EFFECTS OF MYOSMINE ON ANTIOXIDATIVE DEFENCE IN RAT LIVER

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Myosmine [3-(1-pyrrolin-2-yl) pyridine] is an alkaloid structurally similar to nicotine, which is known to induce oxidative stress. In this study we investigated the effects of myosmine on enzymatic and non-enzymatic antioxidative defence in rat liver. Wistar rats received a single i.p. injection of 19 mg kg⁻¹ of myosmine and an oral dose of 190 mg kg⁻¹ by gavage. Nicotine was used as a positive control. Through either route of administration, myosmine altered the hepatic function by decreasing the levels of reduced glutathione, superoxide dismutase, and glutathione peroxidase activities on one hand and by increasing malondialdehyde, catalase, and glutathione reductase activity on the other. Compared to control, both routes caused significant lipid peroxidation in the liver and altered hepatic enzymatic and non-enzymatic antioxidative defences. The pro-oxidant effects of myosmine were comparable with those of nicotine.

KEY WORDS: lipid peroxidation, oxidative stress, rodents, tobacco alkaloid

Myosmine [3-(1-pyrrolin-2-yl) pyridine] is the second tobacco alkaloid to nicotine (1). However, myosmine has a much wider distribution over a wide variety of food products such as maize, rice, wheat, cocoa, and milk in a wide range of mass fractions from 0.03 ng g⁻¹ in carrots up to 6.26 ng g⁻¹ in cream (2). It is therefore not surprising that myosmine has also been detected in human toenail, plasma (up to 5.5 ng mL⁻¹), saliva (up to 4.6 ng mL⁻¹) (3) or breast milk (4). When given orally to Wistar rats, myosmine is metabolised rapidly and completely (5). After nitrosation and/or peroxidation, myosmine bioactivates to 4-hydroxy-1-(3-pyridyl)-1-butanone (HPB) and can form pyridyloxobutylated DNA adducts (6) or N’-nitrosonornicotine (NNN), which is an oesophageal carcinogen (7). In vitro experiments with calf thymus DNA have shown that myosmine is rapidly nitrosated at pH 2 to pH 4 and forms DNA adducts (8). Using the comet assay in human lymphocytes and upper aerodigestive tract epithelial cells, Kleinsasser et al. (9) have demonstrated dose-and time-dependent genotoxic effects of myosmine.

While there are some data about the potential genotoxic and carcinogetic effects of myosmine, little information is available about its toxicity. Organ toxicity of nicotine is known to be related to oxidative stress (10). We assume that because of its structural similarity to nicotine, myosmine might exert similar toxicity by generating reactive oxygen species (ROS).

Zwickenpflug and Tyroller (6) have suggested that because of its imine structure, myosmine seems to be more reactive than other investigated tobacco alkaloids. The authors have shown that when myosmine is mixed with hydrogen peroxide, the latter attacks the carbon nitrogen double bond of the imine
structure, yielding oxaziranes, nitrones, or N-oxides. These metabolic intermediates might be involved in oxidative stress.

The aim of our study was to investigate the effects of myosmine on the enzymatic and non-enzymatic antioxidative defences in rat liver after a single oral and intraperitoneal (i.p.) administration.

MATERIAL AND METHODS

Chemicals and enzymes

Myosmine was synthesised at the Institute of Molecular Biology, Bulgarian Academy of Science (BAS), following the method of Brandänge and Lindblom (11). The product was found to be 99.9 % pure; its identity was confirmed by comparing it with commercial myosmine using ultraviolet and infrared spectral analysis, gas chromatography, and high pressure liquid chromatography. All the other reagents used were of analytical grade. Nicotine dihydrogen ditartrate (CAS 65-31-6), 1-chloro-2,4-dinitrobenzene (CDNB), β-nicotinamide adenine dinucleotide 2' phosphate reduced tetrasodium salt (NADPH), ethylenediaminetetraacetic acid (EDTA), L-glutathione reduced (GSH), L-glutathione oxidised (GSSG), cumene hydroperoxide, thiobarbituric acid (TBA), trichloroacetic acid (TCA), bovine serum albumin (fraction V), glutathione reductase from baker’s yeast (S. cerevisiae) ammonium sulfate suspension, 100 to 300 units mg⁻¹ protein (Biuret) were purchased from Sigma-Aldrich (Taufkirchen, Germany). 2,2'-Dinitro-5,5'-dithiodibenzoic acid (DTNB) was obtained from Merck (Darmstadt, Germany).

Experimental animals

Male Wistar rats weighing (200±10) g were housed under standard laboratory conditions at 20 °C, with 12-h alternating light/dark cycles and free access to food and water. The animals were purchased from the National Breeding Centre, Slivnitzta, Bulgaria. All experiments were performed after at least one week of adaptation to this environment. All procedures were approved by the Institutional Animal Care Committee, and the principles stated in the European Convention for the Protection of Vertebrate Animals used for Experimental and other Scientific Purposes (ETS 123) (12) were strictly followed throughout the experiment.

Experimental design

Earlier studies have shown that the i.p. and oral LD₅₀ of myosmine for rats is 190 mg kg⁻¹ and 1875 mg kg⁻¹, respectively (13). Before starting the experiment, we ran a pilot study to establish the dose-related effect of myosmine on animal behaviour and liver ALT, AST, and LDH (the results are not shown). A single dose of 1/5, 1/10, or 1/20 of LD₅₀ of myosmine was administered i.p. and orally to six groups of three rats per group. The dose of 1/20 of LD₅₀ did not show any statistically significant behavioural or biochemical changes. The dose of 1/5 of LD₅₀ proved very toxic (2 of 3 rats died after i.p. injection and 1 of 3 after oral administration) and the dose of 1/10 of LD₅₀ was well tolerated and at the same time produced significant changes in the investigated parameters. This is why we used the dose 1/10 of LD₅₀ for both routes of administration. For positive control we used nicotine ditartrate in an equitoxic dose of 1/10 of LD₅₀ (14).

The rats were then randomised into six groups with six animals in each. Group 1 were control rats treated with physiological saline i.p. (1 mL kg⁻¹ b. w.). Group 2 were control rats given physiological saline via oral gavage (10 mL kg⁻¹ b. w.). Group 3 were rats injected with nicotine dihydrogen ditartrate i.p. (1 mg kg⁻¹ b. w.). Group 4 were rats administered nicotine dihydrogen ditartrate through oral gavage (6.5 mg kg⁻¹ b. w.). Group 5 were rats injected myosmine i.p. (19 mg kg⁻¹ b. w.), and group 6 were rats administered myosmine through oral gavage (190 mg kg⁻¹ b. w.).

Twenty-four hours after the administration of the single dose, the animals were killed by cervical dislocation. Tissue samples were immediately transferred to ice-cold containers, weighed, and homogenised using appropriate buffers (15).

Preparation of liver homogenates for lipid peroxidation (LPO) assessment

Thiobarbituric acid reactive substances (TBARS), expressed as malondialdehyde (MDA) equivalents, were determined as a marker of lipid peroxidation. The method was described by Polizio and Peña (15). Briefly, 1 mL of homogenate was mixed with 1 mL of 25 % trichloroacetic acid (TCA) and 1 mL of 0.67 % thiobarbituric acid (TBA). Samples were then mixed thoroughly, heated in a boiling water bath for 20 min, cooled down, and centrifuged at 4000g for 20 min. The absorbance of supernatant was measured at 535 nm against a blank that contained all the reagents except tissue homogenate. MDA concentration was
calculated using a molar extinction coefficient of 1.56x10^5 L mol^-1 cm^-1 and expressed in nmol g^-1 of wet tissue.

**Preparation of liver homogenates for reduced glutathione (GSH) assessment**

GSH was assessed by measuring non-protein sulphydryls after precipitation of proteins with TCA, using the method described by Bump et al. (16). Briefly, tissues were homogenised in 5% TCA and then centrifuged at 4000g for 20 min. The reaction mixture contained 0.05 mL of supernatant, 3 mL of 0.05 mol L^-1 phosphate buffer (pH 8), and 0.02 mL of DTNB reagent. The absorbance was determined at 412 nm and the results expressed as nmol g^-1 wet tissue.

**Preparation of liver homogenates for antioxidant enzyme activity measurement**

Rat livers were rinsed in ice-cold physiological saline and minced with scissors. Ten-percent homogenates were prepared in 0.05 mol L^-1 phosphate buffer (pH 7.4), centrifuged at 7000g for 10 minutes, and the supernatants used for antioxidant enzyme analysis. The protein content of liver homogenates was measured using the method of Lowry (17) with bovine serum albumin as a standard.

**Catalase (CAT) activity**

CAT activity was assessed as described by Aebi (18). Briefly, 10 μL of homogenate was added to 1990 μL of H_2O_2 solution (containing 6.8 μL of 30% H_2O_2 + 1983.2 μL of 0.05 mol L^-1 phosphate buffer, at pH 7.4). CAT activity was determined by measuring H_2O_2 decomposition through the decrease in absorbance at 240 nm for 1 min. Enzyme activity was calculated using the molar extinction coefficient of 0.043 L mmol^-1 cm^-1 and expressed as μmol L^-1 min^-1 mg^-1 of protein.

**Superoxide dismutase (SOD) activity**

SOD activity was measured according to Sun and Zigman (19) with slight modifications. The incubation mixture contained 50 mmol L^-1 carbonate buffer with pH 10.2 at 30 °C. The reaction was started by adding epinephrine base (0.001 mol L^-1 in 0.02 mol L^-1 HCl). Epinephrine auto-oxidation was followed spectrophotometrically at 320 nm, using the molar extinction coefficient of 4.02 L mmol^-1 cm^-1. SOD activity is expressed as nanomol of epinephrine that are prevented from auto-oxidation after addition of the sample.

**Glutathione peroxidase (GPx) activity**

GPx activity was measured by NADPH oxidation, using a coupled reaction system consisting of reduced glutathione, glutathione reductase and cumene hydroperoxide (20). Briefly, 100 μL of enzyme sample was incubated at 30 °C for 5 min with 1.5 mL of 0.05 mol L^-1 phosphate buffer (pH 7.4), 100 μL of 1 mmol L^-1 EDTA, 50 μL of 1 mmol L^-1 GSH, 100 μL of 0.2 mmol L^-1 NADPH, and 1 U of glutathione reductase. The reaction was initiated by adding 50 μL of cumene hydroperoxide (1 mg mL^-1) and the rate of disappearance of NADPH over time was determined by monitoring absorbance at 340 nm using an extinction coefficient of 6.22x10^3 L mol^-1 cm^-1. The results are expressed in nmol min^-1 mg^-1 of protein.

**Glutathione reductase activity (GR)**

GR activity was measured spectrophotometrically at 340 nm according to Pinto et al. (21). We followed NADPH oxidation using the extinction coefficient of 6.22x10^3 L mol^-1 cm^-1. The incubation mixture contained 0.05 mol L^-1 phosphate buffer, pH 7.4, 2.5 mmol L^-1 GSSG, and 125 μmol L^-1 NADPH at 30 °C. Enzyme activity is expressed in nmol min^-1 mg^-1 of protein.

**Glutathione-S-transferase (GST) activity**

GST activity was measured using CDNB as the substrate (22). The incubation mixture containing 1.6 mL of 0.05 mol L^-1 phosphate buffer, 100 μL of 1 mmol L^-1 GSH, 100 μL of 1 mmol L^-1 EDTA, and 100 μL of homogenate was incubated at 37 °C for 15 min. After the incubation, 100 μL of 1 mmol L^-1 CDNB was added and the increase in absorbance with time was recorded at 340 nm. Enzyme activity was measured using the extinction coefficient of 9.6x10^3 L mol^-1 cm^-1 and is expressed as nanomol of CDNB-GSH conjugate per minute per milligram of protein.

**Statistical analysis**

The statistical analysis was performed using MedCalc (MedCalc Software bvba, Mariakerke, Belgium). The significance of the data was assessed using the non-parametric Mann-Whitney test. Values of p≤0.05 were considered statistically significant.
RESULTS

The results are expressed as means ±SEM for six rats per group. Figure 1 shows the levels of lipid peroxidation in the liver tissue of control animals and animals treated with myosmine and nicotine. MDA significantly increased in the myosmine and nicotine-treated groups after both i.p. injection (+31 % and +21 %, respectively; p≤0.05) and oral administration (+29 % and +33%, respectively; p≤0.05) in respect to control.

Myosmine and nicotine treatment depleted GSH -36 % and -14 % after i.p. injection and -23 % and -28 % after oral administration, respectively (Figure 2).

The effects on CAT, SOD, and GPx are summarised in Figures 3-5. Significant decreases in the activity of SOD and GPx were noted with both routes of myosmine exposure and were comparable with nicotine. However, myosmine significantly increased CAT activity (24 % after i.p. injection and 19 % after oral administration; p≤0.05), while nicotine did the opposite; it decreased CAT by 22 % and 18 % (p≤0.05) after i.p. injection and oral administration, respectively.

Figures 6 and 7 show the activities of GR and GST after myosmine and nicotine administration. Both significantly increased GR activity through either
route of administration, and to a similar extent, 28% and 35%, respectively (p≤0.05). Neither compound induced significant changes in GST activity after either i.p. injection or oral administration.

Neutralise reactive molecules by deploying their antioxidative defences that include reduced glutathione (GSH), alpha-tocopherol, ascorbic acid, and antioxidant enzymes catalase (CAT), superoxide dismutase (SOD), glutathione peroxidase (GPx), glutathione reductase (GR), and glutathione-S-transferase (GST).

The formation of reactive oxygen species (ROS) is stimulated by a number of xenobiotics and can result in oxidative stress and cellular injury. As myosmine is structurally similar to nicotine, which is known to induce oxidative stress (24), it is expected to act in a similar way. Its proclivity for oxidation reactions (6) may give rise to lipid peroxidation and cellular damage. In our study, a single dose of nicotine decreased GSH, SOD, CAT and GPx activities and increased MDA level and GR activity in rat liver. These results are in good agreement with the results of Sudheer et al. (26), who reported that nicotine subchronic administration resulted in significant decreases of enzymatic and non-enzymatic antioxidative defences in the liver, lung, and the circulation. Myosmine i.p. and oral treatment at equitoxic doses also led to significant increases in MDA and GR and decreased the GSH level and SOD and GPx activities. In contrast to nicotine, myosmine increased CAT activity.

In vivo metabolism of myosmine has been investigated after oral administration in female Wistar rats by Zwickenpflug et al. (5). The authors identified 3-pyridylacetic acid (3-PAA) and 4-oxo-4-(3-pyridyl) butyric acid (keto acid) as the main metabolites and 3-pyridylmethanol, 3-hydroxymyosmine and 4-hydroxy-1-(3-pyridyl)-1-butanone (HPB) as minor constituents.
metabolites. The relatively high amount of 3-PAA (23 %) suggests that myosmine undergoes a biochemical transformation in the rat stomach (27), leading to free radical formation. The increase in MDA level detected in our study may be related to the increase in free radical formation. De Zwart et al. (28) reported that lipid peroxidation produced several toxic byproducts such as MDA, which can attack cellular targets including DNA, inducing mutagenicity and carcinogenicity. The increased MDA level after myosmine treatment, might also contribute to the mutagenic and genotoxic effects of myosmine (9, 29-31). Glutathione is required to maintain the normal reduced state of cells and to counteract the adverse effects of oxidative stress. It is involved in many cellular processes including the detoxification of endogenous and exogenous compounds and can be monitored as a nonspecific indicator of cellular toxicity. The increased lipid peroxidation in myosmine-treated rats can be regarded as a key event for the observed GSH depletion after i.p. and oral administration (see Figure 2). Furthermore, Zwickenpflug and Tyroller (6) suggested that myosmine peroxidation to 3-pyridylmethanol, normicotyrine, HPB, and keto acid produced highly reactive electrophilic intermediates known as epoxides, and we believe that these electrophiles interact with the large nucleophilic pool of GSH in the liver and deplete it.

In addition to increased lipid peroxidation and decreased GSH level, myosmine significantly decreased the activities of the antioxidant enzymes SOD and GPx (see Figures 3 and 5 respectively) and increased CAT activity (see Figure 4). These enzymes act together and constitute the enzymatic antioxidative defence mechanism against reactive oxygen species (32). The SOD dismutates superoxide radicals (O$_2^-$) into hydrogen peroxide (H$_2$O$_2$) and O$_2$. Catalase further detoxifies H$_2$O$_2$ into H$_2$O and O$_2$ (33). Glutathione peroxidase, similar to CAT, detoxifies H$_2$O$_2$. Lower SOD and GPx activities in our study could be attributed to several mechanisms. The first is their overconsumption in inactivating the reactive intermediates generated during the metabolism of myosmine. The second is that oxidative stress could inactivate enzyme proteins through these intermediates or could be a consequence of lower de novo synthesis of enzyme proteins. The increased hepatic CAT activity may be a compensatory mechanism to get rid of excess peroxides due to low GPx activity. The third is that the activation of myosmine by peroxidative or nitrosative mechanisms could lead to pyridyloxobutylation of proteins, DNA, and RNA (6, 8, 30) and could compromise their functioning.

The significant increase in GR (see Figure 6) could be attributed to increased GSSG production, which we have not measured in this study but which is reasonable to assume (23).

Our study confirms that single i.p. and oral administration of myosmine significantly increases lipid peroxidation and compromises the enzymatic and non-enzymatic antioxidative defence systems. Its pro-oxidant effects are comparable with those of nicotine.

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Sažetak

DJELOVANJE MIOZMINA NA ANTIOKSIDATIVNE SUSTAVE U JETRI ŠTAKORA

Miozmin (3-(1-pirolin-2-il)piridin) alkaloid je strukturno sličan nikotinu, za koji se zna da potiče oksidativni stres. Istražili smo djelovanje miozmina na enzimske i neenzimske antioksidativne sustave u jetri štakora. Wistar štakori primili su jednokratno pokusni spoj intraperitonealno u dozi od 19 mg kg⁻¹, odnosno na usta u dozi od 190 mg kg⁻¹. Za pozitivnu kontrolu rabili smo nikotin. Nakon primjene, bez obzira na put, zamijećena je promjena u jetrenoj funkciji u obliku pada razina glutationa, aktivnosti superoksid dismutaze i glutation peroksidaze te rasta razina malondialdehida, aktivnosti katalaze i glutation reduktaze. Ovi nalazi upućuju na to da intraperitonealna i oralna primjena miozmina dovode do značajne lipidne peroksidacije u jetrenome tkivu te promjena u enzimskoj i neenzimskoj zaštiti jetre. Prooksidativno djelovanje miozmina pokazalo se sličnim onom nikotina.

KLJUČNE RIJEČI: duhanski alkaloid, glodavci, lipidna peroksidacija, oksidativni stres

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