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NATURAL DRUGS

PROTECTIVE EFFECT OF THE EXTRACT FROM *ARONIA MELANOCARPA* L. BERRIES AGAINST CADMIUM-INDUCED OXIDATIVE STRESS IN THE KIDNEY: A STUDY IN AN *IN VIVO* EXPERIMENTAL MODEL

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Abstract: The destructive impact of cadmium (Cd) on the oxidative/antioxidative status of the kidney, as well as the possible beneficial effect of co-administration of a 0.1% aqueous extract from *Aronia melanocarpa* L. berries (AM), were studied in a rat model of low-level and moderate general population exposure to this heavy metal (1 and 5 mg Cd/kg feed, respectively, for up to 24 months). Total antioxidative status (TAS) of the kidney and the main indices of the enzymatic (superoxide dismutase - SOD, catalase - CAT, glutathione reductase - GR, and glutathione peroxidase - GPx) and non-enzymatic (reduced glutathione - GSH and thioredoxin - TRx) antioxidative barrier were assessed. The total oxidative status (TOS) and concentrations of hydrogen peroxide (H_2O_2), xanthine oxidase (XOD), myeloperoxidase (MPO), and oxidized glutathione (GSSG) were measured as markers of oxidative status. The oxidative stress index (OSI) was calculated (TOS/TAS) to estimate the intensity of oxidative stress in the kidney. The exposure to Cd, dose- and duration-dependently, weakened the enzymatic and non-enzymatic antioxidative potential of the kidney leading to a decrease in its TAS, as well as enhanced oxidative status of this organ (increased the concentrations of H_2O_2, MPO, and/or XOD, and elevated TOS) resulting in the development of oxidative stress. The administration of AM during the low-level and moderate intoxication with Cd significantly protected from this xenobiotic-induced disruption of the oxidative/antioxidative balance and development of oxidative stress in the kidney. In summary, even low-level long-term exposure to Cd may result in the occurrence of oxidative stress in the kidney, whereas supplementation with chokeberry products may improve the oxidative/antioxidative balance preventing oxidative stress in this organ. Based on the findings it seems possible that the recently noted by us in the experimental model protective effect of the administration of AM against the damaging impact of Cd on the structure and function of the kidney might result, at least partially, from its antioxidative potential and prevention of the development of oxidative stress in the renal tissue.

Keywords: *Aronia melanocarpa* L., cadmium, kidney, oxidative/antioxidative balance, oxidative stress, protection

Abbreviations: ACR - albumin concentration in the urine adjusted for creatinine concentration; ALP - alkaline phosphatase; AM - a 0.1% aqueous extract from *Aronia melanocarpa* L. berries; Bax - Bcl2-associated X protein; b.w. - body weight; CAT - catalase; Cd - cadmium; Cd^{2+} - cadmium ions; Cu - copper; CV - coefficient of variation; ELISA - enzyme-linked immunosorbent assay; Fe^{2+} - divalent iron ions; FR - free radicals; GPx - glutathione peroxidase; GR - glutathione reductase; GSH - reduced glutathione; GSSG - oxidized glutathione; HRP - horseradish peroxidase; H_2O_2 - hydrogen peroxide; KIM-1 - kidney injury molecule 1; MIP1a - macrophage inflammatory protein 1 alpha; Mn - manganese; Mn-SOD - manganese-dependent superoxide dismutase; MPO - myeloperoxidase; NADH - nicotinamide adenine dinucleotide; NADPH - nicotinamide adenine dinucleotide phosphate; NAG - N-acetyl-β-D-glucosaminidase; $^{\bullet}OH$ - hydroxyl radical, OSI - oxidative stress index; $O_2^{\bullet-}$ - superoxide radical; PCR - total protein concentration in the urine adjusted for creatinine concentration; ROS - reactive oxygen species; SD - standard deviation; - SH group - sulfhydryl group; SOD - superoxide dismutase; TAS - total antioxidative status; TOS - total oxidative status; TRx - thioredoxin; w.w., wet weight; XOD - xanthine oxidase; Zn - zinc; β2-MG - beta2-microglobulin

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For a long time, the interest of not only the medical community and scientists but also of the general population, in the possibility of using biologically active substances naturally occurring in some plants and various products based on these plants in the prevention and treatment of many diseases, including civilization diseases, has been observed (for a review, see 1-3). However, recently more and more attention has been paid to the possibility of using plant-based products to prevent health risks posed by exposure to toxic substances (for a review, see 3-6). One of these plants is chokeberry (*Aronia melanocarpa* L.), a deciduous shrub belonging to the family *Rosaceae*. It is found all over the world and is well-known for the medical and culinary properties of its berries (chokeberries). Aronia fruits are a rich source of numerous substances with health-promoting properties and one of the richest sources of polyphenolic compounds characterized by high antioxidative properties (1-3, 7). Chokeberries are also a source of bioelements (e.g. calcium, magnesium, and iron), vitamins (vitamins A, C, E, and K and vitamins from group B), triterpenes, phytosterols, carotenoids (β-carotene), pectins, sugar, sugar alcohols (parasorboside and sorbitol), tannins, dietary fiber, organic acids (L-malic acid and citric acid), carbohydrates, and proteins (3, 7). The components of aronia berries possess powerful antioxidative, antidiabetic, anti-inflammatory, anti-infective, antimutagenic, and cytotoxic properties (1-4, 7). Thus, consuming products from aronia berries, such as dried fruit, juices, jams, preserves, syrups, teas, tinctures, and powdered fruit in the form of supplements has cardioprotective, gastroprotective, hepatoprotective, radioprotective, and immunomodulatory properties, and may effectively protect from the development of some chronic illnesses, including metabolic disorders, cardiovascular diseases, and cancer, and is used to support the treatment of some diseases (1-4, 7). Because of the many health-promoting properties, the inclusion of aronia products into the daily diet is widely recommended (1-4).

Some time ago, considering the beneficial health properties of aronia berries, including especially their high antioxidative potential (1-3, 7) and the ability of polyphenolic compounds to chelate ions of divalent metals (4, 6), we hypothesized that chokeberry-based products may be effective in preventing from unfavourable effects of exposure to cadmium (Cd). This toxic heavy metal belongs to the main chemical pollutants of the natural environment and food in industrialized countries (for a review, see 5, 8, 9). Numerous epidemiological studies provide evidence that the current exposure of the worldwide population to this heavy metal creates a risk to health, including the risk of kidney damage, which is a target organ for this xenobiotic during long-term exposure (10-13). Moreover, forecasts show that exposure to this heavy metal will grow (11). Thus it is very important from the point of view of public health to find an effective strategy against its toxicity.

To investigate the possibility of a protective effect of chokeberry extract during exposure to Cd, we conducted a widely designed study in a female rat model of low-level and moderate lifespan general population exposure to Cd in industrialized countries (the treatment of animals with Cd in the feed at a concentration of 1 and 5 mg Cd/kg, respectively, for up to 24 months). Previously, it was found in the model that the administration of a 0.1% extract from *A. melanocarpa* berries (AM) protected against Cd accumulation in the body, including mainly the liver and kidneys (14), and provided significant protection against this heavy metal-induced damage to the liver (15-17), bones (18, 19), and salivary glands (20) and that these effects resulted, at least partially, from antioxidative properties of AM.

Our more recent study (21) showed that the administration of AM during the exposure to 1 and 5 mg Cd/kg also protected from changes in the morphological structure of the kidney tubules and glomeruli and deterioration of their function. The low-level and moderate treatment with this xenobiotic resulted in damage to the kidney tubules, detected based on enhanced presence in the urine of sensitive biomarkers such as kidney injury molecule-1 (KIM-1), β2-microglobulin (β2-MG), N-acetyl-β-D-glucosaminidase (NAG), and alkaline phosphatase (ALP), as well as to increase in albumin concentration in the urine (ACR) and decreased glomerular filtration rate (estimated based on creatinine clearance) which are clinically relevant early markers of glomerular injury. Pathological changes in the renal tubules such as hyalinization, hyperplasia and hypertrophy of the epithelium of the convoluted tubules, the proliferation of the interstitial kidney tissue, as well as glomerulonephritis and congestion at the cortex/medullary interface were noted at both levels of the 24-month exposure to Cd. Moreover, vacuolization and an extension of the tubular lumen were observed at the low-level treatment and perivascular oedema occurred at the moderate exposure. Inflammatory processes developed in the kidney as well (increased concentrations of chemerin and/or Bcl2-associated X protein - Bax). The toxic impact of Cd on the kidney occurred at

the concentrations of this xenobiotic in the urine (0.0852-0.2820 μg/g creatinine) which were within the lower range of concentrations currently noted in the worldwide general population (for a review, see 10). The co-administration of AM significantly protected from the above-described nephrotoxic action of Cd (21).

The mechanism of the toxic action of Cd, including its nephrotoxicity, is multidirectional and involves its prooxidative action leading to the development of oxidative stress and oxidative damage to the cellular macromolecules and organelles (10, 13, 22). In the view of our previous findings (15-17, 19, 20), we have hypothesized that the protective impact of AM on the kidney (21) may also be related to its antioxidative potential. To investigate this hypothesis, the present study aimed to evaluate the impact of the administration of AM under low-level and moderate exposure to Cd (1 and 5 mg/kg feed) on the oxidative/antioxidative balance in the kidney. For this purpose, total antioxidative status (TAS), and the main markers of the enzymatic (superoxide dismutase - SOD, catalase - CAT, glutathione peroxidase - GPx, and glutathione reductase - GR) and non-enzymatic antioxidative status (reduced glutathione - GSH and thioredoxin - TRx), as well as total oxidative status (TOS) and some indices of the oxidative status such as the concentrations of hydrogen peroxide (H_2O_2), xanthine oxidase (XOD), myeloperoxidase (MPO), and oxidized glutathione (GSSG), in the renal tissue of the female rats administered with Cd and/or AM were determined. The oxidative stress index (OSI), reflecting the extent of destroying the oxidative/antioxidative balance and the level of oxidative stress, was calculated as well. Moreover, the relationships between the main indices of the oxidative/antioxidative status of the kidney (TAS, TOS, and OSI) and already published Cd concentration in this organ (14), as well as markers of its damage (21) were evaluated. To the best of our knowledge, no such research has ever been carried out.

EXPERIMENTAL

Fodder containing Cd

The feed containing Cd at the concentration of 1 and 5 mg Cd/kg was manufactured by Label Food "Morawski" Kcynia by adding cadmium chloride 2.5 hydrate ($CdCl_2$ x 2.5 H_2O; POCh; Gliwice, Poland) to the ingredients of the standard Labofeed H diet (breeding diet ensuring the correct development and growth of young animals) and Labofeed B diet (maintenance diet). Cd content in the fodder was verified in our laboratory by determination of this element concentration (using the atomic absorption spectrometry method). The concentration of Cd in the 1 mg Cd/kg diet was determined at 1.09 ± 0.13 mg/kg (mean ± standard deviation - SD) and 4.92 ± 0.53 mg/kg in the case of the 5 mg Cd/kg diet. Cd concentration in the standard Labofeed diet was 0.0584 ± 0.0049 mg/kg (14).

Extract from the berries of *A. melanocarpa*

A powdered extract from the berries of *A. melanocarpa*, provided by Adamed Consumer Healthcare (Tuszyn, Poland), was used to prepare AM administered to the animals. According to the manufacturer's certificate (Certificate KJ 4/2010), the extract contained 65.74% polyphenols and 18.65% anthocyanins. Other components found in the extract included carotenoids, pectins, sugar, sugar alcohols, phytosterols, triterpenes, as well as minerals and vitamins (producer's data). To prepare the AM that was administered to the animals, 1 g of the powdered extract was dissolved in 1 L of redistilled water every day. The total concentration of polyphenolic compounds and the polyphenolic profile of the extract were estimated by us (18). One litter of the AM contained (mean ± standard error - SE) 612.40 ± 3.33 μg of total polyphenols, including 202.28 ± 1.28 μg of total anthocyanins (80.07 ± 1.05 μg - cyanidin 3-O-β-galactoside, 33.21 ± 0.01 μg - cyanidin 3-O-α-arabinoside, and 3.68 ± 0.01 μg - cyanidin 3-O-β-glucoside), 129.87 ± 1.12 μg of total proanthocyanidins, 110.92 ± 0.89 μg of total phenolic acids, 68.32 ± 0.08 μg of chlorogenic acid, and 21.94 ± 0.98 μg of total flavonoids (18). Cd concentration in AM did not exceed 0.05 μg/L (14).

Animal model

The experimental protocol was approved by the Bialystok Local Ethics Committee for Animal Experiments (Poland; permit number 60/2009 on September 21, 2009). All animal procedures followed institutional guidelines, ethical standards, and the International Guide for the Use of Animals in Biomedical Research. In the study, 192 female Wistar rats (Hannover Wistar rats, bred according to the Charles River International Genetic Standardization Program - Crl: WI (Han)), aged three to four weeks, from a licensed breeding facility (Laboratory Animal House in Brwinów, Poland) were used. The animals were housed throughout the experiment under standard conditions (temperature 22 ± 2°C, relative humidity 50 ± 10%, and a 12-hour light/dark cycle), with free access to feed and drinking fluids (redistilled water or AM). The rats were kept in stainless steel cages (4 rats in each). After

5-day acclimatization to the experimental environment, the rats were randomly assigned to one of six experimental groups, each of 32 animals. One group was given AM (0.1% aqueous solution of the extract from *A. melanocarpa* berries) as the only drinking fluid (AM group), two groups were intoxicated with Cd via the feed at the concentration of 1 and 5 mg Cd/kg (Cd_1 and Cd_5 groups, respectively), and the next two groups were given Cd (1 or 5 mg Cd/kg feed) and AM simultaneously (Cd_1+AM group and Cd_5+AM group) for up to 24 months. The last group was fed redistilled water (containing < 0.05 μg Cd/L) and a standard Labofeed diet (without the addition of Cd) and served as the control group.

The concentration of Cd in the blood, urine, and kidney of the animals in the Cd_1 group (0.1030-0.3240 μg Cd/L, 0.0852-0.2820 μg/g creatinine, and 0.2626-2.8322 μg/g of wet weight - w.w., respectively) and Cd_5 group (0.7350-1.3320 μg Cd/L, 0.2839-0.6949 μg/g creatinine, and 0.9739-14.8705 μg/g w.w., respectively) (14) was within the range of values noted nowadays in the general population non-exposed occupationally to this heavy metal (10, 11), confirming that the used experimental model well reflects current human environmental exposure to this xenobiotic.

The daily intake of aronia extract and polyphenolic compounds in the female rats that received AM as the only drinking fluid ranged from 63.1 to 154.7 mg/kg body weight (b.w.) and from 41.5 to 101.7 mg/kg b.w., respectively (14). The intake of polyphenols was several times higher than the average consumption of these compounds among the worldwide general population (1000 mg/24 h i.e. 14.29 mg/kg b.w. assuming the body weight of 70 kg) (23).

The model has been reported in detail elsewhere (14-21) and thus in the present study, it is only briefly described and together with the range of performed measurements is presented in Figure 1.

The slices of the right kidneys dissected at necropsy after 3, 10, 17, and 24 months of the experiment (14) were used in the present study. The kidneys, immediately after collection, were rinsed with ice-cold physiological saline (0.9% sodium chloride), dried on the filter paper, and weighed with an analytical balance (OHAUS®, Nanikon, Switzerland; accuracy to 0.0001 g). The organs were stored frozen (−70°C) until assayed.

Determination of the indices of oxidative and antioxidative status of the kidney

Preparation of kidney homogenates

Pre-weighted slices of the right kidney were homogenized in a cold potassium phosphate buffer (50 mM, pH = 7.4; prepared by combining 50 mM potassium dihydrogen phosphate and 50 mM dipotassium hydrogen phosphate (POCh; Gliwice, Poland)) to prepare 10% homogenates (weight/volume), with the addition of butyl-hydroxytoluene

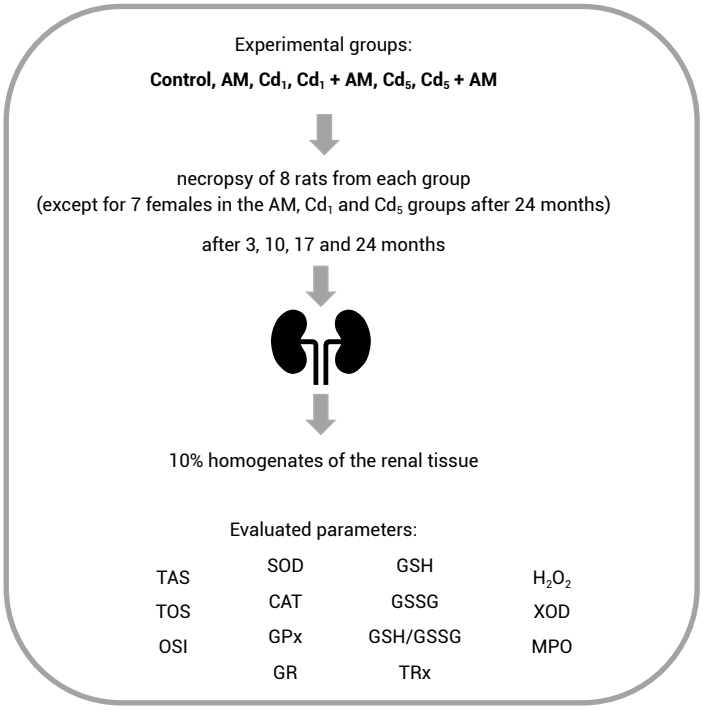

Figure 1. Schematic representation of the experimental protocol.

(Sigma-Aldrich GmbH; Steinheim, Germany) as an antioxidant. Per each 1 mL of the homogenate, 0.01 mL of 0.5 M butyl-hydroxytoluene in acetonitrile (Merck, Darmstadt, Germany) was used. Such prepared homogenates were centrifuged at 10,000 g for 5 minutes at 4°C (24) with a Medical Instruments MPW-350R centrifugator (Warsaw, Poland), and the aliquots were separated and stored frozen (−70°C) until all measurements were performed.

The assessment of the parameters of the oxidative/antioxidative status

The indices of the oxidative/antioxidative status were determined in the aliquots of the kidney homogenates colorimetrically with the use of MULTISCAN GO (Thermo Scientific, Vantaa, Finland) and Epoch (Bio Tek Instruments, Inc, Winooski, USA) spectrophotometers for quantification.

TAS was measured using the Immundiagnostik AG (Bensheim, Germany) ImAnOx (TAS) ELISA kit (Catalogue No. KC 5200). The enzyme-linked immunosorbent assay (ELISA) method is based on the reaction of eliminating a defined amount of H_2O_2 added into the tested sample by antioxidants present in the sample. The remaining H_2O_2 produces compounds that absorb the wavelength of 450 nm. The certified values of TAS in two control samples provided by the manufacturer were within the range of 162-220 μmol/L and 204-276 μmol/L. The values of TAS determined by us were within the reference ranges (184 ± 19 μmol/L and 236 ± 21 μmol/L, respectively, for the first used kit and 183 ± 15 μmol/L and 244 ± 16 μmol/L, respectively, for the second one; mean ± SD). The precision of the measurements of TAS in the aliquots of the kidney homogenates, expressed as the intra-assay coefficient of variation (CV) was < 3%. The inter-assay CV was < 4%.

TOS was evaluated using the Immundiagnostik AG PerOx (TOS) ELISA kit (Catalogue No. KC 5100) based on the determination of total lipid peroxides contained in the studied sample in the reaction with peroxidase at 450 nm. The values of TOS assayed by us in the control samples (190 ± 12 μmol/L and 515 ± 23 μmol/L in the first of the used kits and 204 ± 17 μmol/L and 585 ± 33 μmol/L in the second one; mean ± SD) agreed with the certified values (170-283 μmol/L and 437-728 μmol/L, respectively). The intra-assay CV for TOS measurements in the tested samples was < 6%. The inter-assay CV was < 5%. Based on the determined values of TAS and TOS, OSI was calculated as the ratio of TOS and TAS (OSI = TOS/TAS).

The activity of total SOD was determined using a commercial kit bought from Cayman Chemical Company (Catalogue No. 706002; Ann Arbor, MI, USA). The test detects superoxide radicals ($O_2^{\bullet-}$) produced by xanthine oxidase and hypoxanthine using a tetrazolium salt. The intra-assay CV was < 4% and the inter-assay CV was < 9%. The determination of the activity of CAT was based on the spectrophotometric method by Aebi (25), measuring the quantity of H_2O_2 destroyed by this enzyme. The disappearance of H_2O_2 was seen spectrophotometrically at 240 nm. The intra-assay CV was < 6%. Bioxytech GPx-340 kit (Catalogue No. 21017; Percipio Biosciences (Burlingame, CA, USA) was used to measure GPx activity. This enzyme is used in the assay to catalyze the oxidation of GSH to GSSG, which is begun by the addition of tertbutyl hydroperoxide. The intra-assay CV was < 7%. Determination of the activity of GR was made with the use of the commercial Bioxytech GR-340 kit by Percipio Biosciences (Catalogue No. 21018D; Burlingame, CA, USA). This assay is based on the reduction of GSSG with the use of nicotinamide adenine dinucleotide phosphate (NADPH; one particle of NAPDH is consumed for the reduction of one particle of GSSG), which is oxidized (to nicotinamide adenine dinucleotide - NADH) in the reaction catalysed by GR. The amount of GSSG that is reduced to GSH is estimated indirectly by the assessment of consumed NADH reflected in a decrease in the absorbance measured at 340 nm. The intra-assay CV of this measurement was < 5%.

The concentrations of GSH and GSSG were determined colorimetrically using the Cayman Chemical Company Glutathione Assay Kit (Catalogue No. 703002; Ann Arbor, MI, USA). In order to assay the concentration of GSH, the reaction between GSH and 5,5'-dithio-bis-2-nitrobenzoic acid (DTNB) was performed. Then, the absorbance (at 405-412 nm) of the sample containing the product of this reaction (5-thio-2-nitrobenzoic acid - TNB), was measured. The GSSG was quantified by first derivatizing GSH with 2-vinylpyridine. The intra-assay CV for the determination of GSH and GSSG was < 7%. The inter-assay CV was < 7% for GSH and < 3% for GSSG. Furthermore, the GSH/GSSG ratio was calculated.

The concentration of TRx was assayed with the use of commercial Rat (Trx) ELISA KIT (Catalogue No. 201-11-0445; SunRed, Shanghai, China). The principle of this test was based on the incubation of the sample in wells pre-coated with rat TRx monoclonal antibody, and then the addition of horseradish peroxidase (HRP)-conjugated streptavidin (streptavidin-HRP) in order to obtain immune complex, which then is combined with chromogens.

The chroma of colour is positively correlated with the concentration of TRx, hence it was measured calorimetrically (450 nm) in order to determine this parameter. The intra-assay CV was < 4%, whereas the inter-assay CV was < 8%.

The concentration of H_2O_2 was determined with Bioxytech H_2O_2-560 kit (Catalogue No. 21024; Percipio Biosciences (Burlingame, CA, USA). The assay is based on the reaction of oxidation of divalent iron ions (Fe^{2+}) by H_2O_2 to trivalent iron ions (Fe^{3+}), which binds with xylenol orange, resulting in the formation of a stable, coloured complex measured spectrometrically at 560 nm. The intra-assay CV was < 9%.

XOD in the kidney was measured with the use of Rat (XOD) ELISA KIT (Catalogue No. SRB-T-88306; SunRed, Shanghai, China). The kit used a double-antibody sandwich ELISA method with wells pre-coated with XOD monoclonal antibody. The coloured product of the reaction with chromogen was measured at 450 nm. The intra-assay CV was < 7% and the inter-assay CV was < 4%.

In order to determine the concentration of MPO, Rat (MPO) ELISA KIT (Catalogue No. 201-11-0575; SunRed, Shanghai, China) was used. The determination is based on the positive correlation between the chroma of the colour of complex being a product of the reaction of chromogen with the complex of MPO, streptavidin-HRP, and rat MPO monoclonal antibody measured at 450 nm with the concentration of MPO in an assayed sample. The intra- and inter-assay CV were < 8% and < 4%, respectively.

Statistical analysis

Statistica 13.3 software (StatSoft, Tulsa, OK, USA) was used for all statistical analyses. The results of the determination of the indices of the oxidative/antioxidative status are presented as a median, 25-75% confidence interval, minimum and maximum. The Shapiro-Wilk test was used to evaluate the normality of the data distribution at first. As no normal distribution was noted, a nonparametric signed-rank Kruskal-Wallis with median test (Kruskal-Wallis test) was performed to check if there were statistically significant ($p < 0.05$) differences between the six groups. If statistically significant differences occurred between the six groups, further comparisons were performed to determine statistically significant ($p < 0.05$) differences between particular two experimental groups.

To calculate the mutual dependences between the values of the measured parameters of the oxidative/antioxidative balance, as well as these parameters and already published Cd concentration in this organ (14) and markers of its damage (21), a linear regression analysis was performed. The results of the analysis of regression are shown as the β coefficient, R^2, and the level of statistical significance (p). A dependence between two variables was statistically significant at the value of the β coefficient for which $p < 0.05$.

RESULTS

TAS, TOS, and OSI

The exposure to the 1 mg Cd/kg fed for 3 and 10 months had no impact on TAS, TOS, and OSI in the renal tissue. After 17 and 24 months of the treatment, TAS was decreased (by 33% and 36%, respectively), whereas TOS (6.5- and 7.9-fold, respectively) and OSI (8.6- and 13.9-fold, respectively) were elevated compared to the proper values noted in the control group (Figure 2). At the higher level of exposure to Cd (5 mg/kg feed), TAS dropped already after 3 months (by 42%) and remained at the decreased level (by 30-49%) until the end of the study. Moreover, TOS and OSI in the Cd_5 group were enhanced after 17 (6.6- and 8.5-fold, respectively) and 24 months (7.9- and 13.2-fold, respectively) (Figure 2). There were no differences in the values of TOS, TAS, and OSI in the renal tissue at particular time points between the Cd_1 group and the Cd_5 group, except for lower (by 44%) value of TAS at the higher level of exposure after 3 months of the study (Figure 2).

The administration of AM alone for 3-24 months had no impact on TOS, TAS, and OSI in the renal tissue, whereas its intake during the low-level and moderate exposure to Cd prevented all the above-described changes in the values of TOS, TAS, and OSI, except for TAS in the Cd_5+AM group after 3 and 10 months, that was decreased compared to the control group (by 45% and 41%, respectively) (Figure 2).

Antioxidative enzymes activity

The exposure to Cd resulted in a decrease in the activities of all determined antioxidative enzymes (SOD, CAT, GR, and GPx) in the renal tissue. The effect of Cd depended on the intensity and duration of the treatment (Figures 3 and 4). The activity of SOD was unaffected by the low-level exposure to Cd but at the moderate intoxication for 3-24 months, it was lower (by 30-56%) than in the control group (Figure 3). The activity of CAT, at the exposure to the 1 mg Cd/kg feed was lower

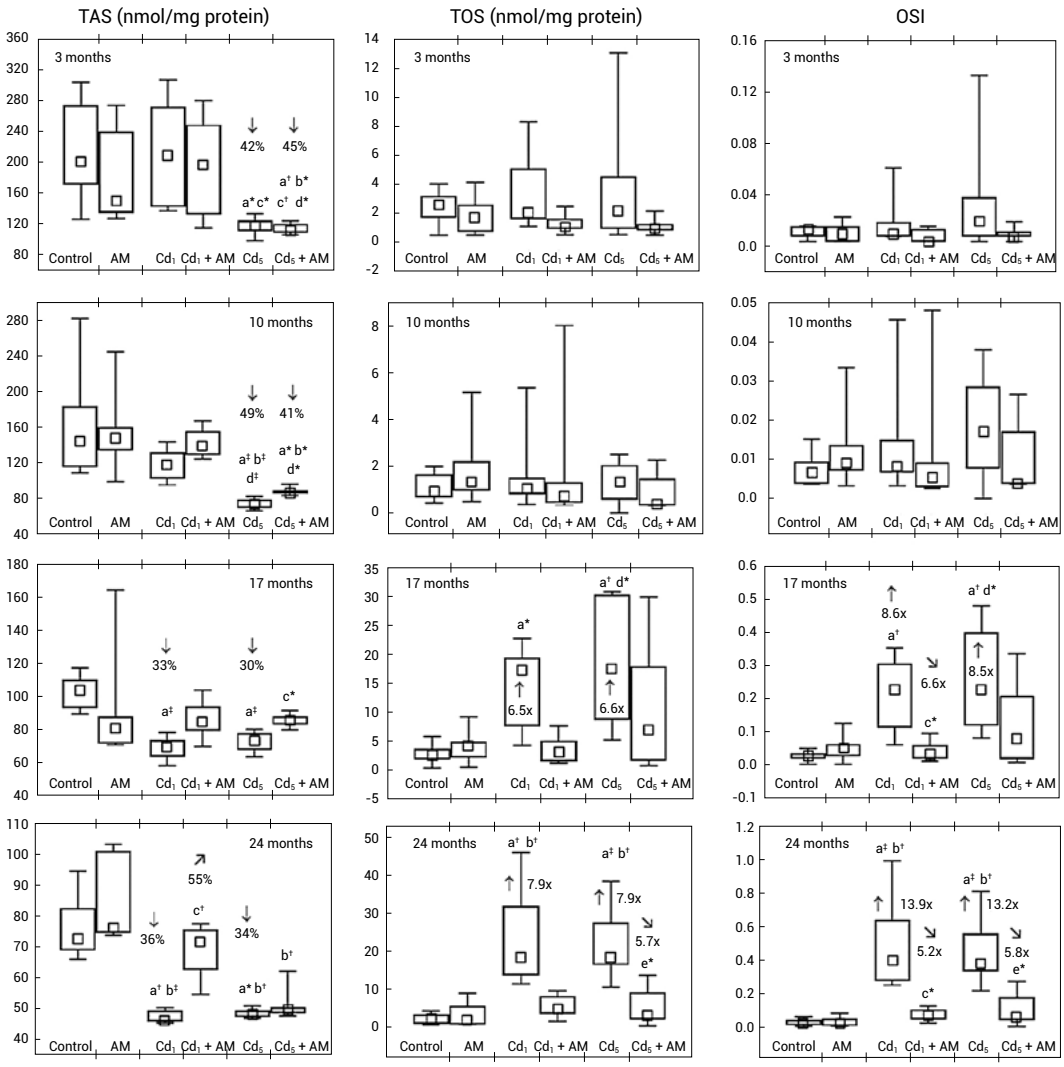

Figure 2. Total antioxidative status (TAS), total oxidative status (TOS), and oxidative stress index (OSI) in the kidney of female rats. The animals were treated with cadmium (Cd) in the feed at the concentration of 0, 1, or 5 mg/kg (Control, Cd_1, and Cd_5 groups) and/or 0.1% extract from the berries of *Aronia melanocarpa* L. (AM, Cd_1+AM, and Cd_5+AM groups) for 3, 10, 17, and 24 months. Data are shown as median, 25–75% confidence interval, and minimum and maximum values for eight animals (except for seven females in the AM, Cd_1, and Cd_5 groups after 24 months). Statistically significant differences (Kruskal-Wallis test) compared to: a – Control group, b – AM group, c – Cd_1 group, d – Cd_1+AM group, and e – Cd_5 group, where $^{*}p < 0.05$, $^{\dagger}p < 0.01$, and $^{\ddagger}p < 0.001$, are marked. The factors of changes or percentage changes compared to the control group (↓, decrease; ↑, increase) or the adequate group treated with Cd alone (↘, decrease; ↗, increase) are indicated by the numerical values below or above the bars.

after 3 and 10 months (7- and 2.7-fold respectively) and unaffected due to the longer treatment, whereas at the exposure to 5 mg Cd/kg feed, it was decreased (3.7-15-fold) throughout the whole experiment (Figure 3). In the Cd_1 group, an increase (6.9 times) in the activity of GR was noted after 24 months, whereas in the Cd_5 group, the activity of this enzyme was enhanced (3 times) after 17 months (Figure 4). Throughout the study, the activity of GPx at both levels of exposure to Cd was lower than in the control group (2-9.2 times in the Cd_1 group and 2.8-8.8 times in the Cd_5 group) (Figure 4). There were no differences in the activities of the measured antioxidative enzymes in the renal tissue at particular time points between the Cd_1 group and the Cd_5 group, except for lower SOD activity after 3 months (by 33%) and GR activity after 24 months (by 71%) and higher (1.8-fold) GR activity after 17 months in the Cd_5 group (Figures 3 and 4).

The supplementation with AM of the animals that were not treated with Cd (AM group) had no impact on the activities of all measured antioxidative enzymes (Figures 3 and 4). The administration of the extract during the low-level and moderate exposure to Cd prevented all changes in the

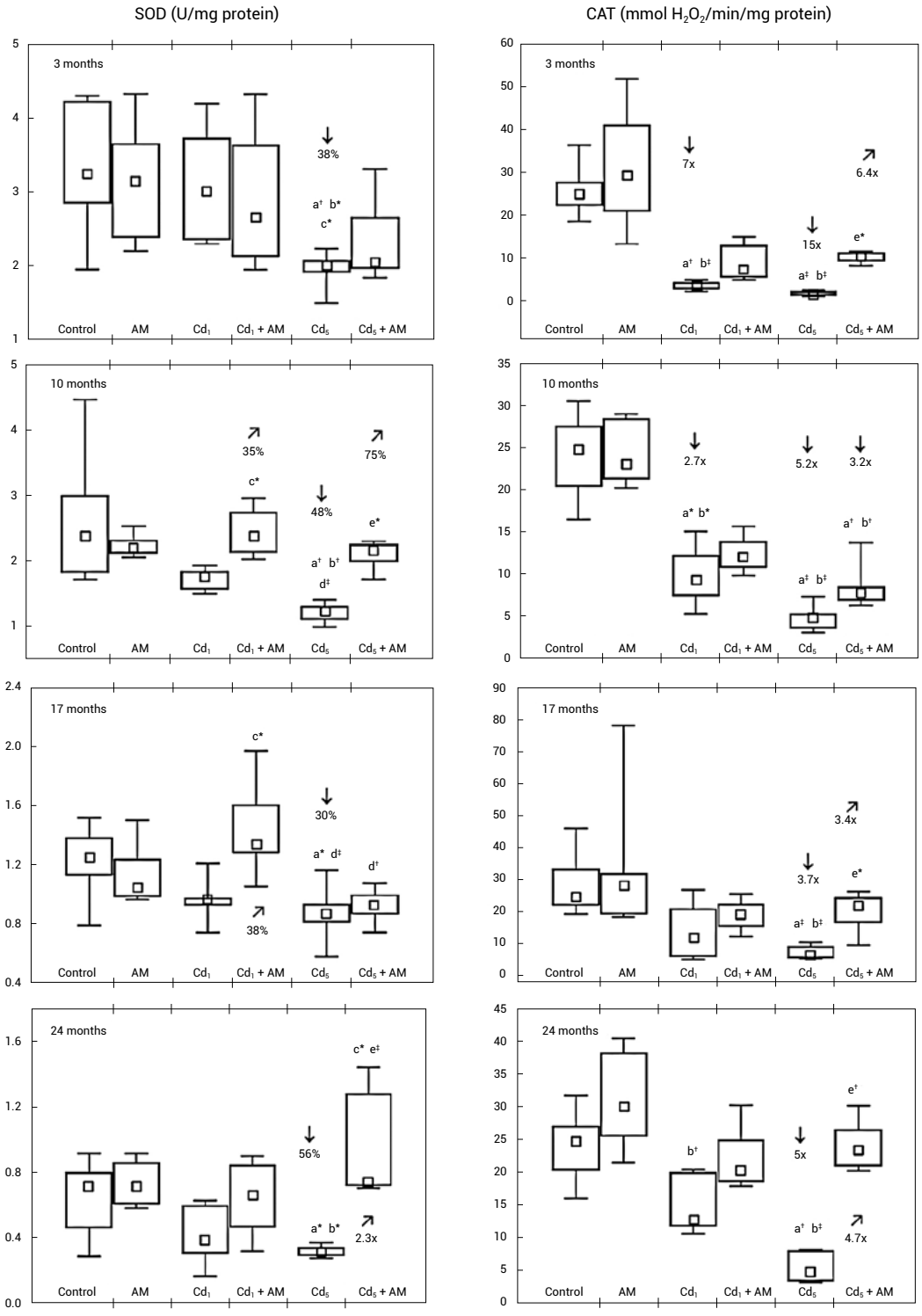

Figure 3. The activity of superoxide dismutase (SOD) and catalase (CAT) in the kidney of female rats. The animals were treated with cadmium (Cd) in the feed at the concentration of 0, 1, or 5 mg/kg (Control, Cd_1, and Cd_5 groups) and/or 0.1% extract from the berries of *Aronia melanocarpa* L. (AM, Cd_1+AM, and Cd_5+AM groups) for 3, 10, 17, and 24 months. Data are shown as median, 25–75% confidence interval, and minimum and maximum values for eight animals (except for seven females in the AM, Cd_1, and Cd_5 groups after 24 months). Statistically significant differences (Kruskal-Wallis test) compared to: a – Control group, b – AM group, c – Cd_1 group, d – Cd_1+AM group, and e – Cd_5 group, where $^{*}p < 0.05$, $^{\dagger}p < 0.01$, and $^{\ddagger}p < 0.001$, are marked. The factors of changes or percentage changes compared to the control group (↓, decrease) or the adequate group treated with Cd alone (↗, increase) are indicated by the numerical values below or above the bars.

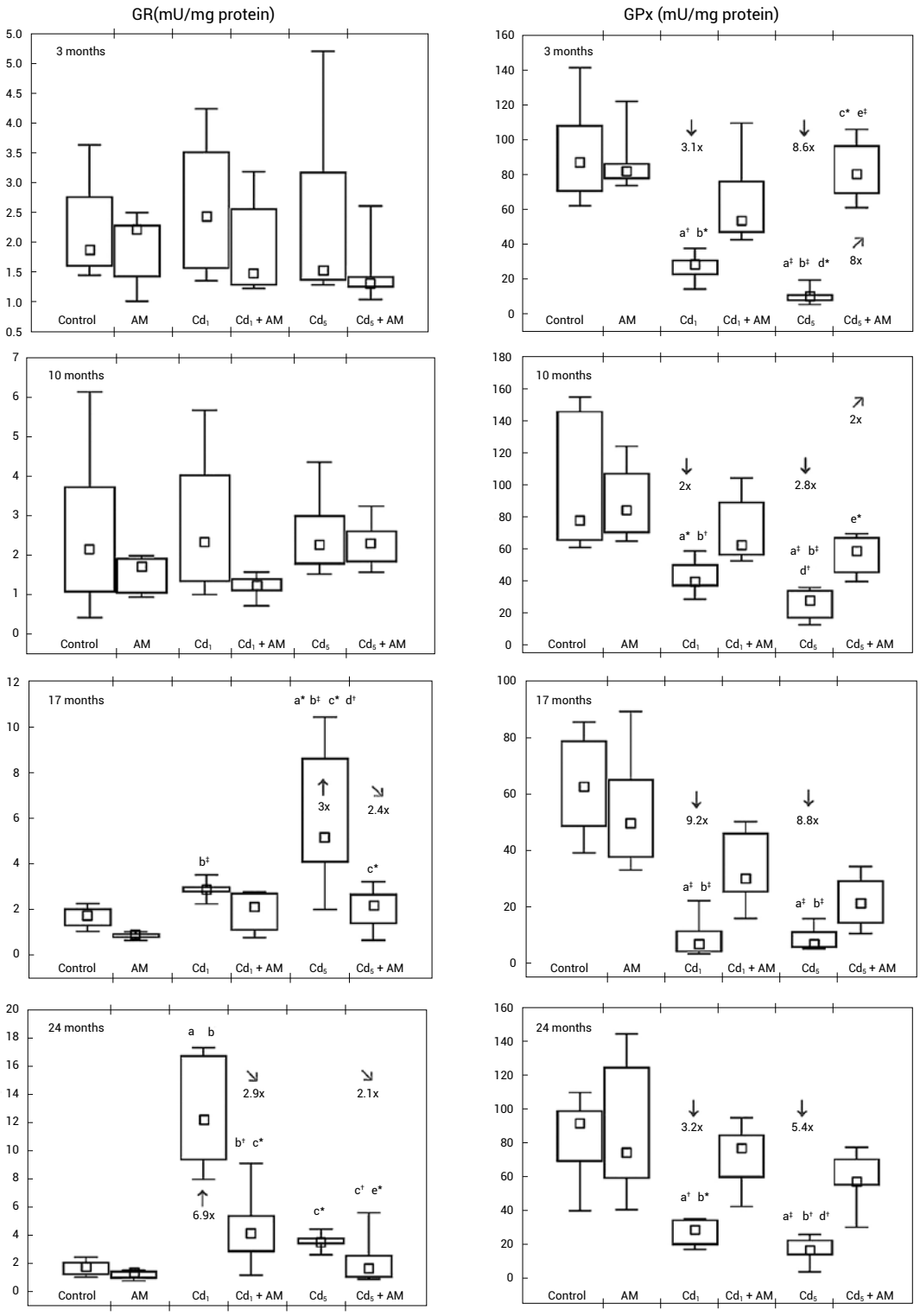

Figure 4. The activity of glutathione reductase (GR) and glutathione peroxidase (GPx) in the kidney of female rats. The animals were treated with cadmium (Cd) in the feed at the concentration of 0, 1, or 5 mg/kg (Control, Cd_1, and Cd_5 groups) and/or 0.1% extract from the berries of *Aronia melanocarpa* L. (AM, Cd_1+AM, and Cd_5+AM groups) for 3, 10, 17, and 24 months. Data are shown as median, 25–75% confidence interval, and minimum and maximum values for eight animals (except for seven females in the AM, Cd_1, and Cd_5 groups after 24 months). Statistically significant differences (Kruskal-Wallis test) compared to: a – Control group, b – AM group, c – Cd_1 group, d – Cd_1+AM group, and e – Cd_5 group, where $^{*}p < 0.05$, $^{\dagger}p < 0.01$, and $^{\ddagger}p < 0.001$, are marked. The factors of changes compared to the control group (↓, decrease; ↑, increase) or the adequate group treated with Cd alone (↘, decrease; ↗, increase) are indicated by the numerical values below or above the bars.

activities of the antioxidative enzymes, induced by this heavy metal, except for CAT activity in the Cd_5+AM group which after 10 months was decreased (3.2-fold) compared to the control group. The administration of AM to the animals treated with the 1 mg Cd/kg feed for 10 and 17 months resulted in an increase in the activity of SOD (by 35% and 38%), respectively, compared to the respective group receiving Cd alone but the activity did not differ compared to the control group (Figure 3). Moreover, the activity of GR in the Cd_5+AM group after 24 months was lower (2.1-fold) compared to the Cd_5 group (Figure 4).

Glutathione homeostasis

The kidney concentration of GSH in the animals fed with the diet containing 1 mg Cd/kg for 17 and 24 months was lower than in the control group (4.7- and 2.4-fold, respectively), whereas at the higher level of exposure, the concentration was decreased, compared to the control group, throughout the whole experiment (2.6-7.3 times) (Figure 5). The concentration of GSSG was increased after 10 months in the Cd_1 group (2.3-fold) and after 24 months in the Cd_1 group and Cd_5 group (3.7- and 3.4-fold, respectively) (Figure 5). At both levels of exposure to Cd, the GSH/GSSG ratio was lower than in the control

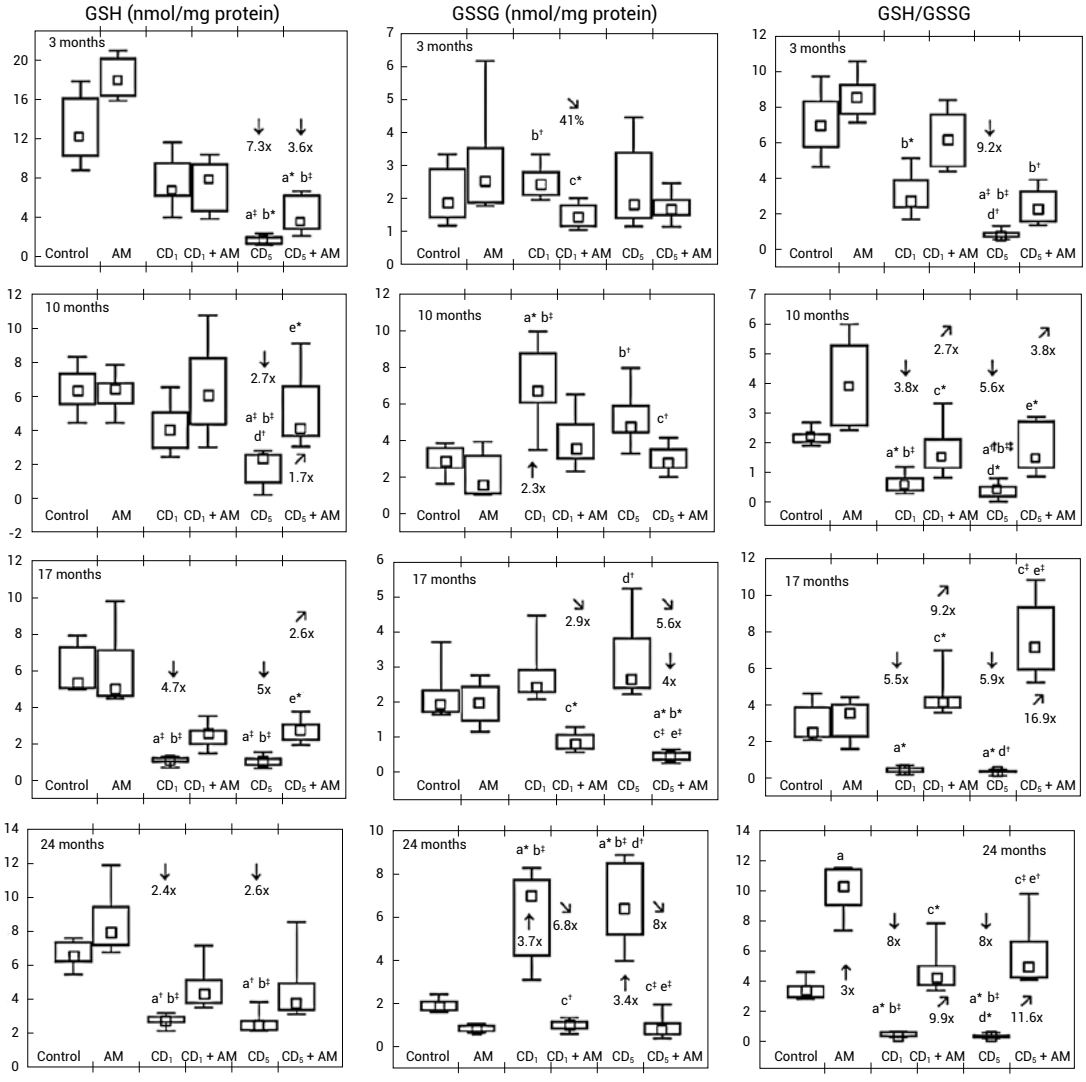

Figure 5. The concentration of reduced glutathione (GSH) and oxidized glutathione (GSSG), as well as their ratio (GSH/GSSG) in the kidney of female rats. The animals were treated with cadmium (Cd) in the feed at the concentration of 0, 1, or 5 mg/kg (Control, Cd_1, and Cd_5 groups) and/or 0.1% extract from the berries of *Aronia melanocarpa* L. (AM, Cd_1+AM, and Cd_5+AM groups) for 3, 10, 17, and 24 months. Data are shown as median, 25–75% confidence interval, and minimum and maximum values for eight animals (except for seven females in the AM, Cd_1, and Cd_5 groups after 24 months). Statistically significant differences (Kruskal-Wallis test) compared to: a – Control group, b – AM group, c – Cd_1 group, d – Cd_1+AM group, and e – Cd_5 group, where $^{*}p < 0.05$, $^{\dagger}p < 0.01$, and $^{\ddagger}p < 0.001$, are marked. The factors of changes or percentage changes compared to the control group (↓, decrease; ↑, increase) or the adequate group treated with Cd alone (↘, decrease(↗, increase) are indicated by the numerical values below or above the bars.

group (3.8-9.2 times) throughout the study, except for the Cd_1 group after 3 months in which the ratio did not differ compared to the control group (Figure 5). There were no differences in the concentrations of GSH and GSSG, and the GSH/GSSG ratio between the Cd_1 group and the Cd_5 group (Figure 5).

The administration of AM alone had no impact on glutathione homeostasis in the kidney estimated based on the concentrations of GSH and GSSG, and their ratio, except for 3-fold higher GSH/GSSG ratio after 24 months. However, the extract administration during the low-level and moderate exposure to Cd prevented this heavy metal-induced disorder in this homeostasis, except for the decrease in GSH concentration in the Cd_5 group after 3 months (Figure 5). Moreover, the concentration of GSSG in the Cd_5+AM group after 17 months was lower compared to both the Cd_5 group (5.6-fold) and the control group (4-fold) (Figure 5).

TRx concentration

The exposure to the 1 mg Cd/kg feed had no impact on the concentration of TRx in the renal tissue (Figure 6). The treatment with the 5 mg Cd/kg feed resulted in a decrease (by 53%) in TRx concentration after 3 months and an increase (by 41%) after 24 months (Figure 6). The concentration of TRx in the Cd_5 group after 3 months was lower (by 44%) compared to the Cd_1 group but after the longer exposure, there was no difference in the value of this parameter between the two groups (Figure 6).

In the Cd_1+AM group, the concentration of TRx after 10 months was lower (by 39%) compared to the control group. After 24 months, it was lower (by 31%) than in the Cd_1 group but did not differ compared to the

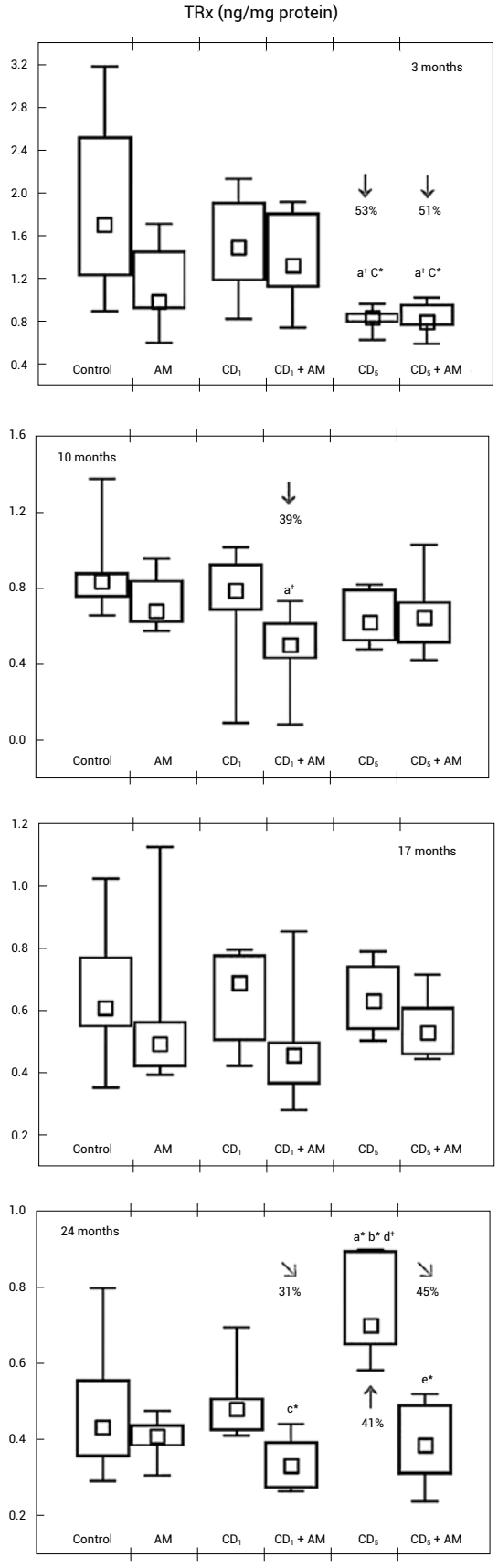

Figure 6. The concentration of thioredoxin (TRx) in the kidney of female rats. The animals were treated with cadmium (Cd) in the feed at the concentration of 0, 1, or 5 mg/kg (Control, Cd_1, and Cd_5 groups) and/or 0.1% extract from the berries of *Aronia melanocarpa* L. (AM, Cd_1+AM, and Cd_5+AM groups) for 3, 10, 17, and 24 months. Data are shown as median, 25–75% confidence interval, and minimum and maximum values for eight animals (except for seven females in the AM, Cd_1, and Cd_5 groups after 24 months). Statistically significant differences (Kruskal-Wallis test) compared to: a – Control group, b – AM group, c – Cd_1 group, d – Cd_1+AM group, and e – Cd_5 group, where $^*p < 0.05$, and $^\dagger p < 0.01$, are marked. The percentage changes compared to the control group (↓, decrease, ↑, increase) or the adequate group treated with Cd alone (↘, decrease) are indicated by the numerical values below or above the bars.

control group. The 24-month administration of AM during the moderate exposure to Cd prevented this heavy metal-induced increase in TRx concentration, whereas its 3-month administration did not counteract the impact of Cd (Figure 6).

H_2O_2 concentration

In the animals maintained on the diet containing 1 and 5 mg Cd/kg for 3, 10, and 17 months, the kidney concentration of H_2O_2 was within the range of the control group; however, after 24 months it was increased (4.9- and 4.6-fold, respectively) (Figure 7). Throughout the study, there was no difference in H_2O_2 concentration between the Cd_1 and Cd_5 groups (Figure 7).

In the Cd_1+AM group and the Cd_5+AM group, the concentration of this compound throughout the study was lower (from 31% to 7.2-fold) compared to the respective group treated with Cd alone, and after 3 months it was even lower (by 54% and 63%, respectively) than in the control animals. The 24-month supplementation with the extract at both levels of exposure to Cd prevented this xenobiotic-induced increase in the concentration of H_2O_2 (Figure 7).

XOD and MPO concentrations

The low-level exposure to Cd had no impact on the concentrations of XOD and MPO, except for an increase in MPO concentration after 17 and 24 months (by 81% and 2.4-fold, respectively) (Figure 8). At the moderate exposure to this xenobiotic, both parameters were unaffected after 3 and 10 months but they increased (74% to 4 times) due to the longer (17 and 24 months) treatment (Figure 8). There were no differences in the concentrations of XOD and MPO between the Cd_1 and Cd_5 groups, except for higher (2.1-fold) XOD concentration in the Cd_5 group after 24 months (Figure 8).

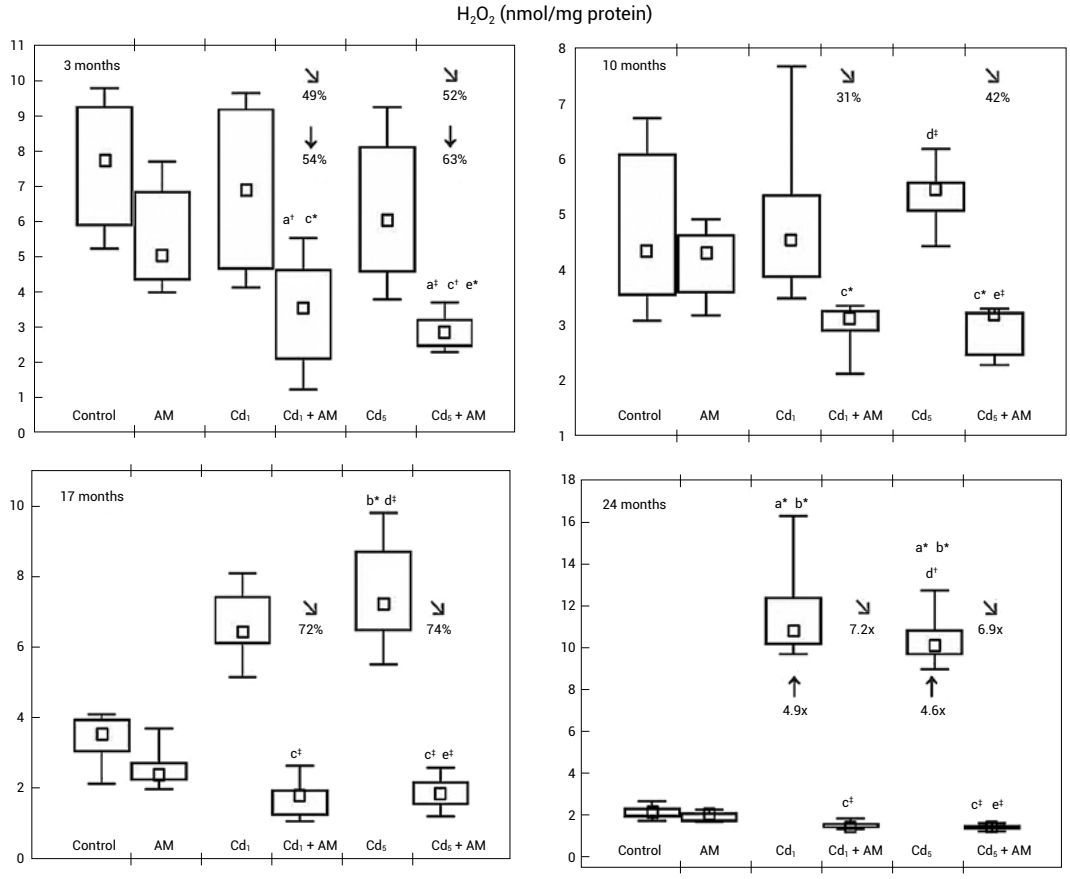

Figure 7. The concentration of hydrogen peroxide (H_2O_2) in the kidney of female rats. The animals were treated with cadmium (Cd) in the diet at the concentration of 0, 1, or 5 mg/kg (Control, Cd_1, and Cd_5 groups) and/or 0.1% extract from the berries of *Aronia melanocarpa* L. (AM, Cd_1+AM, and Cd_5+AM groups) for 3, 10, 17, and 24 months. Data are shown as median, 25–75% confidence interval, and minimum and maximum values for eight animals (except for seven females in the AM, Cd_1, and Cd_5 groups after 24 months). Statistically significant differences (Kruskal-Wallis test) compared to: a – Control group, b – AM group, c – Cd_1 group, d – Cd_1+AM group, and e – Cd_5 group, where $^*p < 0.05$, $^\dagger p < 0.01$, and $^\ddagger p < 0.001$, are marked. The factors of changes or percentage changes compared to the control group (↓, decrease, ↑, increase) or the adequate group treated with Cd alone (↘, decrease) are indicated by the numerical values below or above the bars.

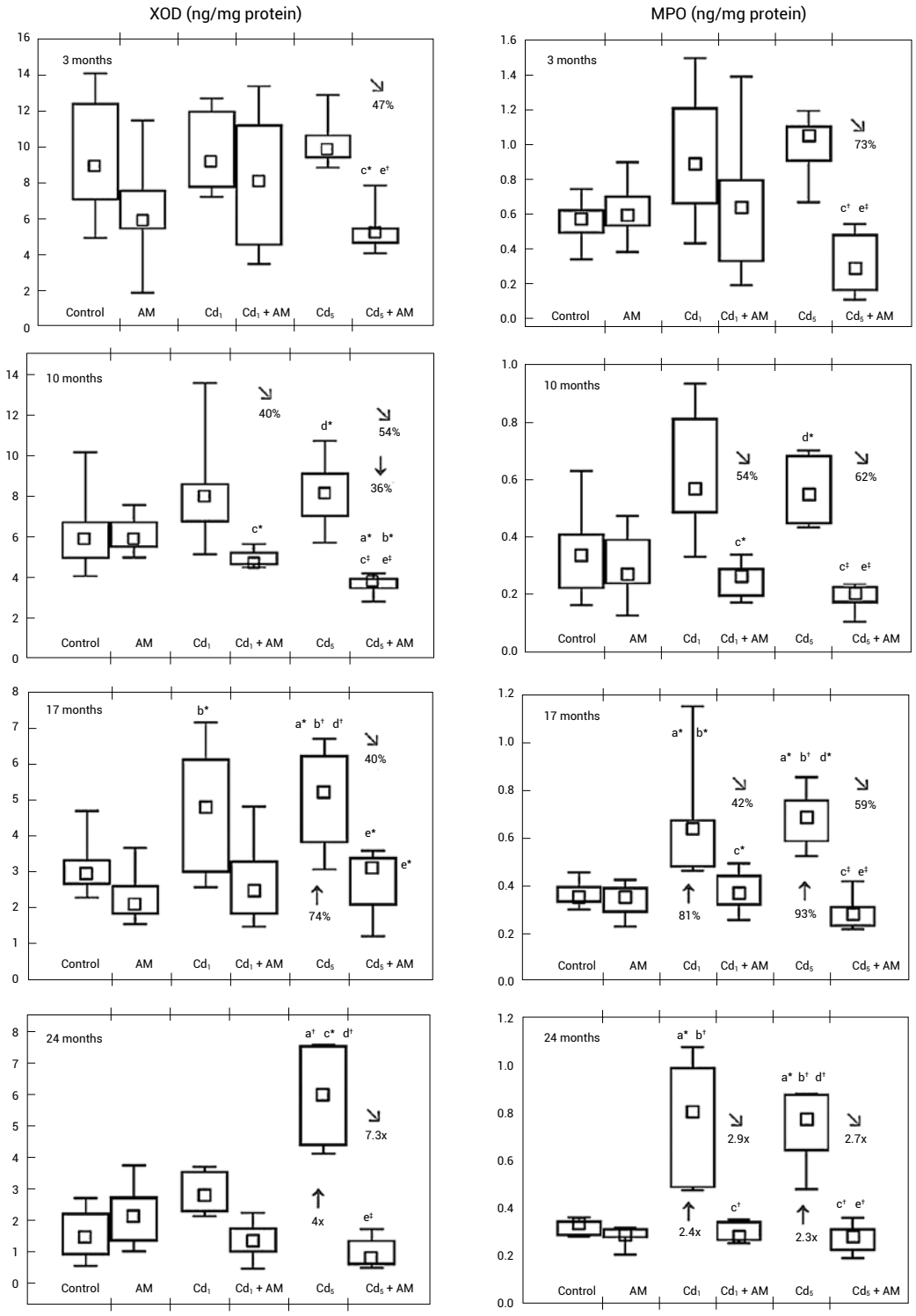

Figure 8. The concentrations of xanthine oxidase (XOD) and myeloperoxidase (MPO) in the kidney of female rats. The animals were treated with cadmium (Cd) in the feed at the concentration of 0, 1, or 5 mg/kg (Control, Cd_1, and Cd_5 groups) and/or 0.1% extract from the berries of *Aronia melanocarpa* L. (AM, Cd_1+AM, and Cd_5+AM groups) for 3, 10, 17, and 24 months. Data are shown as median, 25–75% confidence interval, and minimum and maximum values for eight animals (except for seven females in the AM, Cd_1, and Cd_5 groups after 24 months). Statistically significant differences (Kruskal-Wallis test) compared to: a – Control group, b – AM group, c – Cd_1 group, d – Cd_1+AM group, and e – Cd_5 group, where $^{*}p < 0.05$, $^{\dagger}p < 0.01$, and $^{\ddagger}p < 0.001$, are marked. The factors of changes or percentage changes compared to the control group (↓, decrease, ↑, increase) or the adequate group treated with Cd alone (↘, decrease) are indicated by the numerical values below or above the bars.

The administration of AM prevented the impact of Cd on the concentration of MPO. Moreover, the concentrations of XOD and MPO in the Cd_1+AM group after 10 months and in the Cd_5+AM group after 3 and 10 months were lower (by 40-73%) compared to the respective groups treated with Cd alone and in the case of the concentration of XOD in the Cd_5+AM group after 10 months also compared to the control group (by 36%) (Figure 8).

The relationships between the indices of the oxidative/reductive status of the kidney and Cd concentration in this organ

In the female rats that were not administered AM (the control group and the Cd_1 and Cd_5 groups), negative dependencies were found between Cd concentration in the kidney and TAS, the activities of SOD, CAT, and GPx, the concentrations of GSH and TRx, and the GSH/GSSG ratio in this organ (Table 1). Moreover, positive correlations were noted between this toxic element concentration and TOS, OSI, GR activity, and the concentrations of GSSG and H_2O_2 in the kidney (Table 1). A lack of dependence between Cd concentration and the concentrations of XOD and MPO was found (Table 1).

In the animals receiving AM alone and during the treatment with Cd (AM, Cd_1+AM, and Cd_5+AM groups), negative dependencies occurred between Cd concentration and TAS, the activities of SOD and GPx, and the concentrations of GSH, GSSG, TRx, XOD,

Table 1. Relationships between the investigated biomarkers of the oxidative/reductive kidney status and cadmium (Cd) concentration in this organ of female rats administered or not with a 0.1% extract from the berries of *Aronia melanocarpa* L. (AM)

Parameter	Regression analysis	Cd in the kidney of rats	
		Not administered with AM	Administered with AM
TAS	β^p R^2	–0.437 ‡ 0.182	–0.450 ‡ 0.194
TOS	β^p R^2	0.535 ‡ 0.278	0.443 ‡ 0.188
OSI	β^p R^2	0.483 ‡ 0.225	0.502 ‡ 0.244
SOD	β^p R^2	–0.446 ‡ 0.190	–0.400 ‡ 0.147
CAT	β^p R^2	–0.445 ‡ 0.189	NS
GPx	β^p R^2	–0.494 ‡ 0.236	–0.460 ‡ 0.203
GR	β^p R^2	0.242 * 0.048	NS
GSH	β^p R^2	–0.516 ‡ 0.258	–0.400 ‡ 0.149
GSSG	β^p R^2	0.286 † 0.072	–0.310 † 0.084
GSH/GSSG	β^p R^2	–0.485 ‡ 0.227	NS
TRx	β^p R^2	–0.230 * 0.044	–0.290 † 0.071
H_2O_2	β^p R^2	0.360 ‡ 0.120	–0.430 ‡ 0.173
XOD	β^p R^2	NS	–0.410 ‡ 0.159
MPO	β^p R^2	NS	–0.280 † 0.071

Cd concentrations in the kidney of the rats subjected to necropsy after 3, 10, 17, and 24 months have already been published (14). The results of the analysis of regression are presented as the β coefficient, R^2, and the level of statistical significance (p, where *$p < 0.05$, †$p < 0.01$, and ‡$p < 0.001$). NS, a lack of relationship ($p > 0.05$). All groups not administered with AM were included in the analysis (the control group that received the fodder containing 0.0584 ± 0.0049 mg Cd/kg and the Cd_1 and Cd_5 groups maintained on the feed containing 1 and 5 mg Cd/kg, respectively). All groups administered with AM were included in the analysis (the AM, Cd_1+AM, and Cd_5+AM groups).

MPO, and H_2O_2 in the kidney (Table 1). TOS and OSI positively correlated with the kidney concentration of Cd. Moreover, there were no dependencies between this heavy metal concentration in the kidney and the activities of CAT and GR, and the GSH/GSSG ratio (Table 1).

The relationships between the kidney TAS, TOS, and OSI and markers of this organ damage

Numerous correlations between the main indices of the oxidative/reductive status of the kidney and biomarkers of damage to the tubules and glomeruli, as well as markers of inflammatory processes in this organ were noted in the animals that were not administered with AM (the control group and the Cd_1 and Cd_5 groups) (Table 2). The kidney TAS negatively correlated with the concentrations of KIM-1 and β2-MG, the activities of NAG and ALP, ACR, total protein concentration in the urine (PCR), and urea concentration in the serum, whereas TOS and OSI positively correlated with these markers

Table 2. Relationships between the kidney total antioxidative status (TAS), total oxidative status (TOS), and oxidative stress index (OSI) and biomarkers of this organ damage in the female rats administered or not with a 0.1% extract from the berries of *Aronia melanocarpa* L. (AM)

Parameter	Regression analysis	Not Administered with AM			Administered with AM		
		TAS	TOS	OSI	TAS	TOS	OSI
Markers of tubular damage							
KIM-1 in the urine	β^p R^2	–0.270 † 0.062	0.386 ‡ 0.140	0.333 † 0.101	NS	NS	NS
β2-MG in the urine	β^p R^2	–0.540 ‡ 0.279	0.639 ‡ 0.402	0.662 ‡ 0.432	–0.370 ‡ 0.128	NS	NS
NAG in the urine	β^p R^2	–0.490 ‡ 0.232	0.677 ‡ 0.452	0.793 ‡ 0.625	NS	NS	NS
ALP in the urine	β^p R^2	–0.470 ‡ 0.217	0.469 ‡ 0.212	0.552 ‡ 0.297	–0.250 * 0.053	NS	NS
Markers of tubular damage							
ACR	β^p R^2	–0.570 ‡ 0.318	0.764 ‡ 0.580	0.762 ‡ 0.575	–0.330 † 0.118	0.226 * 0.041	0.233 * 0.044
PCR	β^p R^2	–0.620 ‡ 0.374	0.646 ‡ 0.411	0.705 ‡ 0.491	–0.360 ‡ 0.118	NS	NS
Creatinine clearance	β^p R^2	0.541 ‡ 0.285	–0.330 † 0.096	–0.340 ‡ 0.106	NS	NS	NS
Uric acid in the serum	β^p R^2	NS	0.372 ‡ 0.129	0.342 ‡ 0.107	NS	0.336 ‡ 0.103	0.362 ‡ 0.121
Uric acid in the urine	β^p R^2	NS	NS	NS	NS	NS	NS
Urea in the serum	β^p R^2	–0.250 * 0.050	0.379 ‡ 0.135	0.428 ‡ 0.174	0.220 * 0.318	NS	NS
Urea in the urine	β^p R^2	NS	NS	NS	NS	NS	NS
Inflammatory markers							
Chemerin in the kidney	β^p R^2	NS	0.259 * 0.057	0.278 † 0.067	0.469 ‡ 0.211	NS	NS
MIP1a in the kidney	β^p R^2	0.532 ‡ 0.276	NS	NS	0.866 ‡ 0.747	–0.360 ‡ 0.118	–0.450 ‡ 0.193
Bax in the kidney	β^p R^2	0.374 ‡ 0.130	NS	NS	0.784 ‡ 0.610	–0.210 * 0.032	–0.260 † 0.060

The results of the analysis of regression are presented as the β coefficient, R^2, and the level of statistical significance (p, where *$p < 0.05$, †$p < 0.01$, and ‡$p < 0.001$). NS, a lack of relationship ($p > 0.05$). All groups not administered with AM were included in the analysis (the control group that received the fodder containing 0.0584 ± 0.0049 mg Cd/kg and the Cd_1 and Cd_5 groups maintained on the feed containing 1 and 5 mg Cd/kg, respectively). All groups administered with AM were included in the analysis (the AM, Cd_1+AM, and Cd_5+AM groups). ACR, albumin concentration in the urine adjusted for creatinine concentration; ALP, alkaline phosphatase; Bax, Bcl2-associated X protein; KIM-1, kidney injury molecule 1; MIP1a, macrophage inflammatory protein 1 alpha; NAG, N-acetyl-β-D-glucosaminidase; PCR, total protein concentration in the urine adjusted for creatinine concentration; β2-MG, beta2-microglobulin

of kidney injury. A positive dependence occurred between TAS and creatinine clearance and negative relationships were noted between TOS and OSI and this marker of glomerular function. Moreover, positive dependencies were revealed between the kidney TAS and the concentrations of inflammatory markers such as macrophage inflammatory protein 1 alpha (MIP1a) and Bax in this organ, as well as between TOS and OSI and the concentration of chemerin. In the animals that were supplemented with AM, negative relationships between the kidney TAS and β2-MG concentration in the urine, ALP activity in the urine, ACR, and PCR, and positive dependencies between TAS and urea concentration in the serum, and the kidney concentrations of chemerin, MIP1a, and Bax were noted. Positive correlations occurred between the kidney TOS and OSI and ACR and uric acid concentration in the serum. Moreover, TOS and OSI negatively correlated with the concentrations of MIP1a and Bax in the kidney.

Mutual relationships between the indices of the oxidative/reductive status of the kidney

In both, the rats that were not administered with AM (the control group and the Cd_1 and Cd_5 groups) and those receiving AM alone and during the treatment with Cd (AM, Cd_1+AM, and Cd_5+AM groups), numerous mutual negative relationships were noted between the investigated biomarkers of the antioxidative and oxidative status, including the negative dependencies between TAS of the kidney and TOS and OSI (Table 3).

DISCUSSION

The current study is the first to show that even low-level long-term exposure to Cd leads to disruption of the oxidative/reductive balance and development of oxidative stress in the kidney, whereas consumption of an extract from the berries of *A. melanocarpa* protects against these effects. Since the study was performed in the *in vivo* model well reflecting current exposure of the worldwide general population to Cd, based on the findings it seems possible that environmental exposure to this xenobiotic may disturb the balance between oxidants and antioxidants in the human kidney and lead to oxidative stress in this organ. Furthermore, this article also provides evidence that these outcomes of Cd action may be counteracted by the use of a well-known due to its strong antioxidative properties natural product i.e., extract from *A. melanocarpa* berries.

The results of epidemiological studies (9-13), as well as our recent findings (21) from the research, performed in the experimental model of the current environmental exposure to Cd in industrialized countries used in the present paper, show that the low-level exposure to this toxic element poses a risk of kidney damage. It is known that the mechanism of Cd nephrotoxicity is multidirectional and that oxidative stress is one of the main pathways of the toxic action of this xenobiotic, including damage to the kidney (10, 13, 22). Numerous studies have found, that this heavy metal may induce oxidative stress and oxidative modifications of cellular macromolecules and cellular structures in the kidney, but at higher than in the present study levels of exposure and its higher concentrations in the blood, urine, and/or kidney (26-31). The detailed mechanism of the injurious impact of Cd on this organ at low exposure is still unknown; however, the results of the present study together with numerous dependencies between the main indices of the oxidative/antioxidative status (TAS, TOS, and OSI) and markers of tubular and glomerular damage in the animals exposed to this xenobiotic show that this organ injury may be related to the prooxidative Cd properties and the destruction of the oxidative/reductive balance in the renal tissue.

Under chronic, even low-level, exposure Cd is accumulated in the kidney (10, 14, 32). Initially, it is stored in the epithelial cells of the kidney tubules in the form of unharmful complexes with metallothionein (MT), but these complexes (Cd-MT) are characterized by short half-life and are decomposed with the release of Cd ions (Cd^{2+}) capable of exerting toxic action via various mechanisms, including first of all induction of oxidative stress (10, 33). Although Cd is not a redox-active agent and is unable to generate free radicals (FR) and reactive oxygen species (ROS) directly, this element may destroy the oxidative/reductive balance and lead to the development of oxidative stress indirectly by the pathways such as weakening of the enzymatic and non-enzymatic antioxidative barrier, inducing the activities of oxidases, increasing the concentrations of prooxidants, and causing injury to the mitochondria, which are considered the target cellular organelles for the toxic action of this xenobiotic (10, 13, 17, 22). After penetrating the mitochondrial membranes, Cd^{2+} ions interfere with the electron transport chain, resulting in electron leakage and increased generation of ROS. Moreover, they affect the course of metabolic processes in the mitochondria, such as respiration and the Krebs cycle. As a result, relatively large amounts of H_2O_2 may be released and the oxidative status of cells and tissues can enhance (22). Via the indirect mechanisms, Cd^{2+} ions contribute to the production

Table 3. Mutual relationships between the investigated parameters describing the kidney oxidative/reductive status in the female rats administered (*italic*) or not administered with a 0.1% extract from the berries of *Aronia melanocarpa* L. (AM)

Parameter	Regression analysis	TAS	TOS	OSI	SOD	CAT	GPx	GR	GSH	GSSG	GSH/ GSSG	TRx	H_2O_2	XOD	MPO
TAS	β^p R^2	----	*−0.250 ** *0.051*	*−0.380* ‡ *0.138*	*0.802* ‡ *0.640*	*NS*	*0.367* ‡ *0.125*	*NS*	*0.519* ‡ *0.262*	*0.412* ‡ *0.161*	*NS*	*0.830* ‡ *0.686*	*0.811* ‡ *0.654*	*0.855* ‡ *0.728*	*0.610* ‡ *0.365*
TOS	β^p R^2	−0.440 ‡ 0.186	----	*0.951* ‡ *0.904*	*−0.360* ‡ *0.122*	*NS*	*−0.270* † *0.063*	*0.296* † *0.078*	*NS*	*−0.270* † *0.063*	*NS*	*−0.210 ** *0.035*	*−0.290* † *0.074*	*−0.290* † 0.076	*NS*
OSI	β^p R^2	−0.480 ‡ 0.227	0.971‡ 0.943	----	*−0.460* ‡ *0.200*	*NS*	*−0.280* † *0.069*	*0.352* ‡ *0.114*	*−0.220 ** *0.040*	*−0.340* ‡ *0.107*	*−0.230 ** *0.041*	*−0.310* † *0.085*	*−0.390* ‡ *0.140*	*−0.400* ‡ *0.153*	*NS*
SOD	β^p R^2	0.949 ‡ 0.899	−0.480 ‡ 0.219	−0.520 ‡ 0.261	----	*−0.220 ** *0.038*	*0.300* † *0.080*	*−0.240 ** *0.046*	*0.486* ‡ *0.228*	*0.509* ‡ *0.251*	*NS*	*0.715* ‡ *0.506*	*0.778* ‡ *0.601*	*0.778* ‡ *0.601*	*0.499* ‡ *0.241*
CAT	β^p R^2	NS	NS	NS	NS	----	*NS*	*NS*	*0.314* † *0.088*	*NS*	*0.315* † *0.089*	*NS*	*NS*	*NS*	*NS*
GPx	β^p R^2	0.473 ‡ 0.216	−0.400 ‡ 0.150	0.360 ‡ 0.123	0.442 ‡ 0.187	0.680 ‡ 0.457	----	*NS*	*0.382* ‡ *0.137*	*NS*	*NS*	*0.262 ** *0.059*	*0.393* ‡ *0.145*	*0.402* ‡ *0.152*	*NS*
GR	β^p R^2	−0.270 † 0.060	0.561 ‡ 0.307	0.611 ‡ 0.367	−0.280 † 0.068	NS	NS	----	*NS*	*NS*	*NS*	*NS*	*NS*	*NS*	*NS*
GSH	β^p R^2	0.717 ‡ 0.508	−0.390 ‡ 0.140	−0.380 ‡ 0.132	0.640 ‡ 0.403	0.518 ‡ 0.260	0.685 ‡ 0.463	−0.250 * 0.054	----	*0.369* ‡ *0.127*	*0.434* ‡ *0.179*	*0.465* ‡ *0.208*	*0.671* ‡ *0.445*	*0.376* ‡ *0.132*	*0.485* ‡ *0.227*
GSSG	β^p R^2	−0.330 † 0.100	0.316 † 0.090	0.409 ‡ 0.158	−0.310 † 0.086	−0.270 † 0.064	−0.210 * 0.033	0.326 † 0.097	−0.290 † 0.077	----	*−0.480* ‡ *0.221*	*0.206 ** *0.032*	*0.522* ‡ *0.264*	*0.338* ‡ *0.105*	*NS*
GSH/ GSSG	β^p R^2	0.641 ‡ 0.405	−0.380 ‡ 0.136	−0.390 ‡ 0.144	0.587 ‡ 0.337	0.530 ‡ 0.273	0.615 ‡ 0.372	−0.290† 0.072	0.911 ‡ 0.828	−0.510 ‡ 0.257	----	*NS*	*NS*	*NS*	*0.236 ** *0.046*
TRx	β^p R^2	0.836 ‡ 0.695	−0.210 * 0.033	−0.250 * 0.054	0.793 ‡ 0.625	NS	0.343 ‡ 0.108	NS	0.667 ‡ 0.439	NS	0.574 ‡ 0.322	----	*0.693* ‡ *0.474*	*0.775* ‡ *0.596*	*0.691* ‡ *0.458*
H_2O_2	β^p R^2	NS	0.611 ‡ 0.366	0.636 ‡ 0.398	NS	−0.280 † 0.070	−0.330 † 0.096	0.570 ‡ 0.318	NS	0.382 ‡ 0.137	NS	0.244 * 0.049	----	*0.703* ‡ *0.488*	*0.477* ‡ *0.219*
XOD	β^p R^2	0.617 ‡ 0.374	−0.280 † 0.066	−0.310 † 0.084	0.680 ‡ 0.456	−0.370 ‡ 0.126	NS	−0.210 * 0.034	0.203 * 0.031	NS	NS	0.631 ‡ 0.392	0.215 * 0.036	----	*0.570* ‡ *0.317*
MPO	β^p R^2	NS	NS	NS	NS	−0.560 ‡ 0.301	−0.470 ‡ 0.211	0.212 * 0.035	NS	NS	−0.210 * 0.035	0.247 * 0.051	0.466 ‡ 0.208	0.450 ‡ 0.194	----

The groups not administered with AM included in the analysis were the control group that received the fodder containing 0.0584 ± 0.0049 mg Cd/kg and the Cd_1 and Cd_5 groups maintained on the feed containing 1 and 5 mg Cd/kg, respectively. The groups administered with AM included in the analysis were the AM, Cd_1+AM, and Cd_5+AM groups.
The results of the analysis of regression are presented as the β coefficient, R^2, and the level of statistical significance (p, where *$p < 0.05$, †$p < 0.01$, and ‡$p < 0.001$). NS, *NS* a lack of relationship ($p > 0.05$)

of nitryl, hydroxyl ($^{\bullet}OH$), and $O_2^{\bullet-}$, which initiate further processes of the generation of FR and ROS like the Fenton reaction (22). The Cd-caused weakening of the enzymatic and non-enzymatic defence mechanisms, including a reduction in the content of GSH, favours the development of oxidative stress (10, 22).

The measurements performed in the present study revealed that the low-level and moderate exposure to Cd weakened both the enzymatic and non-enzymatic antioxidative barriers and enhanced the level of prooxidants in the kidney leading to disruption of the balance between the processes of oxidation and reduction and as a result the development of oxidative stress in this organ. Although there was no difference in the values of particular parameters of the oxidative/antioxidative status of the renal tissue between the Cd_1 and Cd_5 groups, with only a few exceptions, and OSI (the main marker of oxidative stress) at both levels of exposure was increased to almost the same extent compared to the control group, the detailed analysis of the results allowed for the conclusion that the prooxidative impact of Cd was dependent on the level of exposure and its duration and the weakening of the antioxidative defence mechanisms preceded the enhancement in the oxidative status of the renal tissue. Numerous negative dependencies between Cd concentration in the kidney and indices of the antioxidative status (TAS, the activities of SOD, CAT, and GPx, the concentrations of GSH and TRx, and the GSH/GSSG ratio), as well as positive relationships between this xenobiotic concentration and the indices of the oxidative status (TOS, OSI, and the concentrations of GSSG and H_2O_2) confirm the dependence between the level of exposure to this heavy metal and the intensity of oxidative stress in this organ. The fact that in the Cd_5 group, TAS of the renal tissue was decreased after 3 and 10 months, while its TOS and OSI were unaffected shows that Cd may first weaken the antioxidative capacity of the renal tissue. Initially, in spite of decreased TAS, the antioxidative barrier was sufficient to remain the oxidative status at the proper level. However, the continuation of the exposure resulted in further weakening of the enzymatic and non-enzymatic antioxidative barrier and enhanced the presence of FR and ROS in the kidney cells leading to destroying the oxidative/antioxidative balance and development of oxidative stress.

Regardless of the cause, a decrease in the activities of antioxidative enzymes such as SOD, CAT, and GPx results in increased concentrations of FR and ROS in the cells. CAT and GPx are accountable for the detoxification of H_2O_2, whereas SOD catalyses the dismutation of $O_2^{\bullet-}$ (16, 27, 34). In addition, GPx together with GR, are enzymes involved in the metabolism of GSH. The first enzyme promotes the oxidation of GSH to GSSG, while the second is responsible for the reduction of GSSG to GSH (34). Thus, the decrease in the activities of antioxidative enzymes noted in the animals exposed to Cd resulted in an enhancement of the amount of FR and ROS in the renal cells, as was reflected in the increased concentration of H_2O_2 and the value of TOS in the Cd_1 and Cd_5 groups. The Cd-induced decrease in the activities of SOD, CAT, and GPx might result from interactions between this toxic element and bioelements such as zinc (Zn), copper (Cu), and manganese (Mn) present in the active centre of SOD (ZnCu-SOD, Mn-SOD), selenium necessary for GPx activity, and iron inhered in the active centre of CAT (34). Such an explanation seems very probable in the case of SOD activity as previous research by our team has revealed that the maintenance of rats on the diet containing 1 and 5 mg Cd/kg disturbed the kidney homeostasis of Cu, Zn, and Mn (17, 35). In discussing the impact of the low-level and moderate exposure to Cd on the activity of SOD in the kidney it is very important to underline that recently, at both levels of the treatment with Cd we have reported an increase in the activity of Mn-SOD in the mitochondria of the kidney, that might result from both an increased concentration of Mn in these organelles and the stimulation of defense mechanisms and/or adaptive response of these cellular structures against Cd-mediated enhanced generation of ROS (17, 34). However, the measurements performed in the present study have revealed that, in spite of the enhanced activity of the mitochondrial Mn-SOD, the total activity of SOD in the kidney cells was unaffected or decreased due to the exposure to Cd, depending on its intensity and duration. The unchanged or even decreased total SOD activity in the kidney despite the enhanced activity of Mn-SOD seems to indicate that the activity of ZnCu-SOD in the kidney might be importantly decreased.

The results of the present study allow for the conclusion that the involvement of Cd in the development of kidney damage at low-to-moderate exposure, including the induction of oxidative stress, may be related to the decreased concentration of GSH, which is the main non-enzymatic antioxidant playing an important role in the inactivation of organic and inorganic FR, ROS and xenobiotics, including prooxidants such as Cd. Due to the presence of the sulfhydryl group (-SH group) in its structure, GSH is capable of complexing Cd^{2+} ions into inert form preventing in this way their toxic action. It is

important to emphasize, that TRx being a protein possessing two -SH groups plays a similar role as GSH (16). The decreased concentration of GSH in the kidney in the Cd_1 and Cd_5 groups might result from the use of this compound in the processes of detoxification of Cd accumulated in the renal tissue and this heavy metal generated FR and ROS. The increased concentration of GSSG and elevated GSH/GSSG ratio confirm enhanced GSH utilization. The increased activity of GR, noted at some time points at both levels of exposure to Cd, might be a compensative mechanism aimed at restoring GSH from its oxidized form.

The increased concentration of H_2O_2 noted in the kidney at both levels of exposure to Cd shows that this compound was not enough sufficiently detoxified due to this heavy metal-induced inhibition of the activities of CAT and GPx. Furthermore, the increase in the concentration of H_2O_2 might result from the increased concentrations of MPO and XOD since reactions catalysed by both enzymes are the source of H_2O_2 (and other reactive oxidants, including $O_2^{\bullet-}$) (16). Enhanced concentration of H_2O_2 in the kidney cells may have very negative consequences because this compound is the source of the generation of extremely reactive $^{\bullet}OH$ in the presence of ions of transition metals such as ion of Cu(I) i.e. Cu^+ and Fe^{2+} (the Fenton reaction) (6, 15, 28) and in this way facilitates the development of oxidative stress as was reflected in the enhanced values of TOS and OSI in the renal tissue.

Because the disruption of the oxidative/antioxidative balance in the cells, regardless of the cause, results in oxidative damage to the critical biological macromolecules (lipids, proteins, and nucleic acids) and structures (including cellular organelles and cellular membranes), ultimately leading to the cell death (9, 10, 22), the revealing in the present study that even low-level exposure to Cd induces oxidative stress in the kidney indicates that such exposure can have a damaging impact on the kidney. Thus, it seems possible that the recently reported by us in the rats exposed to the 1 and 5 mg Cd/kg feed (21) damaging impact of Cd on the structure and function of the kidney might result, at least partially, from its prooxidative action and induction of oxidative stress in the renal tissue. The numerous dependencies noted between the main indices of the oxidative/reductive status such as TAS, TOS, and OSI and markers of tubular and glomerular damage in the animals that did not receive AM under trace-to-moderate-level exposure to Cd (control group, Cd_1 group, and Cd_5 group) indicate the involvement of oxidative stress in the development of this heavy metal-induced kidney damage and this issue will be the subject of our further paper. Moreover, it is worth emphasizing that oxidative stress might also contribute to the development of inflammatory processes noted in the kidney of these animals (21). It is known that there exists a dependence between the oxidative/antioxidative balance and inflammatory processes in the cells (36, 37). Moreover, the coexistence of oxidative stress and inflammation in the kidney leads to stimulating the damage to this organ as a result of synergic deleterious processes (37). The oxidative stress can induce inflammation through the activation of multiple pathways, including up-regulation of the production of proinflammatory cytokines like chemerin and acute phase proteins, whereas the state of inflammation can trigger oxidative stress (36, 37). Thus, the positive correlations between the kidney TOS and OSI and the concentration of chemerin in this organ together with the finding that at both levels of exposure to Cd, the destroying the oxidative/antioxidative balance preceded the increase in chemerin concentration (as well as in Bax concentration) (21) suggest that the prooxidative action of Cd might result in the development of inflammation in the kidney. On the other hand, the positive correlation between the kidney TAS and inflammatory markers such as MIPa or Bax may indicate that Cd-induced mild inflammation might trigger defence mechanisms in this organ, resulting in an increase in the antioxidative capacity.

The current study provided new data on the impact of low-to-moderate exposure to Cd on the oxidative/antioxidative balance in the kidney. The revealing that even low exposure to this xenobiotic, corresponding to the current general population exposure, can induce oxidative stress in the kidney and that under such exposure the antioxidative barrier may be the first target of its toxicity is an important finding. However, our primary interest and thus the most important finding consist in the protective effect of the administration of AM on the oxidative/antioxidative status of this organ during chronic low-level and moderate treatment with Cd.

The fact that in the animals that received AM and were maintained on the fed without Cd addition, the oxidative/antioxidative balance of the kidney was unaffected indicates that the enhanced intake of aronia products in the case of a lack of exposure to an agent characterized by prooxidative capacity did not influence the oxidative/reductive balance in the renal tissue. This confirms the safety of the prolonged supplementation with the AM shown in our previous studies (14-21). The 3-fold higher, compared to the control group, GSH/GSSG ratio in the

AM group after 24 months may imply more effective antioxidative protection of GSH in this organ as a result of the extract intake.

It is very important to emphasize, that the intake of AM in the amount that did not influence the oxidative/antioxidative balance in the absence of exposure to a prooxidative agent such as Cd was effective in the complete counteraction of this toxic element-induced destroying the oxidative/antioxidative balance. The administration of AM during the low-level and moderate repeated exposure to Cd improved both the enzymatic and nonenzymatic (prevented disruption in glutathione homeostasis) antioxidative barrier. It is also interesting to note that the administration of AM under exposure to Cd, which resulted in the increase in TOS and the development of oxidative stress completely prevented these effects. Although not always all parameters of the oxidative/antioxidative status were within the range of proper values in the animals co-administered with Cd and AM, the finding that long-term (17- and 24-month) administration of AM prevented the Cd-induced changes in the values of TAS, TOS, and OSI, which are the best indicators of the oxidative/antioxidative status, allows for the conclusion that an enhancement of daily consumption of aronia polyphenols by several times compared to their average intake among the worldwide general population, may be effective in counteracting oxidative stress in the kidney under low-level and moderate exposure. The extract administration under the exposure to Cd not only improved the antioxidative potential of the kidney but also decreased the concentration of a reactive oxygen compound such as H_2O_2 and the concentrations of oxidases (MPO and XOD) involved in its generation in this organ. It is worth mentioning that the beneficial impact of the co-administration of AM under the treatment with Cd on the oxidative/antioxidative balance in the kidney might be related to the lower concentration of H_2O_2 compared to the animals treated with this toxic element alone. Even if the exposure to Cd alone had no impact on the concentration of H_2O_2, the extract administration resulted in its decrease.

Taking into account the results of the present study and the findings of our previous investigations conducted in these animals, the beneficial influence of AM on the oxidative/reductive status of the kidney under the treatment with Cd may be explained by a direct effect of the extract resulting from its high antioxidative potential (2, 3, 7, 15, 16, 19) and an indirect action related to interactions between the extract ingredients and this toxic element (2, 3, 6, 14). The first effect can be caused, first of all, by a variety of polyphenolic compounds present in the extract, which can act as hydrogen donors capable of inactivating oxidative species and playing a major role in the restoration of nonenzymatic antioxidants. Most common polyphenolic compounds found in AM belong to the group of anthocyanins, proanthocyanidins, phenolic acids, and flavonoids. These compounds are characterized by high antioxidative capacity due to the direct neutralization of FR and ROS by donating an electron or hydrogen atom and acting as radical scavengers of the lipid peroxidation chain reactions by donating an electron to the FR, making radicals less reactive, resulting in the suppression of the chain reactions (1-4, 18). It is important to note that the beneficial effect of AM may also be due to the presence within the extract of components other than polyphenols that have been shown to combat the toxic action of Cd, such as β-carotene, essential microelements, fiber, pectin, triterpenes, and vitamins C and E (1-4, 18).

The protective impact of the administration of AM under the exposure to Cd may also be, at least partially, explained by the indirect action resulting in lower accumulation of this xenobiotic in the kidney (14). It has been noted, that Cd concentration in this organ in the animals supplemented with AM in the case of feeding with the diet containing 1 mg Cd/kg for 3 and 10 months was lower by 29% and 9.5% compared to the animals that did not receive the extract, whereas in the case of the 3-24-month treatment with the 5 mg Cd/kg feed, Cd concentration was lower by 5.6-14% (14). Due to the presence of hydroxyl (–OH) groups, these compounds are capable of chelating ions of divalent metals, including Cd^{2+} ions, and thus may decrease gastrointestinal absorption of this xenobiotic. Moreover, the increased urinary excretion of Cd, previously noted in the animals exposed to 5 mg Cd/kg feed due to the administration of AM (14, 21), shows that polyphenols may also form complexes with Cd^{2+} ions absorbed from the gastrointestinal tract and in this way accelerate their elimination from the body. Due to the lower body burden of Cd, the concentration of this xenobiotic in the kidney was lower, and as a result, the effects of its toxic action, including destroying the oxidative/antioxidative balance, were also less advanced. Because the destructive impact of Cd on the oxidative/antioxidative status is involved in the mechanisms of kidney damage, revealing in the present study that the co-administration of AM prevented oxidative stress in this organ allows for the conclusion that it will also counteract, at least partially, its damage by Cd. The

negative relationships between the kidney TAS and some of the markers of tubular (β2-MG concentration and ALP activity in the urine) and glomerular (ACR and PCR) damage noted in the rats supplemented with AM confirm that the beneficial impact of the extract on the kidneys recently reported by us in these animals (21) might be related to its antioxidative properties.

Although in the available literature, there are no other studies investigating the protective action of natural-based agents against the damaging impact of Cd on the kidney in the animal model well reflecting current exposure of the general population to this heavy metal, there are numerous reports showing protective action of isolated polyphenols (ferulic acid, quercetin) or products rich in these compounds (extract from *Fragaria ananassa* - strawberry, fruit extract from *Cleistocalyx nervosum var. paniala* - Ma Kiang, stem bark extract from *Irvingia gabonensis* - Bush mango, leaf extract from *Coriandrum sativum* - coriander, aqueous extract from the bulb of *Allium cepa* - onion and leaf infusions from *Camelia sinensis* - tea) against Cd-induced disruption of the oxidative/antioxidative barrier and oxidative stress development in this organ (26-31, 38-40).

We are aware of both the scientific value and practical implications of our research, as well as of its limitations. Because females are more susceptible to the toxic action of Cd than males, the experimental model used in our research used female rats, hence the findings apply to the female kidney. Thus, for a more complete investigation of the unfavourable impact of the low-to-moderate exposure to Cd on the kidney and the beneficial effect of AM, it seems necessary to perform a similar study on male rats. Furthermore, it is necessary to investigate the involvement of the ability of AM to counteract Cd-induced oxidative stress in the kidney in the protection from this organ damage. A study of this kind has been carried out by us and the findings will be published shortly.

CONCLUSIONS

The results of the present study provide the first evidence, that moderate and even low-level prolonged exposure to Cd can disrupt the oxidative/reductive balance in the kidney and lead to the development of oxidative stress in the organ. The mechanism of the harmful action of this xenobiotic involves a decrease in the activity of antioxidative enzymes, disruption of glutathione homeostasis, and enhancement of the level of oxidants. Moreover, it has been revealed, for the first time, that products from the berries of *A. melanocarpa*, due to the abundance of compounds possessing antioxidative capacity, can protect the kidney from the development of oxidative stress caused by exposure to Cd. Based on the findings it seems possible that the recently noted by us in the experimental model protective effect of the administration of AM against the damaging impact of Cd on the structure and function of the kidney might result, at least partially, from its antioxidative potential and prevention of the development of oxidative stress in the renal tissue.

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