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Diet supplements, resveratrol and protocatechuic acid, do not disturb wellness and liver morphology in rats*)

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Summary

The aim of the study was to evaluate the influence of a prolonged administration of two natural compounds – resveratrol (20 ppm) and protocatechuic acid (2000 ppm) – on the health status of Wistar rats. The animals were kept on supplemented diets for 7 and 24 weeks. Body weight was assessed weekly, and liver weight during autopsy. Liver morphology was evaluated histologically. The body weight in the 24th week, body weight gain (between the 1st and the 24th week of the experiment), and liver weight were significantly higher for the animals kept on the diet including resveratrol throughout the experiment than for those that had received this diet during the initial 7 weeks. However, differences in these parameters, as well as in the relative liver weight, between the control group and the groups exposed to both compounds were insignificant. Occasionally, mild, mostly reversible, microscopic hepatic changes (i.e., hydropic and fatty changes) were found, and these were slightly more common in the groups kept on supplemented diets. It seems that resveratrol and protocatechuic acid did not significantly disturb the wellness of rats, even after prolonged exposure.

Keywords: resveratrol, protocatechuic acid, liver, rat

A regular daily diet could be insufficient during pregnancy, in early and late periods of life, or in patients suffering from chronic illnesses or malabsorption syndromes. To avoid some mineral or vitamin deficiencies and their clinical consequences on a large population scale, food used in large amounts can be modified (e.g. by the addition of iodine to salt or Fluor to tap water) (2). Another solution, but dedicated for individuals rather than for the whole population, is diet supplementation. It has become very popular during the last decades, especially among teenagers, young adults, and the elderly. Diet supplements are used to complement everyday diet with high concentrations of vitamins, minerals or other compounds commonly derived from plants and characterized by nutritional or other unusual physiological activities, e.g. chemopreventive against malignancies, cardioprotective, or neuroprotective (6). Since the production and marketing of supplements is not subject to highly restrictive regulations, their use is potentially associated with a risk of side effects, mainly secondary to chemical contaminations (by pesticides, mycotoxins, etc.) and pharmacological interactions with co-administered drugs (13).

Resveratrol (trans-3,4',5-trihydroxystilbene; RES) and protocatechuic acid (3,4-dihydroxybenzoic acid; PCA) are active ingredients of many edible fruits and vegetables, their nutritional products (including red and white wines), as well as over-the-counter diet supplements administered mostly because of their wide chemopreventive properties (5, 17, 18). Many epidemiological and experimental studies indicated efficiency of RES in various cardio-vascular disorders and malignancies (5, 18). Similar activity, but confirmed mostly in tumorigenesis models in experimental animals, was also proved for PCA (11, 17). Furthermore, RES exhibits anti-inflammatory activity and positively

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regulates serum glucose level and the metabolism of adipose tissue. Diet rich in the compound promotes longevity and delays neurodegenerative diseases (5).

The aim of the study was to evaluate the influence of a prolonged administration of two diet supplements – resveratrol and protocatechuic acid – on body and liver weight and on hepatic morphology in rats.

Material and methods

The study protocol was approved by the Local Bioethical Committee (493/2004). Adult male albino Wistar CRL:(WI) WUBR rats were obtained from a commercial breeder (Rembertów, Poland) and kept under standard laboratory conditions. Filtrated municipal tap water was provided *ad libitum*. The control group (CON, n = 30) received a standard laboratory rat diet (LSM®; AGROPOL; Motycz, Poland), whereas diets supplemented with resveratrol (Sigma Chemical, USA, purity 99%) and protocatechuic acid (Fluka Chemica, Switzerland, purity 97%) were administered to the study groups in doses of 20 ppm (n = 15) and 2000 ppm (n = 15), respectively, for the first 7 and 24 weeks (the whole experiment). The diets were prepared by AGROPOL. All animals were weighed every week.

At the end of the study (24th week) all rats were sacrificed by intraperitoneal injection of pentobarbital (Morbital; Biowet, Puławy, Poland) and grossly examined during autopsy. The absolute weight of the liver was noted. Liver samples were taken, fixed in 10% buffered formalin and embedded in paraffin blocks. Sections were routinely stained with hematoxylin and eosin (H+E) and evaluated microscopically (Olympus BX45, Japan).

The numerical data obtained were presented by means of

Tab. 1. Basic evaluated parameters for the control rats kept on the basic diet

	n	min	max	M	SD	Me	p
body weight – 1st wk. (g)	30	200.00	246.00	223.93	14.48	225.50	< 0.001
body weight – 24th wk. (g)	30	475.00	760.00	597.67	75.69	582.00	
Δ body weight (%)	30	104.22	233.33	167.70	35.92	161.88	
absolute liver weight (g)	30	11.60	22.19	16.02	2.66	15.49	
relative liver weight (%)	30	2.22	3.15	2.68	0.25	2.64	

Tab. 2. Basic evaluated parameters for the rats exposed to resveratrol (RES)

	n	min	max	M	SD	Me	p
RES – 7 weeks of the exposure							
body weight – 1st wk. (g)	15	200.00	233.00	215.00	12.89	216.00	< 0.001
body weight – 24th wk. (g)	15	462.00	638.00	553.47	61.83	527.00	
Δ body weight (%)	15	126.09	211.22	157.26	22.50	153.51	
absolute liver weight (g)	15	11.80	19.43	14.49	1.97	14.77	
relative liver weight (%)	15	2.36	3.08	2.65	0.25	2.55	
RES – 24 weeks of the exposure							
body weight – 1st wk. (g)	15	202.00	250.00	216.80	14.25	211.00	< 0.001
body weight – 24th wk. (g)	15	475.00	752.00	622.87	78.13	630.00	
Δ body weight (%)	15	132.84	240.27	187.12	29.58	189.11	
absolute liver weight (g)	15	11.60	22.19	17.27	3.02	17.50	
relative liver weight (%)	15	2.24	3.15	2.76	0.22	2.79	
RES – regardless of the period of exposure							
body weight – 1st wk. (g)	30	200.00	250.00	215.90	13.38	213.50	< 0.001
body weight – 24th wk. (g)	30	462.00	752.00	588.17	77.70	581.00	
Δ body weight (%)	30	126.09	240.27	172.19	29.96	169.72	
absolute liver weight (g)	30	11.60	22.19	15.93	2.89	15.53	
relative liver weight (%)	30	2.24	3.15	2.71	0.24	2.74	

Tab. 3. Basic evaluated parameters for the rats exposed to protocatechuic acid (PCA)

	n	min	max	M	SD	Me	p
PCA – 7 weeks of the exposure							
body weight – 1st wk. (g)	15	203.00	250.00	228.00	12.60	226.00	< 0.001
body weight – 24th wk. (g)	15	505.00	700.00	592.67	65.21	597.00	
Δ body weight (%)	15	113.11	201.72	160.45	29.49	166.52	
absolute liver weight (g)	15	11.38	20.95	15.63	2.62	16.08	
relative liver weight (%)	15	2.13	3.15	2.63	0.24	2.62	
PCA – 24 weeks of the exposure							
body weight – 1st wk. (g)	15	200.00	249.00	229.07	16.05	235.00	< 0.001
body weight – 24th wk. (g)	15	540.00	749.00	629.20	61.14	630.00	
Δ body weight (%)	15	132.79	259.50	176.04	34.83	168.09	
absolute liver weight (g)	15	15.89	22.00	17.76	1.95	17.34	
relative liver weight (%)	15	2.44	3.14	2.83	0.20	2.86	
PCA – regardless of the period of the exposure							
body weight – 1st wk. (g)	30	200.00	250.00	228.53	14.19	230.00	< 0.001
body weight – 24th wk. (g)	30	505.00	749.00	610.93	64.83	615.50	
Δ body weight (%)	30	113.11	259.50	168.24	32.68	167.30	
absolute liver weight (g)	30	11.38	22.00	16.69	2.52	16.24	
relative liver weight (%)	30	2.13	3.15	2.73	0.24	2.74	

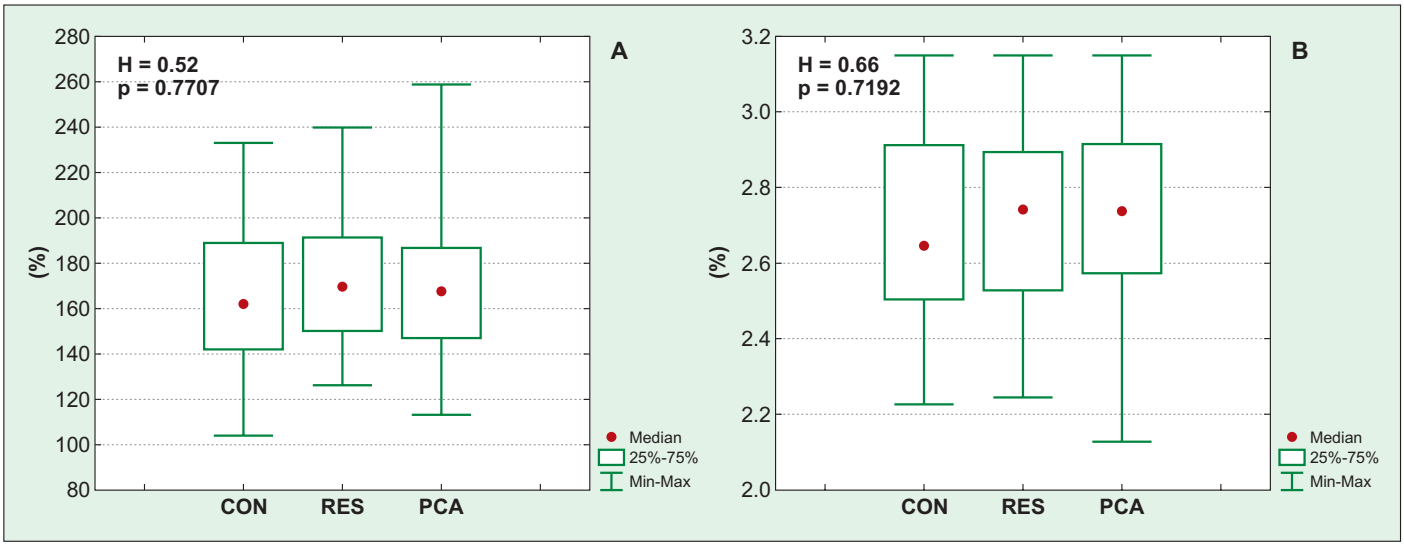

Fig. 1. A: The body weight gain (%) for the whole experiment according to the administered compound. B: The relative liver weight (%) according to the administered compound

arithmetical mean (M), median (Me), minimal-maximal values (min, max), and standard deviation (SD). Data distribution was analyzed by Shapiro-Wilk's W test. The differences between dependent samples were calculated by the T-test or the Wilcoxon test. The differences between groups were analyzed by χ^2, ANOVA or Kruskal-Wallis ANOVA tests. The 0.05 confidence level ($p < 0.05$) was used as the criterion of significance.

Results and discussion

None of the rats had died during the experiment. No significant changes in animal behavior were observed during the whole study. In all evaluated groups significant differences in body weight were noted between the first and the last week of the experiment ($p < 0.001$) (Table 1-3). The body weight in the 24th week and the body weight gain over the whole experiment (Δ body weight between the last and the first week of the experiment) were significantly higher for animals exposed to RES throughout the experiment (24 weeks) than for those that had received this diet only for the initial 7 weeks ($p = 0.0171$ and 0.0051, respectively) (Tab. 2). For PCA-exposed groups, the differences were found to be insignificant (Tab. 3). Moreover, insignificant differences in the body weight in the 24th week and the body weight gain (Fig. 1A) were revealed between the control group and the groups exposed to both compounds.

The absolute liver weight was significantly higher in rats exposed to RES and PCA separately for the whole experiment than for the initial 7 weeks ($p = 0.0071$ and 0.0208, respectively) (Tab. 2, 3). The relative liver weight, as well, was significantly higher for rats kept on the diet including PCA for 24 weeks (Tab. 3). Insignificant differences in the absolute and relative liver weights between the control group and the groups exposed to both compounds were found (Fig. 1B).

Microscopic examination revealed different hepatic lesions that occurred in a limited number of rats and with low intensity (Tab. 4). They included mild hydropic change, mostly in the periportal hepatocytes, randomly distributed macrovesicular steatosis of the hepatocytes (Fig. 2A, 3A), random focal necrosis (Fig. 2B), a few acidophil bodies (apoptotic cells), and mild focal infiltrations composed of lymphocytes and eosinophil, mostly in the portal tracts (Fig. 3B). These lesions were slightly more common in the groups kept on supplemented diets throughout the experiment.

The present study demonstrates that both short- and long-term administration of the two natural supplements – resveratrol and protocatechuic acid – did not significantly influence body and liver weights or hepatic morphology. These findings suggest that the application of both compounds in humans is safe, although it requires additional studies involving a larger number of parameters and non-rodent animal models.

The available literature data on the toxicity of RES are not unequivocal. The compound administered

Tab. 4. Microscopic changes in the liver in the control group (CON) and in the rats exposed to resveratrol (RES) and protocatechuic acid (PCA) for initial 7 and 24 weeks

	CON	RES		PCA	
		7	24	7	24
Hydropic changes in hepatocytes	4 (13.33%)	2 (13.33%)	1 (6.66%)	3 (20.00%)	4 (26.66%)
Fatty changes in hepatocytes	2 (6.66%)	2 (13.33%)	4 (26.66%)	1 (6.66%)	3 (20.00%)
Focal necrosis	2 (6.66%)	–	4 (26.66%)	4 (26.66%)	1 (6.66%)
Acidophil bodies (apoptotic cells)	1 (3.33%)	1 (6.66%)	–	–	–
Inflammatory infiltration	–	–	3 (20.00%)	3 (20.00%)	–

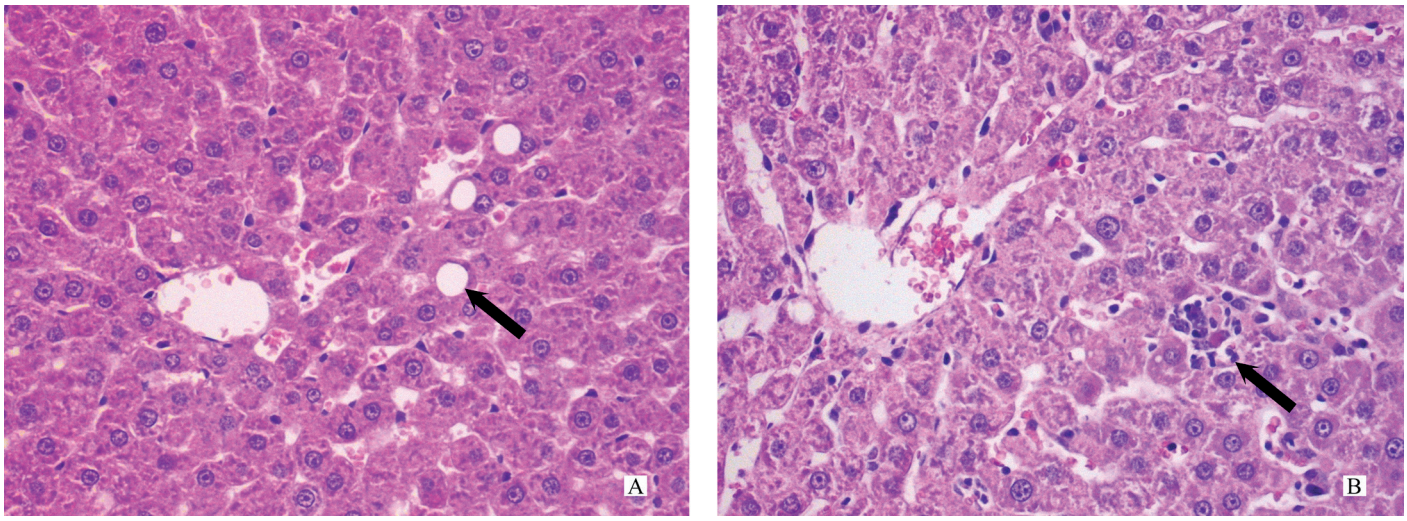

Fig. 2. A: The randomly distributed macrovesicular steatosis of a few hepatocytes (RES, 7 weeks of exposure; H+E, objective magn. 20 ×). B: The focal area of necrosis infiltrated by inflammatory cells (RES, 24 weeks of exposure; H+E, objective magn. 20 ×)

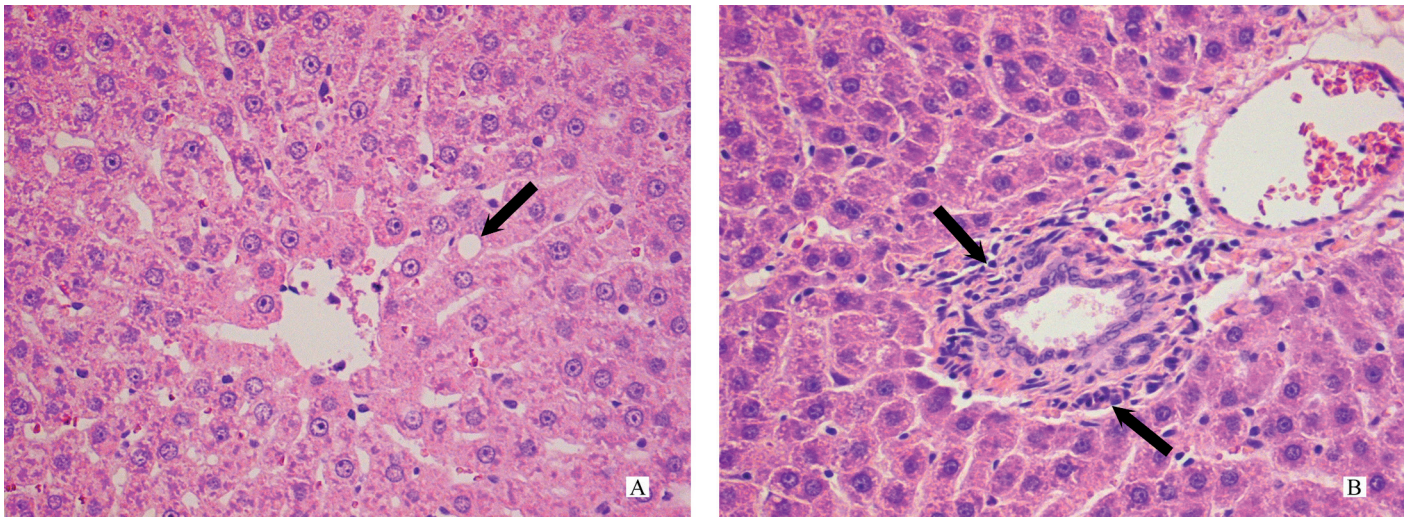

Fig. 3. A: The macrovesicular steatosis of a single hepatocyte (PCA, 24 weeks of exposure; H+E, objective magn. 20 ×). B: The mild infiltration of lymphocytes in the portal tract (PCA, 7 weeks of exposure; H+E, objective magn. 20 ×)

orally in a daily dose of 20 mg/kg to Sprague-Dawley rats for 28 days did not disturb diet consumption, body weight, morphology of the main internal organs, or basic biochemical parameters (7). Such results were confirmed even with much higher doses, e.g., 2000 ppm or 1500 mg/kg, in various chemoprevention studies (18). The no-observed-effect level (NOAEL) for the compound was set at 300 mg/kg (7), but for high purity *trans*-resveratrol (resVida, DSM Nutritional Product Ltd.) it reached 750 mg/kg when administered in the diet or 300 mg/kg after intragastric application (20). Very different data were presented by Li et al. (10), who established the maximum tolerated dose (MTD) of RES for rats at 2.0 mg/kg/day (20 ppm) and reported toxic effects or even death of some animals after a dose of 4.0 mg/kg. Generally, the suggested dose of RES for humans should not exceed 1 g/day, since higher doses may result in adverse effects, such as diarrhea, nausea, and abdominal pain (1). Data on the safe application of PCA are very limited. MTD for rats was fixed at more than 10.000 ppm (19). In most studies on the chemopreventive properties of the compound, doses of 200-2000 ppm were well tolerated (17). No indications regarding the safe dose for humans are available.

The body weight of experimental animals is directly related to their age and available diet. It is assumed that the quality and quantity of diet constituents suitable for young animals should also be adequate for older ones (12). However, permanent access to the diet combined with a relatively low energy demand (limited physical activity, constant external temperature) increased the risk of obesity for animals in long-term experiments. In the present study, the mean body weight of rats in the last week of the experiment was similar to that noted in animals kept on *ad libitum* diet, but it was about 1/3 higher than for rats on dietary restriction (8). Both compounds did not significantly modify the body weight compared with the control group, but a longer administration of RES was associated with increased body weight in the last week of the experiment and the

mean body weight gain over the whole experiment. On the other hand, in many obesity models, a positive effect of RES on the body weight gain, fat tissue distribution, hepatomegaly, and fatty changes was reported, because of its influence on the expression of many genes involved in lypogenesis and lypolysis (4, 21).

In the present study, the morphology of the liver in the groups exposed to RES and PCA was comparable, and mild, mostly reversible changes were observed in all groups. However, it is very likely that such lesions were not related exclusively to xenobiotic administration, but also to the age of the animals. Data on the liver weight and morphology after the administration of RES or PCA to laboratory species, are rudimentary. Kuroiwa et al. (9) noted a significant increase in the relative liver weight in the golden hamster after the administration of RES in a dose of 10 ppm for 14 weeks, but Juan et al. (7) did not observe any changes after the application of the compound in a dose of 20 mg/kg/day for 28 days. Suzuki et al. (16) found increases in body and liver weights in rats kept on a PCA-containing diet (2000 ppm) for 32 weeks. However, the beneficial role of both phytophenols, especially RES, due to their antioxidative properties, is well known in experimentally induced hepatic injuries caused, for example, by metotrexate, ethyl alcohol, lipopolysaccharide or ischemia/reperfusion (3, 17). On the other hand, both compounds in higher doses can have a pro-oxidative effect and stimulate oxidative stress as well (14, 15). This phenomenon may also, at least partly, explain discrete morphological hepatic lesions seen in the present study.

It seems that resveratrol and protocatechuic acid did not significantly disturb the wellness of rats, even after prolonged exposure. Randomly distributed, mild morphological changes in the liver were probably marginally related to the action of both compounds.

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