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Citrus flavanones mildly interfere with pituitary-thyroid axis in old-aged male rats

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ABSTRACT

Citrus flavanones naringenin (NAR) and hesperetin (HES) are potent antioxidants that may contribute to maintenance of health at old age by improving cardiovascular and metabolic status. However, they may also affect thyroid hormone economy. Keeping in mind impaired thyroid function at older age, in this study we tested wheather NAR or HES administration potentiate this decline. NAR or HES were administrated orally (15 mg/kg) to male 24-month-old Wistar rats during 4 weeks. Control groups received vehicle, sunflower oil. Qualitative and quantitative immunohistochemical and immunofluorescent expression of specific proteins and stereological analyses of thyroid tissue were performed. Thyroid stimulating hormone (TSH) and total thyroxine (T_4) concentrations were measured in serum. Thyroid parenchyma of both flavanone-treated groups was characterized by lower ($p < 0.05$) absolute and relative volume of luminal colloid, accompanied by elevated ($p < 0.05$) relative volume of stroma in comparison with the controls. No hypertrophy or absolute thyroid volume change was detected. Intensity of immunopositive signal for thyroglobulin (Tg) and T_4 bound to Tg (T_4-Tg) increased ($p < 0.05$) in the colloid of thyroid follicles after both flavanone treatments. Serum TSH increased ($p < 0.05$) after NAR, while T_4 remained unchanged after both treatments. In conclusion, NAR elevated serum TSH in old-aged males, thus being more potent than HES in altering pituitary-thyroid axis. However, changes in thyroid structure, namely moderate colloid depletion and higher Tg and T_4-Tg protein expressions after both treatments, indicate preserved capacity of the gland to compensate flavanone interfering, and maintain T_4 production in old-aged males.

1. Introduction

Aging is a complex process characterized by a progressive decline in cellular function, overall health and well-being, as well as increased risk of age-related diseases and death. Alterations of the endocrine system and the induction of age-related endocrine diseases significantly contribute to disturbed cellular and molecular metabolic control (Bowers et al., 2013; Chahal and Drake, 2007; Vitale et al., 2013).

Hypothyroidism is very common in patients over 60 years of age and steadily increases with age. The prevalence of thyroid autoimmune diseases and carcinoma increases with advanced age in both sexes (Morganti et al., 2005; Kim et al., 2010; Boelaert 2013). However, according to more recent studies, healthy elderly and centenarians have higher serum thyroid stimulating hormone (TSH) concentrations than younger individuals (Atzmon et al., 2009; Surks and Hollowell, 2007). In male rats, aging is characterized by lower serum thyroid hormone (TH) levels, along with unchanged serum and pituitary TSH level (Cizza et al., 1995; Reymond et al., 1992). Increased number of triiodothyronine (T_3) receptors and deiodinase enzyme (Dio) type 2 activity has been reported in the pituitary of aged male rats (Donda et al., 1990) indicative of adaptation to hypothyroid or hypothyroxinemic alterations.

Abbreviations: NAR, naringenin; HES, hesperetin; TH, thyroid hormones; TSH, thyroid-stimulating hormone; T_3, triiodothyronine; T_4, thyroxine; Tg, thyroglobulin; T_4-Tg, T4 bound to thyroglobulin; NIS, sodium iodide symporter; Dio, deiodinase; IHC, immunohistochemical; IF, immunofluorescent; ROS, reactive oxygen species; DAB, diaminobenzidine tetrahydrochloride.

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Citrus flavanones, nariningenin (NAR) and hesperetin (HES) are bioactive compounds found mostly in grapefruit and orange fruits (Erlund, 2004). Their consumption has been associated with healthy aging and prevention of cardiovascular diseases, metabolic dysfunction and osteoporosis (Cavia-Saiz et al., 2010; Chanet et al., 2012; Galluzzo et al., 2008). Due to their phenolic structures, these flavanones are good blocators (scavenger) of reactive oxygen species (ROS) (Cavia-Saiz et al., 2010). We showed improved antioxidant status and phospholipid composition in the liver of old-aged male rats upon both flavanone treatments, NAR being more potent than HES (Miler et al., 2016).

Besides andtioxidant, flavonoids display diverse biological and pharmacological properties, including antithyroid effects in experimental animals and humans. Namely, NAR was reported to inhibit thyroid peroxidase *in vitro* (TPO; Divi and Doerge, 1996) and 5′-deiodinase *in vivo* (Cody et al., 1986), the key enzymes of TH synthesis and metabolism. Administration of high doses of NAR and HES glycoside precursors (naringin and hesperidin) decreased serum concentration of thyroxine (T_4) and T_3 in hyperthyroid rats (Panda and Kar 2014). However, the intensity of antithyroid effect of polyphenols vary, and depend on animals' diet, dose, age and/or pathological state (Chang and Doerge, 2000; Schmutzler et al., 2004; Šošić-Jurjević et al., 2014).

To the best of our knowledge, there are no data regarding thyroid structure and its functional status in 24-month-old rats after administration of citrus flavanones. In this study we tested if NAR or HES, administered at nutritional doses interfere with pituitary-thyroid axis and potentiate the natural decline of thyroid functioning in our old-aged model.

2. Materials and methods

2.1. Animals and experimental groups

Male Wistar rats (24-month-old) were housed in the unit for experimental animals at the Institute for Biological Research "Siniša Stanković", Belgrade, Serbia. All animals had free access to standard food (Veterinarski zavod, Subotica, Serbia) and water, and were maintained at constant temperature (21 ± 2 °C) and lighting (12 h light: 12 h dark) conditions.

At the beginning of the experiment, the rats were randomly divided in four experimental groups (n = 6 for each group). One group of animals received *per os* (p.o.) 15 mg/kg b.w. of NAR, while the other was treated in a same way with HES (Sigma Aldrich, St. Louise, MO, USA).The citrus flavanones were mixed with sunflower oil (Vital, Vrbas, Serbia), the applied volume of mixture was 0.3 ml *per* animal and the way of application was by syringe directly to the oral cavity. Control group (CON) received the same volume of the vehicle, while old-aged intact controls (ICON) did not receive any treatment. The treatments were administrated daily for 4 weeks.

The animals were decapitated without anesthesia; their thyroid glands including part of the trachea were excised, fixed in 4% paraformaldehyde solution for 24 h and dehydrated in increasing concentrations of ethanol (30–100%) and enlightened in xylene. After embedding in Histowax (Histolab Product Ab, Göteborg, Sweden), each tissue block was serially sectioned at 5 μm thickness on a rotary microtome (RM 2125RT Leica Microsystems, Wetzlar, Germany).

All animal procedures were in compliance with Directive 2010/63/EU on the protection of animals used for experimental and other scientific purposes, and was approved by the Ethical Committee for the Use of Laboratory Animals of the Institute for Biological Research "Siniša Stanković", University of Belgrade (No 2-12/12).

2.2. Histochemical staining procedures

Thyroid sections were stained by routine haematoxylin and eosin (H&E) and Masson trichrome technique for the qualitative and quantitative histological analyses. Masson trichrome staining was applied to get insight into the connective tissue distribution (Ajdžanović et al., 2017). In brief, deparaffinised and rehydrated thyroid sections were incubated in Weigherts' haematoxylin, following acid fuchsine and Ponceau mixture for 5 min, phosphomolybdenum acid for 10 min and aniline blue for 4 min, respectively. In between, the slides were washed in tap or distilled water. Sections were dehydrated and mounted in DPX (Sigma-Aldrich, Barcelona, Spain). All digital images of the thyroid sections regarding light microscopy were made on a DM RB Photomicroscope (Leica, Wetzlar, Germany) with a DFC 320 CCD Camera (Leica) for the images acquisition and analysis.

2.3. Stereological analyses

We performed the stereological measurements on H&E stained thyroid sections as previously reported by Miler et al. (2014). In brief, the measurements were carried out using a newCAST stereological software package (VIS – Visiopharm Integrator System, version 3.2.7.0; Visiopharm; Denmark). Total thyroid volumes (μm^3), as well as the total volume of thyroid tissue phases (epithelium, stroma and colloid; μm^3) were determined using Cavalieri's principle (Gundersen and Jensen, 1987). Thyroid volume (V_{pt}) was then estimated as $\hat{=}a(p) \cdot BA \cdot \sum i = 1nPi$ where *a(p)* is the area associated with each sampling point (208878.02 μm^2), *BA* is the block advance representing the mean distance between two consecutively studied sections (150 μm; Dorph-Petersen et al., 2001), *n* is the number of sections studied for each thyroid, and ΣPi is the sum of points hitting a given target. The same sections were used for estimation of total thyroid volumes as well as the volumes of thyroid tissue phases: follicular epithelium, stroma and colloid. The number of analysed thyroids was 5 *per* group.

Volume density estimation was used to determine the percentage of follicular epithelium, stroma and colloid. Four to five transversal sections from the anterior, central and posterior parts of thyroid (n = 5) were analyzed at objective magnification of x 20. Relative volume densities (V_V) were calculated as the ratio of the number of points hitting each tissue component divided by the number of points hitting the reference space, i.e. analyzed thyroid section: V_V (%) = Pp/Pt × 100 (Pp, counted points hitting the tissue component, Pt, total of points of the test system hitting reference space). Volume density was calculated for each tissue component *per* analyzed section. Then, the average value for all analyzed sections was calculated (for each component separately), representing the relative volume density of the epithelium, stroma and colloid.

2.4. Immunohistochemical and immunofluorescent analyses

The representative sections (5 μm thick, on Superfrost Ultra Plus®, Thermo Scientific Menzel-Gläser manufactured slides), from each previously histochemically and stereologically evaluated thyroid, were stained with immunohistochemical (IHC; Miler et al., 2014; Šošić-Jurjević et al., 2016) or immunofluorescent (IF; Šošić-Jurjević et al., 2015) methods, according to previously described procedures.

Shortly, after tissue deparaffinization, we blocked endogenous peroxidase activity by sections incubation with 0.3% hydrogen peroxide in methanol for 15 min. Afterward, thyroid sections were exposed to heat-induced antigen retrieval to demask target antigens. Slides were placed in a container and covered with 0.1 mol/l

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