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Whey protein protects liver mitochondrial function against oxidative stress in rats exposed to acrolein

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Acrolein (AC) is one of the most toxic environmental pollutants, often associated with incomplete combustion of petrol, wood, and plastic, oil frying, and tobacco smoking, that causes oxidative damage to DNA and mitochondria. Considering that little is known about the protective effects of whey protein (WP) against AC-induced liver toxicity, the aim of our study was to learn more about them in respect to liver mitochondrial oxidative stress, respiratory enzymes, Krebs cycle enzymes, and adenosine triphosphate (ATP). To do that, we treated Sprague Dawley rats with daily doses of AC alone (5 mg/kg bw in 0.9 % NaCl solution), WP alone (200 mg/kg bw, in 0.9 % NaCl solution), or their combination by oral gavage for six days a week over 30 days. As expected, the AC group showed a drop in glutathione levels and antioxidant, transport chain, and tricarboxylic acid cycle enzyme activities and a significant rise in mitochondrial lipid peroxidation and protein carbonyl levels. Co-treatment with WP mitigated oxidative stress and improved enzyme activities. Judging by the measured parameters, WP reduced AC toxicity by improving bioenergetic mechanisms and eliminating oxidative stress.

KEY WORDS: antioxidants; glutathione; oxidative phosphorylation enzymes; tricarboxylic acid cycle enzymes

Acrolein (AC) is a product of incomplete combustion of petrol, wood, and plastic, or is released through frying of food in oil or tobacco smoking (1, 2). It can induce DNA, oxidative, and mitochondrial damage (1–4) to cause genotoxicity (2), cardiotoxicity (5), and dyslipidaemia (1, 6). There are reports of it inducing oxidative stress by increasing thiobarbituric acid reactive substances (TBARS) and protein carbonyl (PC) levels and lowering glutathione (GSH) levels in rat spleen, thymus, and polymorphonuclear leukocytes (PMNs) (2), inducing oxidative stress in red blood cell and brain and heart mitochondria (1, 6–8), and lowering the activity of oxidative phosphorylation (OXPHOS) complexes, tricarboxylic acid cycle (TCA) enzymes, and adenosine triphosphate (ATP) levels and causing mitochondrial dysfunction in rat heart (1).

Considering that oxidative stress seems to be the main mechanism of its action, we wanted to see how would whey protein (WP) fare as protection against its effects, thanks to its many health benefits, such as high free radical-scavenging activity, lipid oxidation inhibition, and antimicrobial, anticancer, antihypertensive, antioxidant, anti-dyslipidaemic, and immunoprotective action (1, 2, 9–14). Furthermore, as limited information is available about the effect of WP on oxidative stress and mitochondrial function in rat liver treated with AC, the aim of our study was to focus on protective effects of WP against AC-induced toxicity in rat liver mitochondria by measuring oxidative stress parameters, mitochondrial enzymes, and ATP levels in the liver.

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MATERIALS AND METHODS

Chemicals

AC was purchased from Sigma-Aldrich (Steinheim, Germany). Unflavoured WP was purchased as food supplement from the General Nutrition Centre (GNC, Istanbul, Turkey). A 1-ounce (28 g) serving of WP contains 21 g of proteins (493 mg of L-arginine, 3558 mg of L-glutamine, 1212 mg of valine, 631 mg of tyrosine, 355 mg of tryptophan, 1350 mg of threonine, 995 mg of serine, 1163 mg of proline, 719 mg of phenylalanine, 443 mg of methionine, 1025 mg of alanine, 2237 mg of aspartic acid, 522 mg of cysteine, 384 mg of glysine, 414 mg of histidine, 1301 mg of isoleucine, 2296 mg of leucine, and 1902 mg of lysine), 1 g of saturated and 2 g of unsaturated fat, 2 g of carbohydrate, and 2 g of sugar. All other (analytical grade) chemicals were purchased from Sigma Chemical Co. (St. Louis, MO, USA).

Animals and treatment

Twenty-four healthy Sprague Dawley male rats, each weighing 250–300 g, were obtained from the Experimental Animal Centre of Ondokuz Mayıs University (Samsun, Turkey). The animals were housed in polycarbonate cages under standard laboratory conditions (21±1 °C temperature, 40–55 % humidity, 12:12 h light-dark cycle)

and had free access to tap water and standard rodent pellet diet throughout the experiment.

For the experiment the rats were randomly divided into four groups of six. The control group was receiving 1 mL of 0.9 % NaCl solution through oral gavage a day because AC and WP were dissolved in 0.9 % NaCl solution for the experimental treatment. The other three groups were orally gavaged with AC alone (5 mg/kg bw a day), WP alone (200 mg/kg bw a day) and AC+WP (5 mg/kg + 200 mg/kg bw a day) in 1 mL of 0.9 % NaCl solution. All groups were receiving treatment six days a week for 30 days. The choice of AC and WP doses was based on maximal daily human consumption as described in our earlier articles (1, 2).

The study was approved by Medical Research Ethics Committee of Ondokuz Mayıs University (HADYEK 2014/24) and all procedures followed the guidelines provided by the National Institutes of Health Guide for the Care and Use of Laboratory Animals (15).

Preparation of liver tissue and isolation of mitochondria

The livers were placed in ice cold 0.15 mol/L NaCI solution, perfused with the same solution to remove red blood cells, blotted on filter paper, weighed, and stored at -80 °C until use. Mitochondria were isolated from the liver by differential centrifugation as described elsewhere (16, 17). Briefly, liver tissues were homogenised in buffer A (250 mmol/L sucrose, 10 mmol/L Tris-HCI, 1 mmol/L EGTA, pH 7.6), centrifuged at 1000 × *g* for 5 min, the obtained supernatant strained through gauze, and then centrifuged again at 7000 × *g* for 10 min. After washing with buffer B (250 mmol/L sucrose, 10 mmol/L Tris-HCI, and 0.1 mmol/L EGTA, pH 7.6), the obtained mitochondrial pellets were then centrifuged twice at 4 °C, first at 1000 × *g* and then 7000 × *g*, both times for 15 min Mitochondrial fractions were stored at -80 °C for further analysis and mitochondrial protein content determined using the method described by Lowry et al. (18).

Determination of mitochondrial oxidative stress, OXPHOS, and TCA enzymes

Mitochondrial super oxide dismutase (MnSOD) activity was determined according to the method described by Beauchamp and Fridovich (19). One unit of SOD activity was defined as the amount of enzyme required to inhibit nitroblue tetrazolium photoreduction rate by 50 %. Glutathione peroxidase (GPx) activity was determined according to Flohe and Gunzler (20). Glutathione (GSH) levels were measured as described by Moron et al. (21) and lipid peroxidation (LP) and protein carbonyls (PCs) levels as described by Esterbauer and Chessman (22) and Levine et al(23), respectively.

Complex I (NADH ubiquinone oxidoreductase) and complex II (succinate ubiquinone oxidoreductase) activities were determined as described by Janssen et al. (24). Complex IV (cytochrome c oxidase) activity was determined as described by Trounce et al. (25). Mitochondrial $NADP^+$-dependent isocitrate dehydrogenase (ICDH)

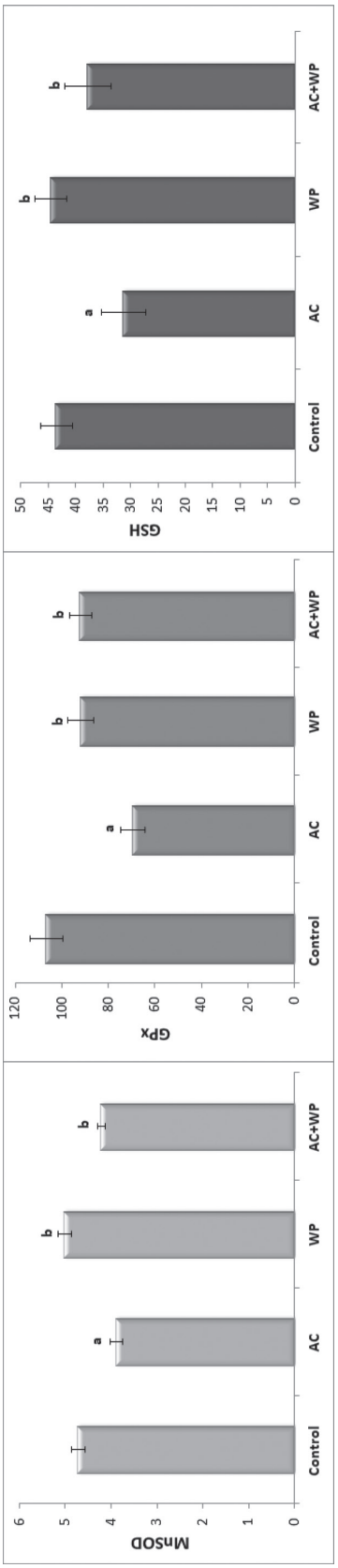

Figure 1 The effects of acrolein (AC) and/or whey protein (WP) on MnSOD (U/mg protein), GPx (nmol of NADPH oxidised/min/mg protein) and GSH (nmol/mg protein) levels. Values are expressed as means ± SD (n=6). (a) significant vs control and (b) significant vs AC ($p<0.05$). GPx – glutathione peroxidase; GSH – glutathione; MnSOD – mitochondrial super oxide dismutase

activity was determined according to Fatania et al. (26). Alpha-ketoglutarate dehydrogenase (α-KGDH) and malate dehydrogenase (MDH) activities were defined as described by Lucas et al. (27) and Gelpi et al. (28), respectively.

Determination of ATP levels

ATP concentrations were determined using the ATP colourimetric/fluorometric assay kit (K354-100, Biovision, Milpitas, CA, USA) and calculated against the ATP standard curve following the manufacturer's instructions. The results are expressed as nmol/mg of protein.

Statistical analysis

Data are expressed as means ± standard deviations (SD). For all group comparisons we used one-way analysis of variance (ANOVA) followed by the *post hoc* Tukey's test for comparison of means, with $p<0.05$ taken as significant difference. All analyses were run on IBM SPSS Statistics for Windows, version 22.0 (IBM Corp., Armonk, NY, USA)

RESULTS

Oxidative stress parameters in liver mitochondria

As expected, AC treatment significantly decreased MnSOD (17.71 %), GPx (32.84 %), and GSH (28.14 %) levels compared to the control group. Compared to the AC group, WP and WP+AC groups had significantly higher respective MnSOD (28.9 % and 8.53 %), GPx (32.27 % and 22.54 %), and GSH (42.38 % and 20.65 %) levels (Figure 1), which clearly shows that treatment with WP improved all these enzyme activities.

Compared to control, AC treatment also significantly increased LP (72.81 %) and PC (29.52 %) levels, but WP, alone or in combination with AC, significantly restored their levels by decreasing LP 35.97 % and PCs 12.50 % compared to the AC group (Figure 2).

Levels of mitochondrial ATP, OXPHOS, and TCA cycle enzymes

Decreases in OXPHOS complex II (38.28 %) and IV (23.82 %) levels in AC-treated rats were significant compared to control, and both were significantly improved by treatment with WP alone or in combination with AC, which increased complex II by 55.48 % and 42.87 %, respectively and complex IV by 43.95 % and 39.84 %, respectively compared to the AC group (Figure 3).

A similar pattern is observed with ICDH (Figure 4), whose activity in the AC-treated group was significantly lower than in control but was restored by WP and AC+WP treatments (38.59 % and 12.86 % higher than in the AC group, respectively). AC did not significantly affect α-KGDH and MDH activity, but WP alone increased both significantly (26.55 % and 48.78 %, respectively) compared to control. In combination with WP it also resulted in significantly higher MDH activity (35.59 %) than in control (Figure 4).

ATP levels dropped significantly (25.95 %) with AC treatment compared to control, whereas WP in combination with AC was not as successful in restoring ATP as it was with other parameters, considering that ATP levels remained 16.27 % lower than control (Figure 5).

DISCUSSION

Mitochondria in hepatocytes can be considered metabolic centres for glucose, lipid, and protein metabolism and energy

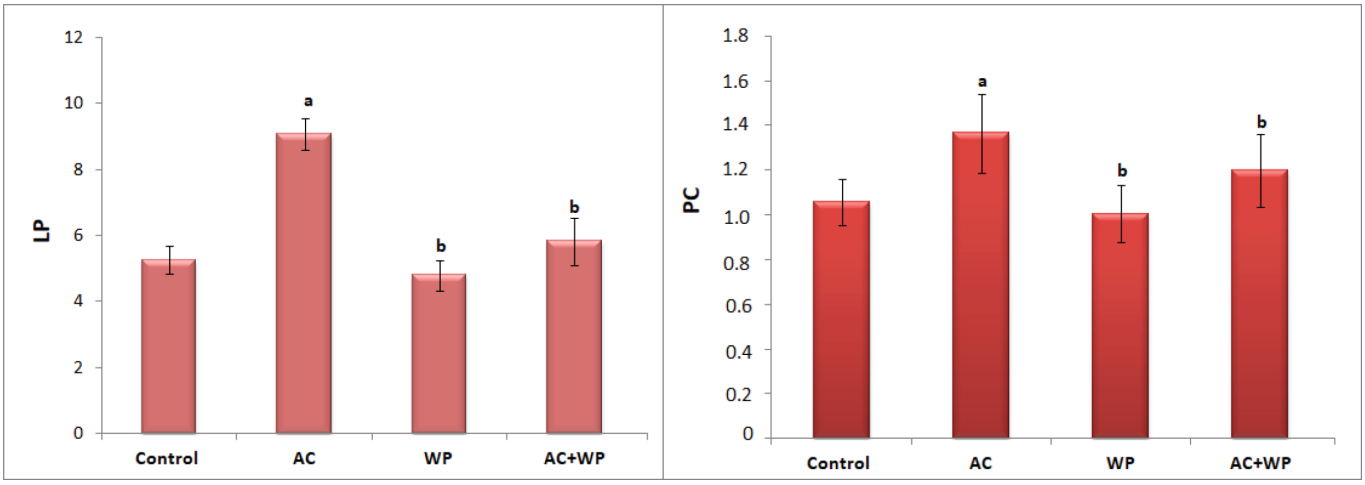

Figure 2 The effects of acrolein (AC) and/or whey protein (WP) on LP (nmol equivalents of malonedialdehyde/mg protein) and PC (nmol DNPH incorporated/mg protein) levels. Values are mean±SD (n=6). Data analysed by one-way analysis of variance (ANOVA) followed by Tukey's test for comparison of means. (a) significant against 0.9 % NaCl control and (b) significant against AC at $p<0.05$. LP – lipid peroxidation; PC – protein carbonyl

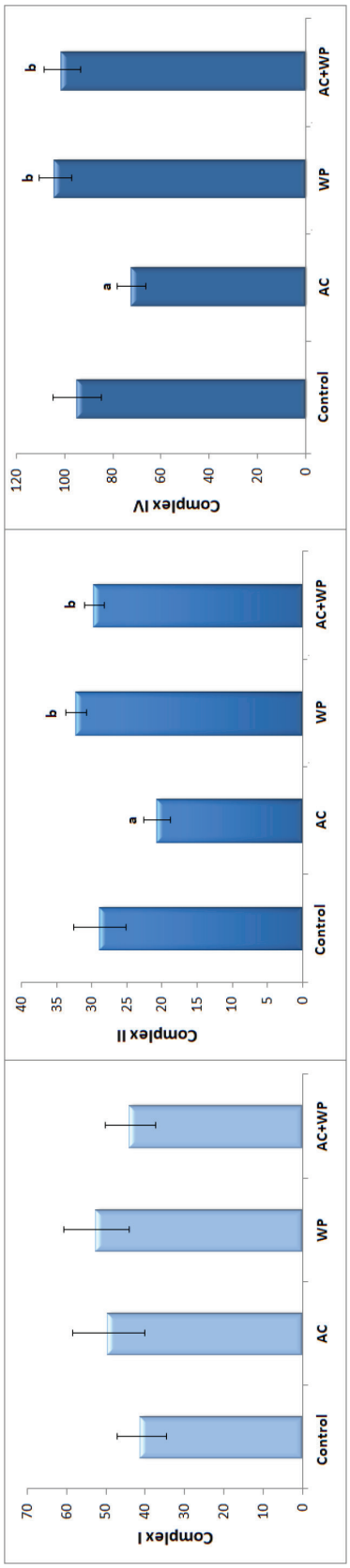

Figure 3 The effects of acrolein (AC) and/or whey protein (WP) on complex I (mmol DCPIP reduced/min/mg protein), complex II (mmol DCPIP reduced/min/mg protein), complex IV (mmol cytochrome c oxidised/min/mg protein) levels. Values are mean±SD (n=6). Data analysed by one-way analysis of variance (ANOVA) followed by Tukey's test for comparison of means. (a) significant against 0.9 % NaCl control and (b) significant against AC at $p<0.05$. Complex I – NADH-coenzyme Q oxidoreductase; Complex II – succinate dehydrogenase-coenzyme Q oxidoreductase; Complex IV – cytochrome c oxidase

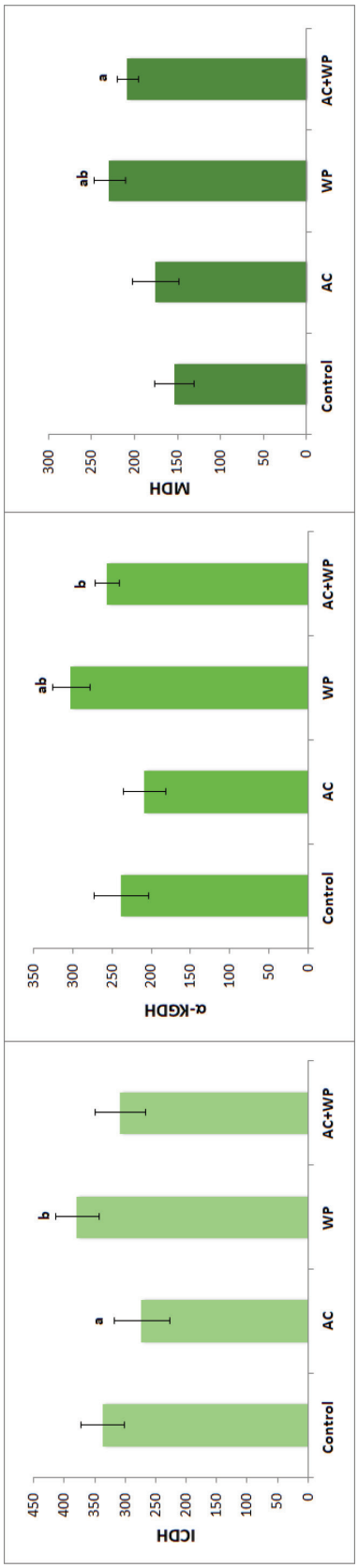

Figure 4 The effects of acrolein (AC) and/or whey protein (WP) on ICDH (mmol $NADP^+$ reduced/min/mg protein), α-KGDH (mmol NAD^+ reduced/min/mg protein), MDH (mmol NAD^+ reduced/min/mg protein) levels. Values are mean±SD (n=6). Data analysed by one-way analysis of variance (ANOVA) followed by Tukey's test for comparison of means. (a) significant against 0.9 % NaCl control and (b) significant against AC at $p<0.05$. α-KGDH – α-ketoglutarate dehydrogenase; ICDH – isocitrate dehydrogenase; MDH – malate dehydrogenase

production. Many chronic liver diseases are associated with the accumulation of damaged mitochondria with abnormal ROS generation, GSH reduction or depletion, increased PC and LP levels, and changes in respiratory complexes. Overproduction of ROS can damage other cellular components such as protein subunits of both mitochondrial and oxidative phosphorylation enzymes, lipid membranes, mitochondrial or nuclear DNA. Consequently, these cellular lesions may contribute to the development of various obesity-related metabolic disorders and hepatocellular carcinoma (29, 30). Similar to earlier reports (1, 4, 8, 31–33), our findings show that AC can damage liver mitochondria by increasing ROS formation in mitochondria (4, 34, 35) and decreasing their overall antioxidant capacity (32, 34).

Our findings have also confirmed the protective effects of WP against oxidative stress reported in several animal studies of different organ and cell mitochondria (1, 2, 9, 10, 13, 36–41), in which WP improved antioxidant defences, mitigated oxidative stress, and mitochondrial dysfunction in spleen, thymus, polymorphonuclear leukocytes, red blood cells, and heart mitochondria.

In animal models mitochondrial antioxidants such as GSH, Mn-SOD, and GPx are effective against liver diseases such as fatty liver caused by mitochondrial damage, as they reduce ROS production and oxidative stress (42). We too have observed that the levels of Mn-SOD, GPx and GSH, which were suppressed by AC, increase after WP treatment, while LPO and PK levels decrease.

As WP is rich in sulphur-containing amino acids, it can improve the levels of some cellular antioxidants such as GSH, CAT, SOD, and GPx by increasing their synthesis (43–45), but high cysteine content in WP limits the rate of GSH synthesis in cells (46). The protective effect of WP may be owed to the restoration of the redox status through increase in GSH (47), and normalised activities of GSH and GPx in our study seem to corroborated this assumption.

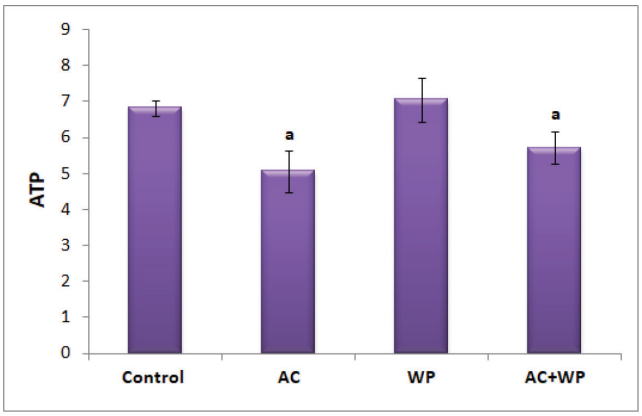

Figure 5 The effects of acrolein (AC) and/or whey protein (WP) on ATP (nmol/mg protein) level. Values are mean±SD (n=6). Data analysed by one-way analysis of variance (ANOVA) followed by Tukey's test for comparison of means. (a) significant against 0.9 % NaCl control and (b) significant against AC at $p<0.05$. ATP – adenosine triphosphate

Furthermore, WP largely eliminated the formation of liver damaging LP, possibly thanks to the radical scavenging activity of DPPH (48).

Other parameters that showed improvement after WP treatment in our study include TCA and electron transport enzymes (complexes I–IV). WP seems to provide substrates for TCA and electron transport via constituent amino acids (37). We believe that higher substrate availability increases NADH metabolism and cellular NAD^+/NADH ratio (49).

ICDH is an enzyme that produces NADPH, which, in turn, helps regenerate mitochondrial GSH and therefore plays a very important role in protecting mitochondria from oxidative damage. Cysteine residues in ICDH are very important for its catalytic function but are inhibited by some lipid peroxidation products (50). In our study, AC may also have suppressed ICDH activity in this way. In line with our other findings, ICDH levels normalised with WP treatment. This can be explained by the ability of WP to inhibit the formation of the membrane LP product 4-hydroxynonenal and H_2O_2, which inhibit this enzyme. In other words, WP may have caused an increase in ICDH activity by increasing the levels of antioxidant enzymes such as mitochondrial GPx or GSH.

α-KGD is a very important TCA cycle enzyme involved in the formation of NADH, and its activity is regulated by the ATP/ADP ratio, NAD^+/NADH ratio, calcium, and substrate. Like ICDH, α-KGD is inhibited by oxidative stress (51), and the improvement of α-KGD activity in our study is most likely related to the elimination of mitochondrial oxidative stress by WP.

ATP, which is the last output of the bioenergetic cycle, was also significantly lower in the AC group for all of the above reasons. However, combined WP+AC treatment could not restore ATP levels to normal. Reasons may be several: (a) insufficient effect of WE on the ATP synthesis mechanisms by substrate-level phosphorylation, (b) AC inhibition of the F0F1-ATPase activity, or (c) failure of the WP dose to normalise/counter this inhibition. Further studies may clarify these reasons.

Considering that oxidative stress in liver mitochondria plays an important role in the pathogenesis of various chronic liver diseases, our results have clearly demonstrated the protective potential of WP against various liver disorders and diseases owed to mitochondrial dysfunction. It will also be important to compare and interpret our *in vivo* findings with future *in vitro* studies using human liver cells.

Conflicts of interest

None to declare.

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Bjelančevine iz sirutke štite mitohondrijsku funkciju jetre od oksidacijskoga stresa u štakora izloženih akroleinu

Akrolein je jedan od najtoksičnijih okolišnih onečišćivača, povezan s nepotpunim izgaranjem nafte i njezinih derivata, drva i plastike te s prženjem u ulju i pušenjem duhana, a uzrokuje oksidacijsko oštećenje DNA i mitohondrija. S obzirom na to da se malo zna o zaštitnom djelovanju bjelančevina iz sirutke protiv toksičnoga djelovanja akroleina u jetri, cilj je ovog istraživanja bio doznati više o tome putem oksidacijskoga stresa mitohondrija u jetri, razinâ enzima potrebnih za stanično disanje i Krebsova ciklusa te razinâ adenozin trifosfata (ATP). U tu smo svrhu štakore soja Sprague Dawley gavažom na usta izlagali samo akroleinu u dnevnoj dozi od 5 mg/kg tj. mase (u fiziološkoj otopini), samo bjelančevinama iz sirutke 200 mg/kg tj. mase (komercijalni proizvod, također u fiziološkoj otopini) ili njihovom kombinacijom šest dana u tjednu tijekom 30 dana. Kao što smo i očekivali, skupina koja je primala samo akrolein iskazala je pad razina glutationa te aktivnosti enzima zaduženih za antioksidaciju, lanac prijenosa elektrona i Krebsov ciklus (ciklus trikarboksilnih kiselina) te značajno povećanje lipidne peroksidacije i razina proteinskih karbonila u mitohondrijima. Kombinirana primjena s bjelančevinama iz sirutke ublažila je oksidacijski stres i poboljšala enzimsku aktivnost. Bjelančevine iz sirutke smanjile su toksično djelovanje akroleina na jetrene mitohondrije u štakora tako što su osnažile bioenergetske mehanizme i uklonile oksidacijski stres.

KLJUČNE RIJEČI: antioksidansi; glutation; enzimi oksidacijske fosforilacije; enzimi ciklusa trikarboksilnih kiselina