it showed no effect on GAG concentration, but incubation of cells in the presence of 100 μM increased their content by 76%.

DISCUSSION

The extracellular matrix is composed mainly of proteoglycans, hyaluronic acid, collagens and other glycoproteins, which all interact with each other. Proteoglycans and their attached glycosaminoglycans are closely associated with collagen fibers and these interactions play a dominant role in the maintenance of tissue structure and cell adhesion [15,16]. GAGs with negatively charged residues can interact with basic amino acids of proteins and change their conformation. Because of their potential reactivity with other components, they play important biological role in cell–cell and cell–matrix interactions during normal and pathological conditions. Furthermore, they have been shown to regulate protein secretion, enzyme activity and gene expression in certain tissues by mechanisms involving both membrane and nuclear events, including the binding of GAGs to transcription factors [3,4,8,9].

GAGs posses the ability to bind water and therefore are responsible for skin hydration, elasticity and firmness. Synthesis and degradation of the main ECM constituents are influenced by a number of internal factors (vitamins, mineral compounds, hormones, metalloproteinase inhibitors - TIMPs) as well as external ones (UV radiation, environment, stress) [17,18]. Skin exposure to UV radiation contributes to cellular imbalance, oxidative stress, stimulation of pro-inflammatory cytokines, increase MMP activity resulted in increased fragmentation of collagen fibres and impaired function of fibroblasts [17]. In these conditions as well as with age the content of glucosaminoglycans and collagen considerably decreases [17-20]. Breen et al. [21] reported that there was an decrease in total glycosaminoglycan (HA and DS) content in adult skin relative to fetal skin. Takeda et al. [22] studied an age-related changes in PG and collagen synthesis in skin fibroblasts of donors in different age. They found decrease in collagen expression at both protein and mRNA level as well as impaired proteoglycan synthesis, whereas overall protein synthesis was slightly affected.

The mostly studied group of natural compounds with great potential antioxidant properties and relatively low toxicity are flavonoids. They are widely distributed in plants. *Cirsium palustre* (L.) Scop., marsh thistle (family Asteraceae, subtribe Cardueae) is a herbaceous biennial plant widely distributed in Europe and found on marshes, hedgerows and moorland pastures. Nazaruk and Jakoniuk [23] demonstrated antimicrobial activity of the polyphenolic constituents extracted from this plant.

In our previous study, we have examined the effect of the compounds C1-C4 obtained from the methanolic extract of *C. palustre* leaves, during multistep chromatographic separations, on collagen expression in human skin fibroblasts [12]. Among them 6-hydroxyluteolin 7-O-glucoside (C2) at concentration of 40 μM and pedalitin (C4) at all used concentrations significantly enhanced amount of collagen in the medium. In current study we showed that the same compounds exerted significant stimulatory impact on GAG content at concentration of 20 and 40 μM. In contrast, eriodictyol 7-O-glucoside (C1) affected these macromolecules differently increasing significantly GAGs (at 20 and 40 μM) without influence on collagen level. Treatment of cells with scutellarein 7-O-glucoside (C3) showed opposite effects with tendency to increase GAG and decrease collagen concentration but the differences in treated and non-treated cells were not significant. The analysis of relationship between the structure of the compounds and their activity suggests that the most important factor influencing the activity seems to be the presence of two hydroxyl groups in the ring B (Fig. 1) since C3 with one –OH group not revealed its activity or to GAGs or to collagen.

Ascorbic acid is a natural anti-oxidant and protects the skin against the harmful effects of free radicals. As a cofactor of prolyl hydroxylase is essential in biosynthesis of collagen and elastin [24]. Except of up-regulation of collagen and collagen-degrading inhibitors (TIMPs), its stimulatory action on GAGs synthesis in fibroblasts was demonstrated [25]. In our study, we used as a positive control. However, flavonoids showed significantly higher stimulatory activity and at lower concentration than ascorbic acid.

Summarizing, in light of these data, flavonoids C1, C2 and C4 may be considered in the future as a therapeutic option in the prevention of biological alterations in the skin aging.

Funding

This study was conducted with the use of equipment purchased by the Medical University of Bialystok as part of the OP DEP 2007-2013, Priority Axis I.3, contract No POPW.01.03.00-20-008/09.

Conflicts of interest

The authors declare that they have no conflicts interests.

REFERENCES

1. Beaty NB, Mello RJ. Extracellular mammalian polysaccharides: glycosamino-glycans and proteoglycans. J Chromatogr. 1987 Jul; 418:187-222 .
2. Hook M, Kjellen L, Johansson S, Robinson J. Cell–surface glycosaminoglycans. Annu Rev Biochem. 1984;53:847–69
3. Comper WD, Laurent TC. Physiological function of connective tissue polysaccharides. Physiol Rev. 1978 Jan;58(1):255–316.
4. Jackson RL, Busch SJ, Cardin AD. Glycosaminoglycans: molecular properties, protein interactions, and role in physiological process. Physiol Rev. 1991 Apr;71(2):481-539.
5. Gallagher JT, Turnbull JE, Lyon M. Patterns of sulphation in heparan sulphate: polymorphism based on a common structural theme. Int J Biochem. 1992 Apr;24(4):553-60.
6. Hardingham TE, Fosang AJ. Proteoglycans: many forms and many functions. FASEB J. 1992 Feb;6(3):861-70.
7. Iozzo RV. Matrix proteoglycans: from molecular design to cellular function. Annu Rev Biochem. 1998;67:609–52.
8. Lyon M, Gallagher JT. Bio-specific sequences and domains in heparan sulphate and the regulation of cell growth and adhesion. Matrix Biol. 1998 Nov;17(7):485-93.
9. Prydz K, Dalen KT. Synthesis and sorting of proteoglycans. J Cell Sci. 2000 Jan;113 Pt2: 193–205.
10. Heim KE, Tagliaferro AR, Bobilya DJ. Flavonoid antioxidants: chemistry, metabolism and structure-activity relationnships. J Nutr Biochem. 2002 Oct;13(10):572-84.
11. Havsteen BH. The biochemistry and medical significance of the flavonoids. Pharmacol Therapeut. 2002 Nov-Dec;96(2-3):67–202.
12. Nazaruk J, Galicka A. The influence of selected flavonoids from leaves of *Cirsium palustre* (L.) Scop. on collagen expression in human skin fibroblasts. Phytother Res. 2014 Sep;28(9):1399-405.
13. Farndale RW, Sayers CA, Barrett AJ. A direct spectrophotometric microassay for sulfated glycollsaminoglycans in cartilage cultures. Connect Tissue Res. 1982;9(4):247-8.
14. Barbosa I, Garcia S, Barbier-Chassefiere V, Caruelle JP, Martelly I, Papy-Garcia D. Improved and simple micro assay for sulfated glycosaminoglycans quantification in biological extracts and its use in skin and muscle tissue studies. Glycobiology 2003 Sep; 13(9):647-53.
15. Scott JE. Proteoglycan-fibrillar collagen interactions. Biochem J. 1988 Jun;252(2):313-23.
16. Munakata H, Takagaki K, Majima M, Endo M. Interaction between collagens and glycosaminoglycans investigated using a surface-plasmon resonance biosensor. Glycobiology. 1999 Oct;9(10):1023-7.
17. Fisher GJ, Wang ZQ, Sibhash SC, Varani J, Kang S, Voorhees JJ. Pathophysiology of premature skin aging induced by ultraviolet light. N Engl J Med. 1997 Nov;337(20):1419–28.
18. Tanaka H, Okada T, Konishi H, Tsuji T. The effect of reactive oxygen species on the biosynthesis of collagen and glycosaminoglycans in cultured human dermal fibroblasts. Arch Dermatol Res. 1993;285(6):352-5.
19. Schachtschabel DO, Wever J. Age-related decline in the synthesis of glycosaminoglycans by cultured human fibroblasts (WI-38). Mech Ageing Dev. 1978 Oct;8(4):257-64.
20. Lee DH, Oh JH, Chung JH. Glycosaminoglycan and proteoglycan in skin aging. J Dermatol Sci. 2016 Sep;83(3):174-81.
21. Breen M, Johnson RL, Sittig RA, Weinstein HG, Veis, A. The acidic glycosaminoglycans in human fetal development and adult life: Cornea, sclera and skin. Connect Tissue Res. 1972 Jul;1(4):291–303.
22. Takeda K, Gosiewska A, Peterkofsky B. Similar, but not identical, modulation of expression of extracellular matrix components during in vitro and in vivo aging of human skin fibroblasts. J Cell Physiol. 1992 Dec;153(3):450–9.
23. Nazaruk J, Jakoniuk P. Flavonoid composition and antimicrobial activity of Cirsium rivulare (Jacq.) All. flowers. J. Ethnopharmacol. 2005 Nov;102(2):208-12.
24. Nusgens BV, Humbert P, Rougier A, Colige AC, Haftek M, Lambert CA, Richard A, Creidi P, Lapiere CM. Topically applied vitamin C enhances the mRNA level of collagens I and III, their processing enzymes and tissue inhibitor of matrix metalloproteinase 1 in the human dermis, J Invest Dermatol. 2001 Jub;116(6):853–9.
25. Kao J, Huey G, Kao R, Stern R. Ascorbic acid stimulates production of glycosaminoglycans in cultured fibroblasts. Exp Mol Pathol. 1990 Aug;53(1):1-10.