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Age-related Changes in Rat Thymus Connective Tissue Influenced by Aronia Melanocarpa

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The purpose of this study is to determine the influence of Aronia melanocarpa (AM) on macrophage and mast cell quantity and collagen fibres distribution in age-related tissue remodeling of rat thymus. Two control groups, young (CY) – 2 month-old and mature (CO) – 12 month-old, have been put on a standard diet. The rats in the experimental group (A) received 10 *ml/kg* AM juice daily. Histological, immunohistochemical, morphometric and statistical assays were performed. Supplementation with juice from AM resulted in a significant decrease in the amount of collagen fibres, the number of mast cells in interlobular connective tissue and the number of CD68 positive cells in the medulla of rat thymus. Our results show for the first time the effect of AM on the age remodelling of connective tissue in the thymus. These results support the beneficial potential of the nutrient treatment of age-related diseases.

Key words: Aging, thymus, stromal elements, Aronia melanocarpa, rats

Introduction

Aging is a continuous and slow process compromising the morphofunctional characteristics of different organs and systems both in humans and in animals [14]. Chronic, low-grade, systemic inflammation is the primary risk factor for major human chronic diseases, including cardiovascular disorders, cancer, type 2 diabetes and neurodegenerative disease. Increased production of inflammatory mediators which accompanies this process is referred to as "inflammaging"[8].

Regression of the thymus leads to a decline in naive T cells output modifying the composition of the peripheral T cells pool and altering T cells' phenotype and function.

These changes are believed to contribute significantly towards the clinical features of immunosenescence [14].

With age, the thymus suffers changes in its architecture, losing a clear demarcation between cortex and medullary regions, which is related to lymphocyte cell death. Other changes observed in the aging thymus include a decrease in the number and activity of thymic epithelial cells, reducing growth factors production. Moreover, there is a progressive increase of collagen and adipose tissue deposition in the capsule and septa regions. A large corpus of evidence supports the notion that the stromal population in the thymus is a primary target of age-associated thymic dysfunction [2].

Mast cells (MCs) are strategically located at host/environment interfaces and also populate connective tissue in association with blood and lymphatic vessels and nerves. In the last few years literature data has clearly shown that the function of MCs is not limited to acting as first line of defence against invading pathogens or as effector cells in allergy. MCs play a critical role in tissue remodelling, tissue matrix turnover and renewal [15]. In the thymus, MCs are localized in the connective tissue of the capsule and interlobular septa, and inside the thymic lobules. MCs synthesize and release a large panel of growth factors and cytokines, including interleukin (IL) IL-1, IL-2, IL-3, IL-4, IL-6, TNFα, granulocyte-monocyte colony stimulating factors (GM-CSF), and nerve growth factor (NGF), which stimulate thymocyte and thymic epithelial cell functions. In human and chicken thymus, MCs are restricted to the medulla and to connective tissue septa and their number increases in the adult thymus when compared to a foetal thymus [15].

Macrophages are a type of thymic stromal cell involved in phagocytosis, antigen presentation and production of cytokines, which influence T cells proliferation and maturation [10].

Certain plants and spices containing flavonoids have been used for thousands of years in traditional Eastern medicine. Moreover, the inhibitory action on inflammatory cells, especially mast cells, appears to surpass any other clinically available compound. Evidence suggests that only activated cells are susceptible to the modulating effects of flavonoids. The stimulated activities of numerous cell types, including mast cells, basophils, neutrophils, eosinophils, T and B lymphocytes, macrophages, platelets and others, can be influenced by particular flavonoids [9]. Flavonoids are powerful antioxidants and anti-allergic nutrients which inhibit the release of chemical mediators, synthesis of Th2 type cytokines, such as IL-4, and CD40 ligand expression by high-affinity immunoglobulin E (IgE) receptor-expressing cells, such as mast cells and basophils [17]. The anthocyanin fruit AM is ranked first in its antioxidant potential as confirmed by several different methods. A number of *in vitro* and i*n vivo* studies have demonstrated the wide range of applications of the juice extracts or the dry substance of the fruits of AM, as anti-inflammatory, anti-mutagenic, anti-cancerogenic, lipidlowering, antidiabetic, antihypertensive, hepatoprotective, immunomodulatory effects, etc.[4].

Scientific literature provides only limited data on the effect of its application on aging and in particular on the age-related thymus changes.

Aim

The purpose of this study is to determine the influence of AM on macrophages and MCs quantity and collagen fibres distribution in relation with the age-related tissue remodelling of rat thymus.

Material and Methods

The study included 18 male Wistar rats, 12 of them – 9 months of age with initial body weight 350g ± 50; and 6 animals aged 2 months with body weight 100g ± 10. The animals were provided by and bred in the vivarium of the Medical University-Plovdiv under standard laboratory conditions. The rats were divided into 3 groups. Two control groups, defined as young (CY) – 2 month-old and mature (CO) – 12 month-old, have been put on a standard diet and tap water ad libitum. The rats in the experimental group (A) were age matched to the mature controls and received AM juice diluted 1:1 in drinking water in dose 10ml/kg. The experiment lasted for 90 days. The functional beverage from AM fruits was supplied by „Vitanea" Ltd and ITC – Innovative-Technological Centre Ltd., Plovdiv, Bulgaria.

Table 1. Anthocyanin and polyphenol content and antioxidant activity of Aronia melanocarpa juice. Data from the Innovative-Technological Centre Ltd.

	Antocyanins (mg/l)	Polyphenols (mg/l)	ORAC (μmol TE/l)
Aronia 100%	57	4772	55307

The experimental protocol was approved by the Committee on Ethical Treatment of Animals from the Bulgarian Agency for Food Safety (№102/10.07.2014). All animals received humane care in compliance with the "Principles of laboratory animal care" formulated by the National Society for Medical Research and the "Guide for the care and use of laboratory animals" prepared by the National Institute of Health (NIH publication No. 86-23, revised 1996). At the end of the experimental period the animals were euthanized with i.m. Ketamin (90 mg/kg) + Xilazine (10mg/kg). The whole thymus was dissected and was fixed in 10% neutral formalin for further histological examination. Thymus samples were subjected to routine paraffin embedding, cutting and staining with Toluidine blue and Azan (Heidenchain).

Immunoreaction for CD68 was performed using an automated Leica BOND-MAX system. Tissue sections of 4 μm thickness were incubated with mouse anti CD68 (ready to use) monoclonal antibody (Leica Biosystems Newcastle Ltd, United Kingdom).

The areas for morphometric analysis were the interlobular septa of the thymus on tissue sections of 4 μm thickness. On the Azan stained slices a measurement of the relative distribution of the collagen fibers in the interlobular thymic septa per unit area was performed. Slices with Toluidine bluestaining were used to determine the mean distribution of mast cells per unit area, by counting only the mast cells, located in the territory of the interlobular septa. Slices with CD68 immunoreaction were used to determine the mean distribution of CD68 positive cells per unit area, by separately counting the CD68 positive cells in medulla and cortex of the thymus. In all three studies five slices per animal were examined. The measurements were done with the help of software "DP – Soft" 3.2, Olympus, Japan. Microphotographs were performed with Nikon Microphot SA microscope (Japan), combined with Camedia-5050Z digital camera (Olympus, Japan).

Statistical analysis was performed using SPSS software (version 18.0). All data were presented as means ± standard error of mean (SEM) and analysed by one-way ANOVA, followed by Tukey's post hoc test. $P < 0.05$ was considered as statistically significant.

Results

The Azan-stained collagen fibres of the interlobular septa were blue in contrast to the red colour of the thymus parenchyma. In the young control group (CY), the amount of connective tissue in the septa was scarce in contrast to the adult controls (CO) in which septal and perivascular enlargement and the presence of adipose tissue were observed in addition to its increased amount. In the Aronia group (A), the septa were narrower and adipose tissue was almost absent (**Fig. 1**).

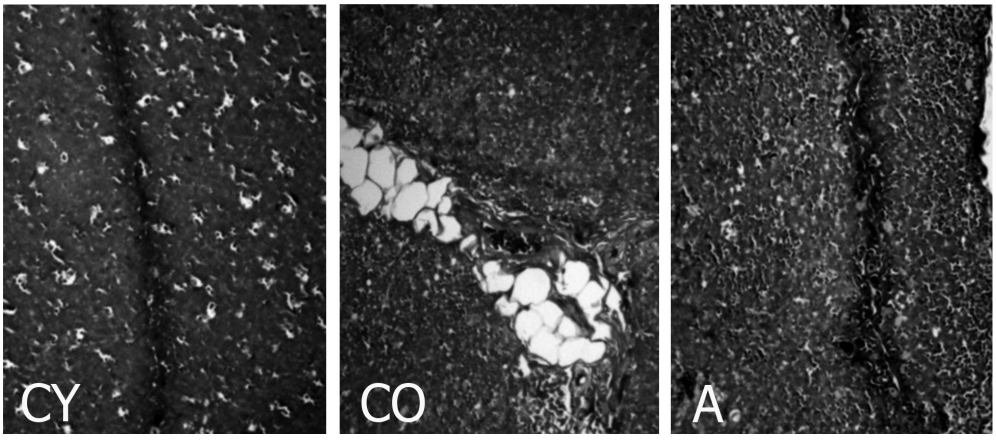

Fig. 1. Histological assay of thymus. Azan (Heidenhain) staining (x 200). Young controls (CY), mature controls (CO), AM supplemented group (A)

Toluidine blue-stained deep purple metachromatic granules were used as a hallmark of mast cells (**Fig. 2**).

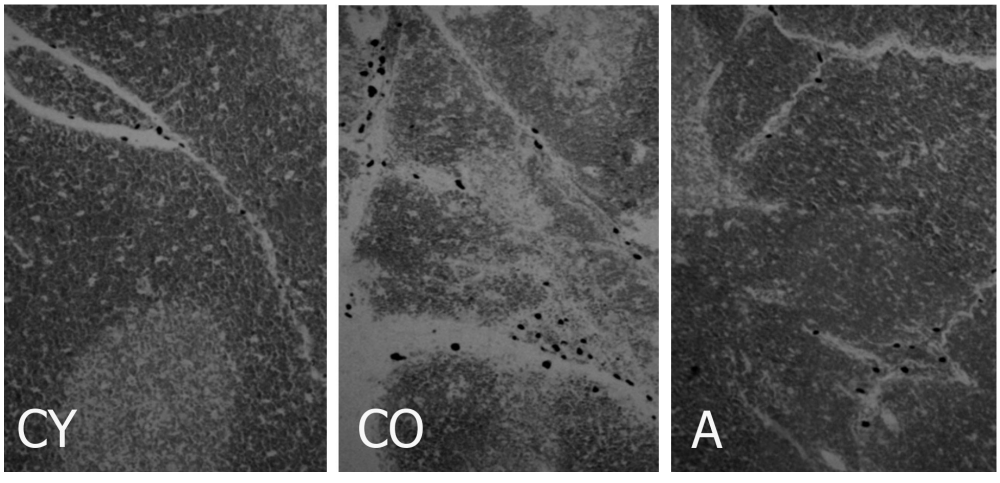

Fig. 2. Histological assay of thymus. Toluidine bluestaining (×100).Young controls (CY), mature controls (CO), AM supplemented group (A)

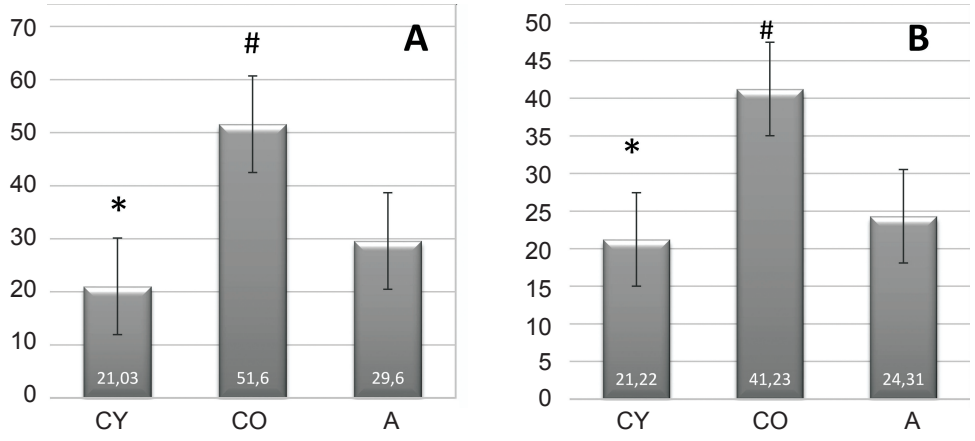

Fig. 3. Results from morphometric analysis: A: Relative distribution of connective tissue in thymus septa, * CY v/s CO, ($p<0.05$), # CO v/s A, ($p<0.05$).B: Mean distribution of mast cells in thymic septa. * CY v/s CO, ($p<0.05$), #CO v/s A, ($p<0.05$).

The morphometric evaluation demonstrated that the MCs number increased significantly in adult thymus compared with young thymus (**Fig. 3 B**). As a result of the supplementation, the number of mast cells has decreased significantly in the experimental group (A) compared to the mature controls (CO). Decrease of the amount of collagen connective tissue in the interlobular thymus septa corresponds with the lower number of MCs (**Fig. 3 A**).

Fig. 4 demonstrates CD68 positive cells with the cytoplasm of a dark-brown color. The immunohistochemical reaction represents macrophages on the background of the hematoxilin countrastained thymocyte nuclei.

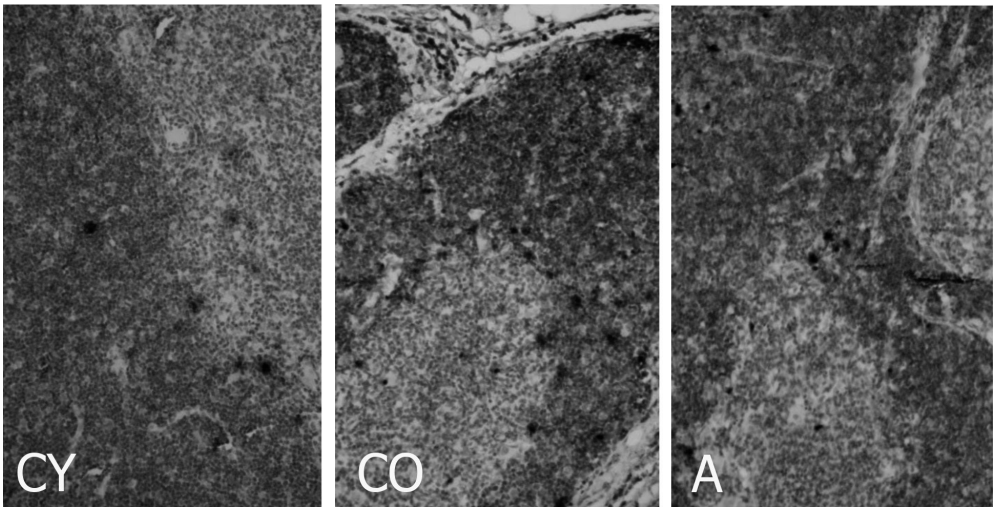

Fig. 4. CD68 immunoreaction in rat thymus (x 400).Young controls (CY), mature controls (CO), AM supplemented group (A)

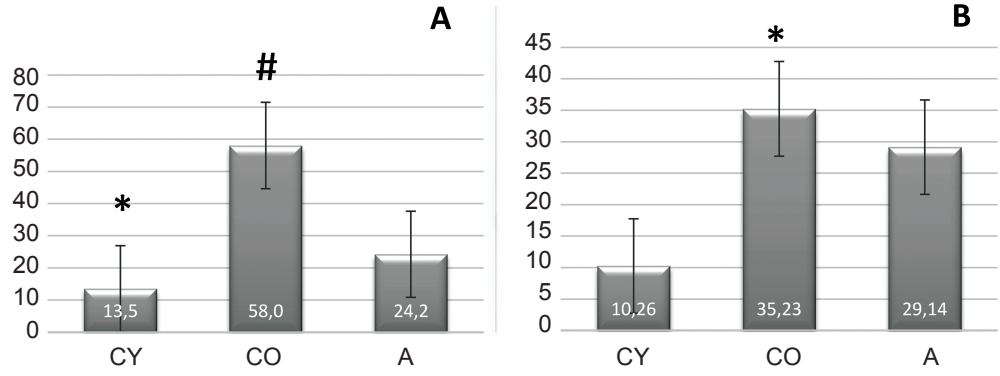

Fig. 5. Mean distribution of CD68 immunopositive cells in thymus. A: CD68 in thymic medulla *CY v/s CO, ($p<0.05$)#CO v/s A, ($p<0.05$) B: CD68 in thymic cortex *CO v/s CY, ($p<0.05$)

The morphometric evaluation demonstrated that the number of CD68 immunopositive cells in thymic medulla increased significantly in adult thymus compared with young thymus (**Fig. 5A**). As a result of the supplementation, the number of CD68 immunopositive cells has decreased significantly in the experimental group (A) compared to the mature controls (CO), ($p<0.05$).

In adult controls thymic cortex (CO), (**Fig. 5 B**) the number of CD68 immunopositive cells has increased significantly compared to young controls (CY), ($p<0.05$).The group of AM supplemented animals (A) showed a decreased number of CD68 immunopositive cells in thymic cortex, but differences with adult controls (CO) didn't reach significance.

Discussion

Our results showed that the MCs number has increased significantly in an adult thymus compared to a young thymus and coincide with the studies of other authors [13]. The decrease in the amount of collagen connective tissue in the interlobular thymus septa of the supplemented group (A) corresponds with the lower number of MCs in the same group. According to Langhi et al. [11] the mast cells have the ability to secrete pro-inflammatory cytokines and affect the activity of macrophages even without signs of degranulation, thus sustaining minimal levels of inflammation that lead to fibrosis. Pal et al. [12] in their study discovered that in the mesenteric lymph nodes the number of mast cells increased significantly with age while their function worsened which resulted in an increase in the oxidative stress in aged mesenteric tissue. In his study Gruber [7] demonstrated that mast cells interact with fibroblasts which changes their activity thus affecting extracellular fibrosis. Mechanistic links between aging, thymic adiposity, and thymic atrophy have been revealed by recent works showing that lipotoxic "danger-associated molecular patterns" (DAMPs), such as ceramide and free cholesterol, increase with aging [3, 19], and can initiate NLRP3 inflammasome signaling and IL-1β production in thymic myeloid cells [19]. These studies suggest that lipotoxic DAMPs inhibit thymus function via IL-1β signaling in thymic epithelial cells [19]. In experimental models of inflammation Borissova [1] and Valcheva-Kuzmanova [18] demonstrated the anti-inflammatory effect of flavonoids present in the juice from AM. Ghante et al. [5] in an experiment with plant extracts from Randia dumetorum (RD) fruits rich in al-

kaloids, flavonoids, polyphenols etc. proved that the bronchorelaxant potential of RD fruit extracts is well supported by an anti-inflammatory effect, stabilization of mast cell membranes and scavenging of different free radicals. In his study Shirley [16] showed that resveratrol at low concentrations exerts its anti-inflammatory properties by preferentially targeting the arachidonic acid pathway.

Our results showed for the first time that supplementation with AM juice leads to a significant decrease in the amount of collagen fibres, the number of mast cells in interlobular connective tissue and the number of CD68 positive cells in thymic medulla in rat thymus.

Conclusion

The decrease in the amount of collagen fibres, the number of mast cells in interlobular connective tissue and the number of CD68 positive cells in thymic medulla demonstrate that a functional drink of AM may counteract age-matched thymic connective tissue remodelling. These results support the beneficial potential of the nutrient treatment of age-related diseases.

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