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Letter to the Editor

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Old age-associated impairment of the rat liver antioxidant defense system: the basis for affirmation of the experimental model

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To the Editor,

Keeping in mind our recent breakthrough in the field of biomedical effects of citrus flavanones, achieved by using an insufficiently exploited rat experimental model of old age (1,2), we consider it important to highlight the status of the antioxidative defense system in this animal model, as it is a crucial physiological definer. In general, aging represents a complex process characterized by the deterioration of overall health, slowing of the metabolism, and accumulation of numerous mutations (3). The United Nations World Population Aging Report in 2015 suggested that 12.5% of the entire human population is older than 60 years, while some demographic assessments of the World Bank predict it to reach 17% older than 65 years by 2050. Aging is associated with elevated incidences of chronic diseases, such as diabetes, hypertension, dyslipidemia, liver function disturbances, endocrine issues, osteoporosis, sarcopenia, and cancer (3,4). Changes in the antioxidant defense system (ADS), detoxification mechanisms, and cell reparation underlie the majority of the mentioned diseases (5). Namely, as a consequence of the decrease in ADS and cell reparation capacities during aging, the rate of oxidative damage increases. If reactive oxygen species (ROS) are not efficiently removed from the milieu, accumulation of oxidized and glycoxidized products can subsequently cause cellular senescence (6). It is well known that antioxidant enzymes such as superoxide dismutase (SOD) 1 and 2, catalase (CAT), glutathione peroxidase (GPx), and glutathione reductase (GR), as well as nonenzymatic glutathione (GSH), eliminate ROS such as superoxide anion (O^{2-}) or hydrogen peroxide (H_2O_2). As a consequence of the lack of their efficient removal, this can lead to damage of cellular proteins, lipids, and organelles. Oxidative metabolism in the liver causes the production of highly reactive ROS, whereby 90% of these molecules are generated in the mitochondria during the process of aerobic metabolism, but also during the detoxification of drugs and other xenobiotics (5). Additionally, thyroid hormones directly elevate mitochondrial respiration and produce large amounts of ROS in the liver (7). Impairment of the liver's ADS may also cause lipid accumulation, as well as nonalcoholic fatty liver disease or other liver pathologies, or can even lead to cancer (6). In support of this thesis, in advanced age the liver is subjected to a progressive decrease in the intensity of blood flow, while sinusoidal capillaries become thicker, less porous, and defenestrated, thus affecting chylomicron uptake, drug elimination, and the accumulation of harmful macromolecules (8).

In our experiments, 24-month-old male Wistar rats were used, and in order to define the animal model, we compared properties of their antioxidant defense system in the liver (given its importance as mentioned above) to the adequate systems of 4-month-old (adult) rats. All the procedures related to the care and use of our experimental rats were in accordance with the ethical standards, as already described in detail (1,2). It was reported that the concentration of free testosterone decreases in males with advanced age, while this change directly correlated to a lower detoxification capacity and pharmacokinetics of xenobiotics in the liver (9,10). Presumably, our experimental animals were unable to eliminate various deleterious products of metabolism and damaged macromolecules in an adequate manner. Although the liver has a highly performing ADS in comparison with the modest defense in other organs (e.g., in the brain), old-aged liver is also susceptible to oxidative damage. Namely, in the livers of old rats we observed decreased activity of SOD1, CAT, and GR by 53%, 47%, and 39% respectively, compared to the same parameters in young rats' livers (Figure 1A). Additionally, gene expression of *SOD1* and *GR* were decreased by 33% and 28%, respectively (Figure 1B). Protein expression analysis showed a significant decrease

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in the expression of GR, by 141%, and a substantial increase in SOD2 expression by 124%, all in comparison to the observed parameters in young rats' livers (Figure 1C). A decrease in the enzymatic activity of SOD1 and its gene expression, coupled with an increase in SOD2 protein expression, affects the capacity and efficiency of these enzymes to protect mitochondria from detrimental O^{2-}, keeping in mind that mitochondrial ROS leakage is

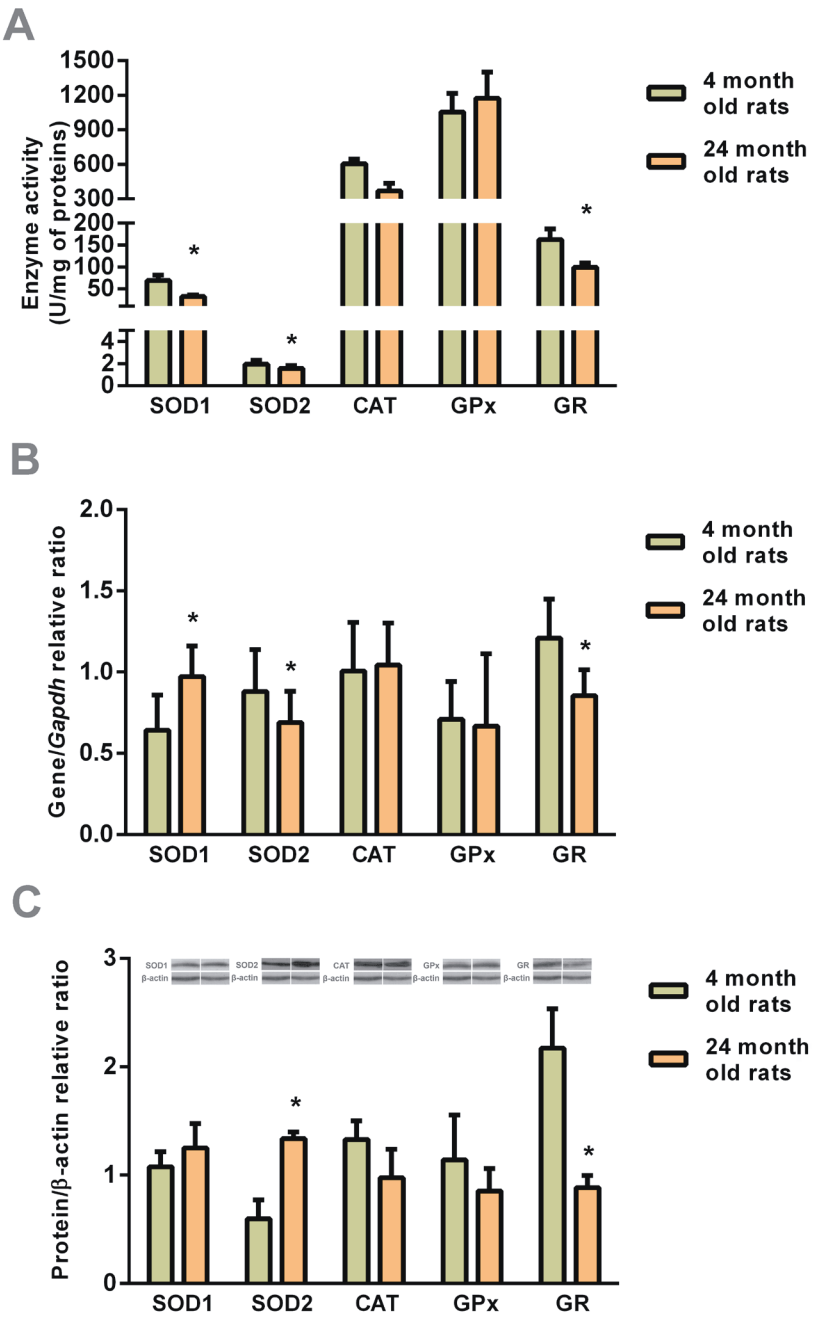

Figure 1. Comparison of the liver antioxidant enzyme activities (A) and gene (B) and protein (C) expression in Wistar male rats between 4 months and 24 months old. SOD1 - Superoxide dismutase 1, SOD2 - superoxide dismutase 2, CAT - catalase, GPx - glutathione peroxidase, GR - glutathione reductase. All values are the means ± SD, n = 4 animals per group. *P < 0.05 vs. 4-month-old rats. Methodological and statistical tools used for obtaining these results were described in detail in our previous work (1).

one of the main causes of oxidative stress in the cell (9). GR activity, as well as its gene and protein expression, was significantly decreased (Figure 1), corresponding to the decrease in the intracellular GSH pool in the liver of older animals (Table). These findings implicate that the liver is incapable of eliminating H_2O_2, given that the activity of CAT, an enzyme also involved in H_2O_2 elimination, is lower (Figure 1A). Taken together, the listed changes reflect a disturbed ADS in older male rats in comparison to young adult rats. Moreover, this animal model can also be useful in testing and screening the antioxidant potential of plant-derived compounds. Polyphenols, plant secondary metabolites, act as antioxidants scavenging free radicals and activating antioxidant enzymes, and as such their full capacity can be evaluated in such a model of old-aged rats.

In addition to these results, body mass gain and absolute liver weight were expectedly significantly higher in older animals, by 66% and 46%, respectively, in comparison to young animals, while the relative liver weight remained unchanged in the same context (Table). The evaluation of the functional status of the liver was achieved by measuring the concentration of alanine aminotransferase (ALT), aspartate aminotransferase (AST), and gamma-glutamyl transpeptidase (GGT) in the serum of young and old rats. The results obtained displayed an old age-associated 4-fold increase in GGT concentration when compared to young rats (Table). The observed unchanged ALT and AST concentrations are in line with the lack of anatomical and histological changes in older rat livers. However, an increase in serum GGT with aging is associated with higher consumption and depletion of intracellular GSH, indicating oxidative stress in the liver.

From the perspective of thyroid hormone status, old rats represent a good experimental model as the concentration of TSH remains unchanged while T_4 concentration in serum decreases (Table). It is apparent that T_4 has no input towards the metabolism increase and elevation of ROS production in the liver, also keeping in mind its lower serum concentration and the conversion into bioactive T_3. Finally, the total cholesterol in the serum

Table. Comparison of measured relevant biometric, biochemical, and hormonal parameters between 4-month-old and 24-month-old Wistar male rats.

Measured relevant parameter	4-month-old rats	24-month-old rats	Reference
Body mass and liver weights (g)			
Body mass	463 ± 40	770 ± 44*	(1)
Absolute liver weight	14.2 ± 1.2	20.8 ± 1.6*	(1)
Relative liver weight	3.2±0.2 (g/b.m.)	2.9 ± 0.2 (g/b.m.)	(1)
Liver GSH concentration (nM/mg)			
GSH	17.29 ± 0.86#	13.61 ± 0.94*	Unpublished result (1)
Serum aminotransferase levels (U/L)			
ALT	62.6 ± 5.86	58.5 ± 4.95	(1)
AST	283.00 ± 11.68	245.00 ± 23.39	(1)
GGT	1.06 ± 0.19	4.25 ± 0.50*	(1)
Lipid status (mmol/L)			
CHOL	1.61 ± 0.10	2.67 ± 0.64*	Unpublished results
LDL	0.51 ± 0.08	0.69 ± 0.23	Unpublished results
HDL	1.48 ± 0.09	1.55 ± 0.39	Unpublished results
TG	1.60 ± 0.17	1.23 ± 0.47	Unpublished results
Serum hormone levels (ng/mL)			
TSH	1.74 ± 0.20	1.88 ± 0.28	(2)
T_4	32.99 ± 8.00	19.85 ± 2.30*	(2)

GSH - Glutathione, ALT - alanine aminotransferase, AST - aspartate aminotransferase, GGT - gamma-glutamyl transpeptidase, CHOL - total cholesterol, LDL - low-density lipoprotein, HDL - high-density lipoprotein, TG - triglycerides, TSH - thyroid-stimulating hormone, T_4 - thyroxine. All values are means ± SD, n = 4–6 animals per group. *$P < 0.05$ vs. 4-month-old rats. #Methodological and statistical tools used for obtaining these results were described in detail in our previous work (1).

of 24-month-old rats increased by 73% in comparison to the same parameter in young controls (Table), which agrees with the observed lower T_4 concentrations and dyslipidemia seen in advanced age.

Besides all the positive aspects of this animal model, it has a few disadvantages that deserve attention. First, it is a rather expensive model and breeding and growing Wistar rats to an advanced age is a very long process and demands constant attention. Second, the mortality rate due to natural causes is between 30% and 50%; as such, the initial pool of animals should be double or triple that needed for the experiment at hand. Third, these fragile animals should be handled with additional caution, triggering minimal stress conditions. Namely, old animals are very prone to stress and should not undergo oral gavage application or other aggressive methods of treatment administration (e.g., intraperitoneal or intramuscular). Accordingly, the citrus flavanones used in the main experiments performed, pertinent to aging, were initially mixed with sunflower oil and directly applied into the rats' oral cavities (1,2).

In conclusion, this generally underused rat model can provide a very useful tool in the identification of molecular and cellular mechanisms of antioxidative actions of different phytochemicals by the investigation of the functionality of the liver ADS. Furthermore, various indirect effects of natural compounds, potentially therapeutic during aging, could be observed considering the antioxidative system's input in metabolism, endocrine-related events, or even immune response.

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