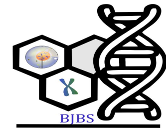

Possible Participation of Glycosaminoglycans' Concentrations in Age-Related Inversion of Structural and Functional Properties of Connective Tissues in Postnatal Ontogenesis

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Abstract

Concentrations of the different types of glycosaminoglycans in most parts of postnatal ontogenesis rises; this is considered as one of the response mechanisms of adaptation of connective tissue mechanical properties to an increase in body weight and muscular strength of an organism in the course of its growth and development. Investigations were carried out using the skin of twenty (20) 1-, 3-, 12- and 24-months old male Wistar rats, 5 in each age group. The aim of the study was to investigate the metabolic justifications (changes in glycosaminoglycans' concentrations) to the decrease in the structural stability of matrix sub-molecular complexes of the connective tissue in early postnatal ontogenesis. The different glycosaminoglycan fractions were estimated using ion exchange chromatography. It was found out that, concentrations of the different types of these connective tissue macromolecules continuously increased. It was therefore, concluded that, because of the various dynamics of their metabolism in ontogenesis, at the age of 3-months, changes led to increases in the contents of glycosaminoglycans, on which to a large extent, depend the viscous properties of tissues.

Key words: Skin, Glycosaminoglycans, Structural Stability, Ontogenesis.

Introduction

Glycosaminoglycans (GAG) (essentially hyaluronic acid) form a large group of polysaccharide-rich and/or protein-rich macromolecules that are located in the strongly hydrated matrix ground substance in basement membranes, at cell surfaces, and even in sub-cellular compartments; they are unbranched heteropolysaccharides that are built through a standard principle; they consist of repeating disaccharide derivatives, including amino sugars, hexosamines (sulphated and acetylated) and uronic acids (D-glucuronic or L-iduronic). Galactose, galactosamine, N-acetylgalactosamine-4-sulphate and galacturonic acid are also common components of GAGs. Sulphate groups in sulphated amino sugars can be attached to glycosaminoglycans via an oxygen atom (O-Sulphation) or via a nitrogen atom (N-Sulphation).[1,2,3] The number of repeated disaccharides varies and therefore, the different forms of connective tissues have varying proportions of glycosaminoglycans. The determination of the collagen and glycosaminoglycan (GAG) contents in native and bioengineered tissues is of considerable interest because the collagen-to-GAG ratio determines the water content of the tissue, which is crucial regarding its mechanical properties, such as mechanical resilience and shock absorption (cartilage) function; it also explains the lubricating properties of certain GAGs (hyaluronic acid, synovial fluid).[2,4] Such complexes of polysaccharides and polypeptides are used in the

Date received-30/08/2016 Date accepted- 20/10/2016

cosmetic industry as compounds of emulsion cosmetic for the skin and hair.[2,4]

Age-related peculiarities in the content of structural biopolymers and the entire structure of the extracellular matrix eventually lead to changes in the functional properties of all types of connective tissue.[5] Not only changes in the concentrations of collagen and elastin determine the structure and functional properties of connective tissues, polysaccharides (glycosaminoglycans), some parts of which are structural components of proteoglycans, and rest part, are present in the amorphous ground substance, also do contribute in the determination of structure and functional properties of connective tissues.[6] Thus, concentration of the major connective tissue macromolecules - collagen; content of hydroxyproline in individual collagen and elastin molecules; separate fractions of glycosaminoglycans – hyaluronic acid, chondroitin-4-sulphate, chondroitin-6-sulphate, heparan sulphate, and dermatan sulphate; degree and types of collagen cross covalent bondings as well as its visco-elastic properties and thermostability are the basic parameters of structural stability of matrix sub-molecular collagen complexes.[5]

Hydroxyproline is a major component of collagen, comprising around 13.5% of its amino acid composition;[15,16,17] concentration of this imino acid was determined using a calibration graph. Content of *de novo* synthesized collagen was calculated using the conversion factor 13.5.[19]

The aim of this study was to investigate the metabolic justifications (changes in glycosaminoglycans' concentrations) to the decrease in the structural stability of matrix sub-molecular complexes of the connective tissue in early stages of postnatal ontogenesis. There is a small period in the beginning of this developmental moment, when the structural stability of connective tissue formations discussed, does not increase with age but rather decreases and afterwards continuously rises. It is therefore, expedient to deeply study the aforementioned phenomenon as well as the consequential biological role in early ontogenesis. To achieve this aim, the following tasks were set as objectives:

- Determine the content of collagen bound to glycosaminoglycans in the skin of rats in each age group.
- Determine the concentration of the different types of glycosaminoglycans (Hyaluronic acid, chondroitin, dermatan and heparan sulphates) in the skin of rats in each age group by way of ion exchange chromatography.
- Calculate collagen:glycosaminoglycan ratio in each age group.

Materials and Methods

Design of the Study

Investigations were carried out using the skin of twenty (20) male Wistar rats[5] that were divided into four (4) groups:

Group 1 (Control) consisted of five (5) 1--month old male rats.
Group 2 consisted of five (5) 3--months old male rats.
Group 3 consisted of five (5) 12--months old male rats.
Group 4 consisted of five (5) 24--months old male rats.

Experiments were carried out in the Department of Human and Animal Physiology of the V. N Grazing Kharkov National University, Ukraine from where clearance on the rules contained in treating animals was obtained, in accordance with International principles of the European Convention «On protection of vertebrate animals used in experiments and other scientific works».[7] Standards of biomedical ethics, in accordance with the Law of Ukraine «On protection of animals from man-handling» were as well followed.[8]

Briefly after obtaining informed consent, Wistar rats were lulled to sleep by intravenous injection of 0.5cm^3 of 0.4% solution of sodium thiopental[9] and then decapitated. Incubated samples were cleaned up from hair and dermal fatty layer using acetone and diethyl ether for 24 hours in each case, and subsequently ground in liquid nitrogen to powder form.

Extraction of Glycosaminoglycans (GAGs)

Glycosaminoglycans were isolated by enzymatically digesting powdered skin samples by type IA collagenase (from *Clostridium histolyticum*, Sigma,

USA) in sodium phosphate buffer solution, pH 7.4.[10] Content of collagen-bound GAGs was determined through extracting type I collagen from obtained skin powder by I.0 M solution of NaCl[11, 12] by way of its hydroxyproline concentration after its precipitation by cold (up to 4°C) acetone. After hydrolysis, GAGs were precipitated by adding (1:10) 2% cetylpyridinium chloride (Merck, USA) to the supernatant.

Analytical Determination of Collagen Concentration

Although other techniques using assay kits are mostly used nowadays, we estimated the content of hydroxylproline using a relatively rapid quantitative oxidation and condensation method earlier proposed by[13] as modified by.[14] This is a method based on the oxidation of free hydroxyproline by chloramine 'B' to pyrole; as well as the reaction of pyrole with para-dimethyl-amino-benzo-aldehyde (DABA) to produce a pink coloured complex. Samples were digested in glass ampoules in which equal volumes of 12 N solution of HCl were added. Ampoules were sealed and hydrolyzed at 130^0C for 6 hours.[15,16] The resulting hydrolyzates were neutralized with 1N solution of NaOH, monitoring the pH with universal indicator paper. Neutralized hydrlyzates were used to carry out colour reaction.

Colour Reaction

Reagents

№ 1 - Stock Hydroxyproline Standard

1mmol of hydroxyproline (13.113mg) was in $100cm^3$ of water and stored at 4°C. Working standards ranging from 0.05 to 2.0 mmol were prepared immediately before use.

№ 2 – Stock (Initial) Buffer Solution for Chloramine B

50g of citric acid, 120g of sodium acetate, 34g of NaOH were weighed and diluted to the $100cm^3$ mark of one litre volumetric flask with distilled H_2O.

№ 3 - Working Buffer Solution

This was freshly prepared by mixing $500cm^3$ of the stock buffer solution, $150cm^3$ of n-propanol, 84 cm^3 of 96% ethanol, and $100cm^3$ of H_2O.

№ 4 – Solution of Chloramin B

Chloramine B was prepared immediately before use: 1.41g was dissolved in a mixture of 10 cm^3 of n-propanol, $84cm^3$ of working buffer solution, № 2 and $10cm^3$ of H_2O.

№.5–Para-dimethyl-amino-benzo-aldehyde (DABA) was prepared by weighing and dissolving 15mg of DABA in $26cm^3$ of 60% perchloric acid and n-propanol, all adjusted to $100cm^3$ with n-propanol. This solution was immediately prepared prior to carrying out colour reaction.

Procedure for the Determination of Hydroxyproline

$1cm^3$ of chloramin B solution was added to $2cm^3$ of sample solution containing neutralized hydrolyzate, mixed well and allowed to stand for 20 minutes at room temperature. During this time, $0.5cm^3$ of n-propanol were added to resulting mixtures and agitated thoroughly. After 20 minutes, $1cm^3$ of DABA, and hydrogen perchlorate solutions were added, and the resulting solutions were thoroughly mixed and heated in a water bath (ultra-thermostat U-5) for 20 minutes at 60°C. The obtained coloured solutions were cooled to room temperature and optical densities measured on a colorimetric apparatus KFK-2MP by recording the absorption difference at λ = 540nm between the sample and the reference, in cuvetes of 0.5cm thick.

Fractionation of Isolated Glycosaminoglycans (GAGs)

Fractionation of isolated GAGs was carried out by ion-exchange chromatography in thermostated titrating column, which was filled with ion-exchange resins (Dowex 1x 2 in Cl-form, 200–400 mesh, Sigma- Aldrich, USA).

Procedure for the Fractionation of Glycosaminoglycans (GAGs)

Each glycosaminoglycan was fractionated by $20cm^3$ of Nacl solution, and $2cm^3$ of eluate was each time collected in a test tube. Hyaluronic acid and chondroitin sulphate contents were determined by way of L-iduronic acid concentration in aliquots using «carbasole reaction». Dermatan sulphate content was determined by way of L-iduronic acid concentration but using «orcine method». [17] After drying samples at room temperature, filters containing them were put in

Beckman's flask and to which toluene scintillators were carefully added. The radioactivity of all samples was estimated on Beckman's counter LC-7800.

Statistical analyses of results were performed using the software package 'Origin Pro 7.5; as well as Chi square test in which *P* value of 0.05 was considered significant.

Results and Discussion

Results of the study are represented in Tables 1, 2 and 3.

Analytical Determination of Collagen and Glycosaminoglycans' Concentrations

Concentrations of the main supporting protein, collagen; as well as that of glycosaminoglycans, which are constituted in proteoglycans and the ground substance of the dermis of the skin, continuously increased in ontogenesis (Tables 1 and 2).[18] This increase in concentrations of all, without exception, structural biopolymers is one of the main reasons of the increase in skin's stiffness and rigidity with ageing. However, an absolute increase in concentrations does not explain the nature of the increase in the elasticity of the skin from 1 to 3 months of life. Similar changes in the mechanical properties of the different types of connective tissue in early postnatal ontogenesis were earlier noticed but were not at all experimentally explained.[12]

As of the relation, total collagen concentration to that of glycosaminoglycans (Table 3), a higher relative content of glycosaminoglycans per cell and the ratio *collagen/GAG*, which is less than one, prove a more intensive synthesis of glycosaminoglycans by fibroblasts compared to collagen.[5,19]

Table 1 Age-related Changes in the Contents of Collagen in Rats' skin (mg/g of fresh tissue).[18]

Protein Type	Groups of Animals			
	1 (Control)	2	3	4
Collagen	9.40±1.63	12.62±0.95*	23.30±1.4*	31.73±2.8*
Elastin	0.79±0.18	1.53±0.25*	2.31±0.41	2.40±0.43

Note. * = significant (p <0.05) relative to control (1 month).

Table 2 Age-related Changes in the Contents of GAGs in Rats' Skin (mg/g of fresh tissue).[18]

Type of GAG	Groups of Animals			
	1 (Control)	2	3	4
Hyaluronic Acid	3.77 ± 0.14	5.07 ± 0.37*	7.84 ± 0.27*	8.60 ± 0.22*
Heparan Sulphate	3.02 ± 0.14	3.73 ± 0.33*	7.19 ± 0.14*	11.14 ± 0.47*
Chondroitin Sulphates	4.83 ± 0.09	5.34 ± 0.55	7.80 ± 0.29*	12.81 ± 0.4*
Dermatan Sulphate	1.64 ± 0.07	3.53 ± 0.29*	5.57 ± 0.5*	6.38 ± 0.21*
Keratan Sulphate	1.14 ± 0.04	3.67 ± 0.24*	5.57 ± 0.31*	9.43 ± 0.32*
Total GAG Concentration, Σ	14.40 ± 0.03	21.63 ± 1.5*	33.97 ± 1.0*	48.36 ± 0.75*

Note. * = significant (p <0.05) relative to control (1 month).

Table 3 Age-related Changes in Concentration Ratios of Total Collagen to Total Glycosaminoglycans in rats' skin (mg/g of fresh tissue).

Parameters	Groups of Animals			
	1	2	3	4
ΣCollagen:ΣGAGs	0.65	0.58	0.61	0.66

Conclusion

Because of the various dynamics of their metabolism in ontogenesis, at the age of 3-months, changes led to increases in the contents of glycosaminoglycans, on which to a large extent, depend on the viscous properties of tissues.

The biological role or significance of this inversion or reversal of the direction of age-related changes in the metabolic properties of the connective tissue lies in the fact that, during this period of development, after a slowdown in growth of the feotus before birth, in early postnatal ontogenesis, during the period up to 3 months, there is a rapid growth of the newly born organism, and intercellular complexes with high structural stability and rigidity are favourable for cellular differentiation and migration, and successful morphogenesis.

Acknowledgements

We are grateful for and acknowledge the tremendous assistance of the technical staff, Department of Human and Animal Physiology of the V. N. Grazing Kharkov National University, Ukraine while carrying out most of the experiments. We are also grateful to Mohammed Sani Bichi, a senior Technologist in the Department of Human Physiology, Faculty of Basic Medical Sciences of the Bayero University, Kano, Nigeria, for the technical support given in carrying out conclusive parts of the experiments.

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