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Does ADMA affect the oxidant/antioxidant balance in rats?

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Aim: Asymmetric dimethylarginine (ADMA) is an inhibitor of nitric oxide synthase (NOS). Oxidative stress might be defined as an imbalance between protein oxidation and antioxidants. Our aim was to determine in vivo whether ADMA causes oxidative damage.

Materials and methods: Thirty rats were divided into 3 equal groups: a control group, a group administered 1 mg/kg ADMA, and a group administered 2 mg/kg ADMA. ADMA was administered intraperitoneally for 7 days. Malondialdehyde (MDA), protein carbonyl (PC) content, and nitrate+nitrite concentrations were measured with serum samples. Superoxide dismutase (SOD), catalase, and glutathione peroxidase (GSH-Px) activities were analyzed with plasma samples.

Results: A significant increase in MDA concentration was observed in the ADMA groups, but this increase was not dose-dependent. However, no significant changes in PC content or nitrate+nitrite concentration were observed. Furthermore, catalase, SOD, and GSH-Px activity was suppressed in the ADMA groups. Suppression of GSH-Px activity was dose-dependent.

Conclusion: ADMA results in oxidative damage in vivo with lower doses than in NOS inhibition. ADMA has more of an oxidative effect on lipids than it does on proteins. Antioxidant enzymes must be consumed in significant amounts to remove the stress produced by ADMA.

Key words: Asymmetric dimethylarginine, malondialdehyde, superoxide dismutase, glutathione peroxidase

1. Introduction

Asymmetric dimethylarginine (ADMA) is a competitive inhibitor of endothelial nitric oxide synthase (eNOS) (1). ADMA is a methylated arginine derivative generated by the addition of methyl groups in arginine residue in proteins through protein arginine methyltransferase 1 (PRMT1), and it is secreted by proteolysis (2). ADMA is either excreted by urine or metabolized by dimethylarginine dimethylaminohydrolase (DDAH) in the kidneys, liver, pancreas, and endothelium (3). A close relationship between ADMA and many diseases, such as cardiovascular system disease (1), preeclampsia (4), and Behçet's disease (5), has been identified.

ADMA causes eNOS uncoupling and leads to decreased NO concentration and increased production of superoxide (6). Furthermore, an increase in systemic oxidative stress causes oxidation of tetrahydrobiopterin, which is an eNOS cofactor in endothelial cells, and which decreases the amount of NO and makes a contribution to eNOS uncoupling (7). Superoxide radicals react with NO, resulting in the production of peroxynitrite, which is an important mediator in tissue damage and dysfunction induced by inflammation (8). Excessive production of peroxynitrite leads to oxidative damage of carbohydrates, lipids, proteins, and DNA (9).

Malondialdehyde (MDA) is a carbonyl compound resulting from lipid peroxidation caused by oxidative stress. MDA is found in the body in free form (fMDA) or bound to –SH or $-NH_2$ groups of nucleic acids, proteins, and lipoproteins (bMDA). Total MDA (fMDA+bMDA) is frequently measured to show the existence of lipid peroxidation; total MDA (tMDA) indicates total damage. tMDA results in a reaction of thiobarbituric acid (10).

When oxidative stress is increased, ADMA levels may increase, due to a decrease in DDAH activity (11). An increase in PRMT1 expression is observed in parallel with increased lipid peroxidation (12,13). Not only oxidative stress, but also an increase in nitrosative stress, leads to

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nitrosylation of DDAH enzymes and causes increased ADMA concentration (14). There are many studies in the literature defining the relation between ADMA and oxidative stress. However, it is not clearly known, particularly within in vivo systems, whether this increase in ADMA concentration is causal or casual.

Catalase, superoxide dismutase (SOD), and glutathione peroxidase (GSH-Px) are the primary antioxidant enzymes. SOD is an active enzyme in the detoxification of superoxide radicals ($O_2^{\cdot-}$), and it transforms the $O_2^{\cdot-}$ radical into hydrogen peroxide (H_2O_2). However, hydrogen peroxide is also a strong oxidizing agent. This agent is detoxified by catalase enzyme, which is another antioxidant enzyme, and transformed into water and free oxygen (15). GSH-Px enzymes catalyze oxidation of glutathione in the presence of increased H_2O_2, and H_2O_2 is transformed into water and rendered harmless (16).

The aim of this study was to determine in vivo whether ADMA causes oxidative damage, and to contribute to the discussion of whether ADMA is the result of or the reason for oxidative stress. For this purpose, we analyzed MDA, protein carbonyl (PC) content, and nitrate+nitrite concentrations, which are markers representing oxidative stress, as well as SOD, catalase, and GSH-Px activity, which represent antioxidant capacity.

2. Materials and methods

This study was carried out with the approval of the Ondokuz Mayıs University Local Ethics Committee for Animal Experiments (HADYEK-2010/45). In total, 30 male Wistar rats, weighing 250–300 g, were bought from the Ondokuz Mayıs University Laboratory Animals Research and Application Center. All experimental studies were carried out at the same center, with the exception of biochemical analysis.

2.1. Animals and study groups

The animals were kept in light for 12 h and in dark for 12 h, and the temperature in the animal cages was maintained at 21–23 °C.

The 30 rats were divided into 3 equal groups: a control group in which 0.9% isotonic NaCl was administered, a group in which 1 mg/kg ADMA was administered, and another group in which 2 mg/kg ADMA was administered.

ADMA (Cat. No.: D4268, Sigma-Aldrich, USA), dissolved in 0.9% isotonic NaCl, was intraperitoneally administered once a day for 7 days.

2.2. Sample collection

The animals were euthanized after administration of ADMA for 7 days; 100 mg/kg ketamine hydrochloride and 10 mg/kg xylazine were administered intraperitoneally on day 8. Blood was taken directly from the heart and transferred into tubes with potassium EDTA, with no anticoagulant. The tubes were centrifuged at 3000 × *g* for 10 min. The serum and plasma samples were stored at –80 °C until biochemical analysis was performed.

2.3. Biochemical analysis

A day before analysis, the samples were brought to 4 °C. They were placed in a room-temperature environment on the day of the study and left to thaw.

2.3.1. Determination of MDA

MDA concentration was analyzed with serum samples (17). First, 0.5 mL of serum and 2.5 mL of 8.3% trichloroacetic acid were mixed and incubated at 90 °C for 15 min. Tubes were cooled and centrifuged at 3000 × *g*, 4 °C, for 20 min, after which 2 mL was taken from the supernatant and mixed with 1 mL of 0.225% thiobarbituric acid (TBA). This mixture was incubated at 90 °C for 15 min. The absorbance of the color formed after cooling the tubes was measured at 532 nm. MDA values were calculated using the extinction coefficient of the TBA–MDA complex (1.56×10^5 cm^{-1} M^{-1}). The results are presented in μmol/L.

2.3.2. PC content

PC content was analyzed with serum samples. 2,4-Dinitrophenylhydrazine solution, which was prepared in hydrochloric acid, was reacted with carbonyl and then washed 3 times with an ethanol/ethyl acetate mixture. The residue was dissolved in 100 mM NaOH solution and measured at 360 nm (18). The results are presented in nmol/mL.

2.3.3. Determination of NO

Concentrations of NO produced endogenously were detected by measuring the concentrations of nitrate (NO_3^-) and nitrite (NO_2^-). NO_3^- and NO_2^- concentrations in serum samples were determined with a Griess reaction after deproteinization (19). NO_3^- in the samples was transformed into NO_2^- in a reaction environment with cadmium fragments. The reaction of the naphthylethylenediamine and sulfanilamide mixture with NO_2^- was measured at 545 nm. The results are presented in μmol/L.

2.3.4. Total SOD activity

Total SOD activity was measured in plasma (20,21). An ethanol–chloroform (5 volumes of ethanol to 3 volumes of chloroform) mixture was prepared. In an Eppendorf tube, an ethanol–chloroform mixture (1 volume of serum to 1 volume) was mixed and centrifuged at 3000 × *g* for 40 min. The ethanol phase on the top was used for determination of activity. Next, 100 μL of ethanol phase and 50 μL of xanthine oxidase were pipetted into 2.85 mL of solution [containing 0.12 mM xanthine, 0.12 mM 2Na.EDTA, 30.6 μM nitro blue tetrazolium, and 0.06 g/L bovine serum albumin (BSA)] and incubated at 25 °C for 20 min. The reaction was stopped with 1 mL of 0.02 mM $CuCl_2$. Absorbance of the samples was measured at 560 nm. The results are presented in U/mL.

2.3.5. GSH-Px activity

GSH-Px activity was measured in plasma (22). First, 2.65 mL of phosphate buffer (44.3 mM, pH 7, 4.4 mM EDTA),

100 µL of 5 mM reduced glutathione (GSH), 100 µL of 0.13 mM reduced nicotinamide adenine dinucleotide phosphate (NADPH), 10 µL of glutathione reductase, 10 µL of 3.3 mM NaN_3, and 20 µL of serum were pipetted, respectively, into tubes in which GSH-Px activity was measured. This was incubated at 25 °C for 30 min. Next, 100 µL of 0.07 mM H_2O_2 was pipetted, and the change in absorbance at 340 nm was recorded. The results are presented in U/dL.

2.3.6. Catalase activity

Plasma catalase activity was measured by the Aebi method (23). Destruction of hydrogen peroxide by catalase was monitored at 240 nm and 100 µL of plasma was pipetted into 50 mM phosphate buffer (pH 7) containing 2.99 mL of 18% hydrogen peroxide. Kinetic measurement was performed using destruction of hydrogen peroxide. The results are presented in kat/dL.

2.4. Statistical analysis

SPSS 15.0 (SPSS Inc., Chicago, IL, USA) was used for statistical analysis. A Shapiro–Wilk test was performed to determine whether the data were in compliance with a standard distribution. A Kruskal–Wallis test was applied to determine whether there were any differences between groups. Afterwards, a Mann–Whitney U test was used for the binary group comparisons. The results are presented as mean ± standard deviation (SD). Statistical significance was set at $P < 0.05$.

3. Results

3.1. Lipid peroxidation

MDA concentrations of the control group, the 1 mg/kg ADMA group, and the 2 mg/kg ADMA group were 2.61 ± 0.17 µmol/L, 3 ± 0.25 µmol/L, and 3.33 ± 0.51 µmol/L, respectively. When compared with the control group, MDA concentration was significantly higher in the groups in which both 1 mg/kg and 2 mg/kg of ADMA were administered ($P < 0.001$ and $P < 0.001$, respectively). However, no significant difference was observed when the MDA concentration of the group in which 2 mg/kg of ADMA was administered was compared with the MDA amount of the group in which 1 mg/kg of ADMA was administered ($P = 0.089$) (Figure 1).

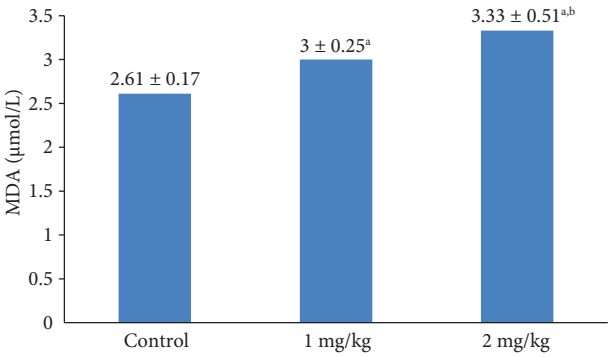

Figure 1. Comparison of MDA levels between groups. MDA, malondialdehyde. a: $P < 0.001$ compared with control group. b: $P = 0.089$ compared with 1 mg/kg ADMA group.

3.2. Protein oxidation and nitrate/nitrite concentration

There were no significant differences in either nitrate/nitrite or PC content concentrations compared with nitrate/nitrite and PC content concentrations between the groups (Table).

3.3. Antioxidant capacity

3.3.1. SOD activity

SOD enzyme activity was 4.58 ± 1.31 U/mL in the control group and 4.78 ± 1.27 U/mL and 3.58 ± 0.43 U/mL, respectively, in the 1 mg/kg and 2 mg/kg ADMA groups. A significant difference was observed when comparing the control group with the 2 mg/kg group ($P = 0.009$), while no significant difference was observed when comparing the control group with the 1 mg/kg group ($P = 0.353$). Furthermore, there was a significant difference between the 1 mg/kg group and the 2 mg/kg group ($P = 0.023$) (Figure 2).

3.3.2. Catalase activity

Catalase activity was 2.15 ± 1.8 kat/dL in the control group, 0.76 ± 0.58 kat/dL in the 1 mg/kg ADMA group, and 0.63 ± 0.22 kat/dL in the 2 mg/kg ADMA group. There were significant differences between the control group and the 1 mg/kg and 2 mg/kg ADMA groups ($P = 0.019$ and $P = 0.009$, respectively). However, there was no significant difference between the 1 mg/kg ADMA group and the 2 mg/kg ADMA group ($P = 0.796$) (Figure 3).

Table. Nitrate/nitrite and PC content concentrations of groups. The results are presented in mean ± standard deviation.

	Nitrate/nitrite (µmol/L)	PC content (nmol/mL)
Control	84.52 ± 13.23	738.33 ± 78.13
1 mg/kg ADMA	88.41 ± 16.17	700.36 ± 60.62
2 mg/kg ADMA	91.98 ± 9.73	666.7 ± 78.27

ADMA, asymmetric dimethylarginine; PC, protein carbonyl.

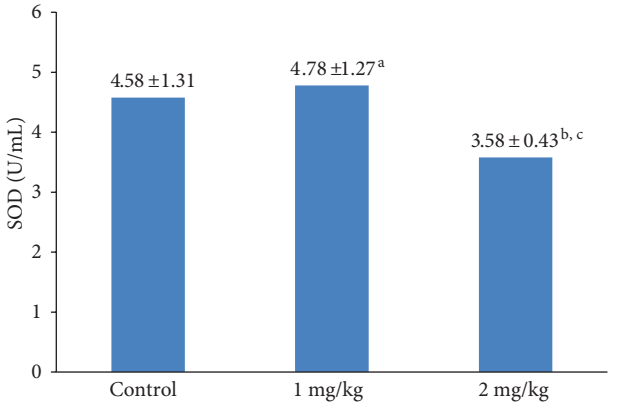

Figure 2. Comparison of SOD levels between groups. SOD, superoxide dismutase. a: P = 0.353 compared with control group. b: P = 0.009 compared with control group. c: P = 0.023 compared with 1 mg/kg ADMA group.

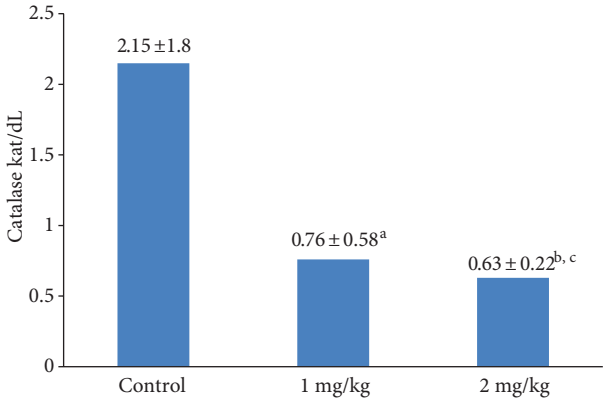

Figure 3. Comparison of catalase levels between groups. a: P = 0.019 compared with control group. b: P = 0.009 compared with control group. c: P = 0.796 compared with 1 mg/kg ADMA group.

3.3.3. GSH-Px activity

GSH-Px concentrations in the control group and the 1 mg/kg and 2 mg/kg ADMA groups were 126.22 ± 13.15 U/dL, 112.31 ± 8.74 U/dL, and 100.92 ± 6.95 U/dL, respectively. There were significant differences between the groups when comparing the control group with the 1 mg/kg ADMA group and with the 2 mg/kg ADMA group (P = 0.009 and P < 0.001, respectively). There was a significant difference between the 1 mg/kg ADMA group and the 2 mg/kg ADMA group (P = 0.003) (Figure 4).

4. Discussion

In this study, lipid peroxidation increased when 1 mg/kg and 2 mg/kg ADMA were administered; however, protein oxidation did not change. Furthermore, it was observed that those doses of ADMA reduced antioxidant capacity through decreased SOD, catalase, and GSH-Px activity.

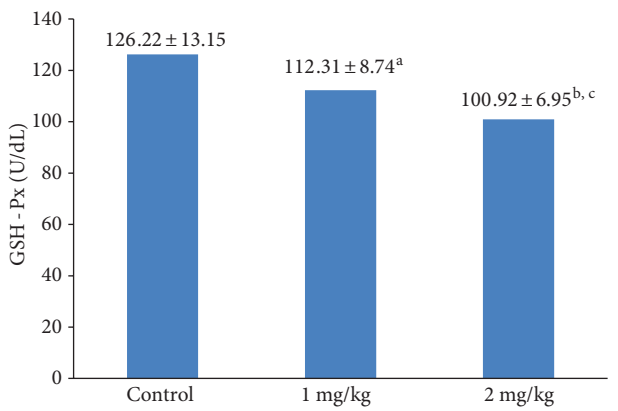

Figure 4. Comparison of GSH-Px levels between groups. a: P = 0.009 compared with control group. b: P < 0.001 compared with control group. c: P = 0.003 compared with 1 mg/kg ADMA group.

In the study by Yang et al., plasma and liver ADMA levels were high in bile duct ligation (BDL)-induced cirrhotic rats, liver thiobarbituric acid reactive substances (TBARS) levels were high, and liver SOD activity was lower than in the control group. In the same study, a decrease in ADMA and TBARS levels and an increase in SOD activity were observed when vitamin E was administered (24). In our previous study, we found that MDA, ADMA concentration, ADMA/arginine rate, catalase, GSH-Px, and SOD activities were elevated in preeclamptic patients (4). In another study, by Tain et al., phenazine methosulfate and 2,3-dimethoxy-1,4-naphthoquinone, which are H_2O_2 and superoxide donors, were put in a medium containing hepatic clone 9 cells, and PRMT1, DDAH1, and DDAH2 expression were monitored. DDAH activity decreased over time. It was shown that oxidative stress had an effect on enzyme activity in degrading ADMA rather than producing it. In the same study, melatonin prevented reduction in DDAH2 activity induced by H_2O_2 (25). Reactive oxygen species (ROS) and ADMA increased when bovine retinal capillary endothelial cells were incubated with high-dose glucose. PRMT-1 expression increased, and DDAH activity and DDAH2 expression decreased. When benazepril, telmisartan, diphenyliodonium (NADPH oxidase inhibitor, DPI), and N-acetyl-L-cysteine (antioxidant and free radical scavenger, NAC) were added to the media, all the observed effects were reversed (26). All the results discussed show that there is a strong relation between ADMA and oxidative stress. In this study, we observed a significant increase in MDA levels in the groups in which ADMA was administered, but this increase was not dose-dependent. However, there was not a significant change in PC content concentration. It can be stated that ADMA results in increased ROS concentration and causes more damage to lipids than to proteins.

ADMA causes eNOS uncoupling and leads to decreased NO concentration and increased production of superoxide (6). In addition, an increase in systemic oxidative stress causes oxidation of tetrahydrobiopterin, which is an eNOS cofactor in endothelial cells; it also decreases the amount of, and makes a contribution to, eNOS uncoupling (7). In the study by Wells et al., there was a dose-dependent decrease in nitrite concentration when ADMA was applied in LA-4 (murine airway epithelial cell line) cells. A 75% decrease in NO concentration was observed 48 h after 500 μM ADMA was administered (27). However, in our study, even 2 mg/kg, the maximum dose, did not cause a decrease in nitrate/nitrite concentration and, thus, eNOS uncoupling. Therefore, we assert that NOS inhibition occurs at much higher ADMA concentrations, which are necessary for lipid peroxidation.

Many enzymes cause ROS production in mammals. Mainly, these enzymes are mitochondrial respiratory chain enzymes, lipoxygenases acting in arachidonic acid metabolism, cyclooxygenases, cytochrome P450 system, xanthine oxidase, peroxidase, and hemoproteins. The superoxide that forms has many effects, such as transformation of low-density lipoprotein (LDL) into oxidized LDL and activation of redox-sensitive, proinflammatory signal pathways (28,29). In addition, superoxide radicals react with NO and produce peroxynitrite, which is an important mediator in tissue damage and dysfunction induced by inflammation (8). Excessive production of peroxynitrite leads to oxidative damage of carbohydrates, lipids, proteins, and DNA (30). Superoxide radicals are transformed into hydrogen peroxide (H_2O_2) by a reaction catalyzed by SOD. H_2O_2 is degraded into water and oxygen by a reaction catalyzed by catalase (31). Cao et al. observed vasoconstriction when administering ADMA on descending vasa recta; these authors tested whether this constriction was caused by superoxide. For this purpose, they added the SOD mimetic agent tempol, and NAD(P)H oxidase inhibitor apocynin, to the bath containing ADMA and observed a reduction in vasoconstriction (32). In another study, an increase in alveolar permeability of glomeruli was observed when ADMA was applied. This increase was considered to be due to superoxide radicals. A decrease in superoxide radicals and permeability was observed when tempol, a SOD mimetic agent, was administered. In the same study, aprotinin, which is a NAD(P)H oxidase inhibitor, was applied together with ADMA¸ and albumin permeability of the glomeruli decreased. This result shows that one of the superoxide resources is NAD(P)H oxidase (33). In our study, the activities of catalase, SOD, and GSH-Px were suppressed in the groups in which ADMA was administrated. The activity of GSH-Px was suppressed dose-dependently, but this was not observed in SOD and catalase. These results show that antioxidant enzymes were used in high amounts in order to equalize the increased ROS amount in the environment.

Studies on treatments to reduce ADMA concentration are ongoing. PRMT, CAT, and DDAH, which play a part in ADMA metabolism, are the focus of these treatments. For instance, melatonin has an effect that changes DDAH activity (25). In the study by Yang et al., administration of vitamin E in 3T3-L1 adipocytes caused decreased ROS and increased DDAH activity, causing reduced ADMA concentration (34). In another study, ADMA levels were significantly reduced when acetylsalicylic acid was given to healthy volunteers (35). In our study, antioxidant capacity decreased. Administration of antioxidant substances externally to increase antioxidant capacity can be a significant factor in preventing damage caused by ADMA.

A lower amount of ADMA causes more oxidative damage in vivo than its NOS inhibitory concentration. ADMA has a devastating effect on lipids, more so than on proteins. Antioxidant enzymes are consumed in significant amounts to remove the stress produced by ADMA

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