Effects of ibogaine *per os* application on redox homeostasis in rat liver and erythrocytes

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Abstract: Ibogaine, administered as a single oral dose (1-25 mg/kg body weight), has been used as an addiction-interrupting agent. Its effects persist for up to 72 h. *Ex vivo* results showed that ibogaine induced cellular energy consumption and restitution, followed by increased reactive oxygen species production and antioxidant activity. Therefore, the aim of this work was to explore the effect of a single oral dose of ibogaine (1 or 20 mg/kg body weight) on antioxidative defenses in rat liver and erythrocytes. Six and 24 h after ibogaine administration, histological examination showed glycogenolytic activity in hepatocytes, which was highest after 24 h in animals that received 20 mg/kg ibogaine. There were no changes in the activities of superoxide dismutases, catalase, glutathione peroxidase, glutathione reductase and glutathione-S-transferase in the liver and erythrocytes after ibogaine treatment, regardless of the dose. Hepatic xanthine oxidase activity was elevated in rats that received 20 mg/kg compared to the controls (p<0.01), suggesting faster adenosine turnover. TBARS concentration was elevated in the group treated with 1 mg/kg after 24 h compared to the controls (p<0.01), suggesting mild oxidative stress. Our results show that ibogaine treatment influenced hepatic redox homeostasis, but not sufficiently to remodel antioxidant enzyme activities at 6 and 24 h post-ibogaine application.

Keywords: ibogaine; glycogen; antioxidant enzymes; lipid peroxidation; xanthine oxidase

INTRODUCTION

Ibogaine is the main alkaloid in the root bark of the plant *Tabernanthe iboga* [1], which has been used by the natives of western-central Africa to overcome fatigue, hunger and thirst, and at higher doses, to provoke dreamlike hallucinations for spiritual rituals [2]. In the "ibogaine medical subculture", predominantly in Europe and America, it is used to facilitate abstinence from a variety of addictive substances (such as cocaine, heroin, methadone and alcohol), when ibogaine is most frequently administered as a single oral dose in the range from 1-25 mg/kg of body weight (b.w.) [2-4]. In the most recent publication [5] in the Clinical Guidelines for Ibogaine-Assisted Detoxification, published by the Global Ibogaine Therapy Alliance (GITA), it is suggested that dosing should never exceed

24 mg/kg in a 24 h interval. It is also suggested that a single initial booster dose (threshold dose, usually 1 to 5 mg/kg) is administered before the single larger treatment dose which should not be administered 2 or 3 h after the initial dose, but at least within the 24-36 h time frame after application of the initial booster dose. Alper et al. [6] reported that a subset of 33 individuals was treated for the indication of opioid withdrawal with a single dose of ibogaine averaging 19.3 mg/kg. The U.S. Food and Drug Administration (FDA) Advisory Panel formally approved human trials with 1, 2, and 5 mg/kg of ibogaine, and clinical trial (also approved by the FDA) in 1995 recommended similar doses [7]. Based on existing animal data, Schep et al. [8] suggested that appropriate safety factors should be applied, including a maximum oral dosage limit of less than 1 mg/kg. Therefore, for our experiment

we chose to explore the effects of a single oral dose of either 1 or 20 mg/kg b.w.

Ibogaine's anti-addictive activity is achieved through its effects on receptors (N-methyl-D-aspartate [9], nicotinic [10], 5-hydroxytryptamine 2A (5-HT$_{2A}$), muscarinic [1] and kappa, sigma-2 and mu opioid receptors [1]), inhibition of monoamine oxidase [1], antagonism of 5-HT and dopaminergic transporters [1], and through adaptive changes in ATP-related cell energy homeostasis [11-13]. Paškulin et al. [14] showed an increase in the production of energy-related enzymes in rat brain after intraperitoneal (i.p.) injection of 20 mg/kg b.w., and in yeast *Saccharomyces cerevisiae* [11] cultivated with ibogaine at a concentration of 1 mg/L, which corresponds to the dose in the brain. It was also shown that ibogaine caused rapid depletion of ATP that was accompanied by increased production of reactive oxygen species (ROS) [13] and activation of antioxidant defense enzymes in yeast [11]. While ibogaine *in vitro* did not show any significant antioxidant properties *per se* [12], the observed ibogaine-associated increase in activities of antioxidant enzymes (cytosolic copper-zinc superoxide dismutase (SOD1), glutathione reductase (GR) [13] and catalase (CAT) [15]) in different *in vitro* [12] and *ex vivo* [13] model systems supported ibogaine as a pro/antioxidative agent. In previous work on yeast [11], elevated expression of metabolic enzymes involved in glycolysis and of SOD protein were detected after 5 h at a dose of 1 mg/L ibogaine (equivalent to ibogaine levels found in brains of rats that received 20 mg/kg b.w., i.p.). However, among the proteins identified by matrix-assisted laser desorption/ionization-time of flight mass spectrometry (MALDI-TOF MS) that were induced after 24 and 72 h in brains of rats (that received 20 mg/kg b.w., i.p.), SOD was not listed [14], suggesting species- and tissue-specific effects of ibogaine. Therefore, in our experiments we measured the activities of the antioxidant enzymes, SOD1, mitochondrial manganese superoxide dismutase (SOD2), CAT, glutathione peroxidase (GSH-Px) and GR after ibogaine *in vivo*, *per os* (p.o.) application (1 or 20 mg/kg b.w.) after 6 and 24 h. Ibogaine has a blood half-life in humans ranging between 4-8 h, and the elimination half-life of ibogaine in humans is from 4-7 h [7,16-18]. Thus, the time point of 6 h in our experiments was the mean time of its half-life used to examine the expression of antioxidant enzymes in yeast (5 h).

In mammals, ibogaine is converted to its active metabolite, noribogaine (12-hydroxyibogamine). Studies in human subjects after single oral ibogaine administration showed its complex pharmacokinetic profiles. Ibogaine is cleared rapidly from the blood, and replaced by noribogaine [18]. However, ibogaine metabolism in the rat is different when compared to the human. Ibogaine is eliminated faster than noribogaine [1] and 24 h after p.o. administration about 65% of the ibogaine in rats [19], and even 90% in humans [7], can be eliminated via the urine and feces. According to Bauman et al., 24 h after intravenous (i.v.) administration of ibogaine, noribogaine still persists in rat blood, unlike ibogaine, which can no longer be detected [20]. Therefore, in the present work, we also measured the concentration of both ibogaine and noribogaine in blood plasma.

Having in mind ibogaine pharmacokinetics, as well as its influence on both energy metabolism and the activity of antioxidant enzymes explored *ex vivo* [11,12,15] and *in vitro* [13] using different doses and experimental models, herein we examined the effects of ibogaine *in vivo* after application of a single dose (1 or 20 mg/kg b.w., p.o.) on rat liver and erythrocytes after 6 and 24 h. Histological examination of the liver was performed in order to (i) visualize the potential changes in the amount of glycogen (as representation of energy metabolism), and (ii) to examine the possible morphological effects on hepatocytes. In the light of the documented effect of ibogaine on cellular energetics, we measured blood glucose concentration. Food and water consumption and the specific gravity of the urine were also monitored. The metabolic function of the liver was estimated by measuring the activity of xanthine oxidase (XOD; purine turn-over) and glutathione S-transferases (GST), as well as the level of bilirubin and urobilinogen in the urine. Furthermore, thiobarbituric acid reactive substances (TBARS) were measured as markers of possible oxidative damage to the liver and total and non-protein free sulfhydryl (-SH) groups in the liver as the marker of thiol-based redox state. The general systemic oxidative effect of ibogaine was assessed by monitoring the changes in activities of antioxidative enzymes in the liver and erythrocytes.

MATERIALS AND METHODS

Animals

All procedures complied with the EEC Directive on the protection of animals used for experimental and other scientific purposes and were approved by the Ethical Committee for the Use of Laboratory Animals of the Institute for Biological Research "Siniša Stanković", University of Belgrade, No. 02-03/16. Male Wistar rats, 3-months of age, b.w. 290-320 g, were housed individually under standard laboratory conditions day/night 12 h/12 h, at 22°C with free access to food and water.

Design of the experiment

Ibogaine hydrochloride (PubChem CID: 197059, purity 98.93%) was a kind gift from the Sacrament of Transition, Maribor, Slovenia; ibogain hydrochloride (Remøgen, Phytostan Enterprises Inc., Canada, purity 99.9%) was provided by Mara Bresjanac from the LNPR project funded by the ARRS Program P3-0171. Ibogaine was dissolved in dH_2O by strong vortexing (at a stock concentration of 2 mg/mL) and maintained in the dark until use. The animals were divided into six groups (6 animals per group) and treated with one dose of ibogaine p.o. by gavage (1 or 20 mg/kg b.w.). The control groups were gavaged with an equal amount of dH_2O. All animals received 1 mL of liquid (dH_2O or ibogaine solution of appropriate concentration) per 100 g b.w. by gavage. The experimental groups were as follows: C6 – control treated with dH_2O, decapitated after 6 h; C24 – control treated with dH_2O, decapitated after 24 h; L6 – low dose ibogaine (1 mg/kg b.w.), decapitated after 6 h; L24 – low dose ibogaine (1 mg/kg b.w.), decapitated after 24 h; H6 – high dose ibogaine (20 mg/kg b.w.), decapitated after 6 h; H24 – high dose ibogaine (20 mg/kg b.w.), decapitated after 24 h.

Blood preparation

When the rats were killed, blood was collected in transparent heparinized tubes (500 I.U. per mL of blood). Blood plasma was separated from erythrocytes by centrifugation (MiniSpin, Eppendorf, at 3000 x g, for 10 min) and stored at -20°C until analysis. Erythrocytes were washed three times with saline (0.9% w/w)

and stored at -20°C until use. Antioxidant enzymes and XOD were measured in erythrocyte hemolysates (hemolyzed by ice cold dH_2O).

Determination ibogaine and noribogaine concentrations in blood plasma

A validated liquid chromatography-tandem mass spectrometry (LC-MS/MS) method [21] was used for the determination of ibogaine and noribogaine in blood plasma samples. Sample pretreatment consisted of simple protein precipitation with a double volume of acetonitrile. Plasma calibration standards were prepared by adding the appropriate amounts of working standard solutions and prazepam (internal standard) to drug free plasma. All samples and standards were treated adequately to prevent decomposition of ibogaine and noribogaine due to light exposure. The compounds (ibogaine, noribogaine and prazepam) were separated with gradient elution on Zorbax XDB-CN (75 mm x 4.6 mm i.d., 3.5 μm). The LC-MS/MS instrument consisted of the Agilent 1100 HPLC system and the tandem quadrupole mass spectrometer, Quattro micro™ API, from Waters, with an electrospray operating in positive ionization mode. The software used was MassLynx 4.1. Two SRM transitions were acquired for ibogaine (311→174, 122), noribogaine (297→160, 122) and for the internal standard (325→105, 271). The calibration curves for both compounds in the plasma were linear in a prepared range from 0.1 to 100.0 ng/mL, with a correlation coefficient of at least 0.996. The limit of quantification was 0.2 ng/mL.

Liver preparation

After decapitation, the liver was perfused with cold saline (0.9% w/w) and excised. A portion of the left lobe of the perfused liver was fixed in 4% paraformaldehyde solution in phosphate buffered saline pH 7.4 for 24 h. The remaining liver tissue was frozen in liquid nitrogen and stored at -70°C.

Preparation of micrographs

After 24 h of tissue fixation, the liver samples were dehydrated by increasing the concentrations of ethanol and xylol, and then embedded in Histowax (Histolaboduct

AB, Göteborg, Sweden). Thin sections (5 μm) were cut on a rotary microtome (Leica, Germany), caught on glass slides and prepared for hematoxylin eosin (H&E) and Periodic Acid-Schiff (PAS) staining [22]. Hematoxylin dyes the nucleus blue, while the eosin dyes the cytoplasmic proteins, collagen and erythrocytes a reddish color. Glycogen granules in the cytoplasm are colored purple-magenta by the PAS staining method. Microscopic liver slides were examined using a Leica DM LS2 light microscope, and original photomicrographs were captured using a Canon PowerShot S70.

Qualitative analysis of the liver

The qualitative H&E analysis included histomorphological liver examination of the hepatic lobules, dilatation of the central vein, dilatation of portal vein branches, hepatocyte edema, the presence of necrosis, portal infiltration of lymphocytes and plasma cells, hyperplasia of Kupffer cells and other pathological changes.

To compare the presence of glycogen in hepatocytes of animals from all experimental groups (quantitative analysis), the exact number of glycogen-positive cells was counted using a high-power field (HPF) at 400x magnifications of the areas (5 per each sample) around the central vein with the strongest glycogen signal. The presence of glycogen was expressed as the number of cells that contain glycogen per 100 cells.

Since the amount of glycogen in glycogen-positive cells can vary, in order to additionally clarify the obtained results, we classified each sample into one of three classes according to the average intensity of PAS staining (i.e. the amount of glycogen) in all cells in the examined fields of individual sample as follows: + (weak staining) – the amount of glycogen <33.3% of the maximum; ++ (medium staining) – the amount of glycogen 33.3-66.7% of the maximum; +++ (strong staining) – the amount of glycogen >66.7% of the maximum (semiquantitative analysis). The scale is in the range between 0%, which represents cells negative to PAS staining, and 100%, which represents cells with the maximal observed intensity of PAS staining in hepatocytes of all groups (cells full with glycogen), with boundaries placed on one third and two thirds of the scale. To ensure objectivity, all PAS staining slides were interpreted by three different coauthors, with the mean of their findings as the final result.

Determination of concentration of TBARS and -SH groups

For measuring the level of TBARS and total and non-protein free -SH groups, the perfused liver tissue was homogenized (3x10 s) and sonicated (3x15 s, at 10 kHz) in 0.05 M tris(hydroxymethyl)aminomethane (Tris), 1 mM ethylenediaminetetraacetic acid (EDTA) buffer, pH 7.4. The sonicates were centrifuged (MiniSpin, Eppendorf) for 15 min at 9000 x g. The level of lipid peroxidation in liver was estimated according to the concentration of TBARS measured in liver sonicates according to the method of Rehncrona et al. [23]. The supernatants were mixed with equal volumes of 0.6% 2-thiobarbituric acid and the mixtures were heated for 10 min at 95°C. The absorbance of the supernatant was measured at 532 nm spectrophotometrically on a Shimadzu UV-160 (Shimadzu Scientific Instruments, Shimadzu Corporation, Kyoto, Japan). The amount of TBARS was calculated using malondialdehyde as a standard.

The concentrations of total -SH groups were measured according to Ellman's protocol customized for a microtiter plate [24]. Supernatants were mixed directly on a microtiter plate with 0.1 M potassium phosphate buffer (pH 7.3) and 3 mM 5,5'-dithiobis-(2-nitrobenzoic acid (Ellman's reagent). Measurements were performed 10 min after development of the yellow color. Absorbance was measured at 412 nm (Multiskan Spectrum, Thermo Fisher Scientific Oy, Vantaa, Finland). For determination of non-protein free -SH groups, sulfosalicylic acid was added before centrifugation and measurements were performed in the supernatant.

Determination of the activities of antioxidant enzymes, XOD and GST

For determination of antioxidative enzyme, XOD and GST activities, liver samples were homogenized and sonicated as described above in 0.25 M sucrose Tris-EDTA buffer pH 7.4, and centrifuged at 105000 x g at 4°C for 90 min. The enzyme activities were measured in supernatants spectrophotometrically using a Shimadzu UV-160 spectrometer. The activities of SOD1 and SOD2 were determined by the adrenaline method [25]. One SOD unit (U) is defined as the amount of enzyme required to decrease the rate of adrenaline

autooxidation by 50%. CAT activity was measured by monitoring H_2O_2 consumption at 230 nm, according to Beutler [26]. Glutathione peroxidase (GSH-Px) activity was measured using t-butyl hydroperoxide as a substrate, by a modification of the assay described by Paglia and Valentine [27]. After the reaction of the substrate with glutathione (GSH) by sample GSH-Px, the remaining oxidized glutathione (GSSG) in the reaction mixture is reduced by GR that consumes NADPH. The method is based on the follow-up of the absorbance at 340 nm (absorbance peak of NADPH). The activity of GR was determined using the method based on NADPH oxidation and GSSG reduction [28]. The activity of GST was determined using 1-chloro-2,4-dinitrobenzene and GSH as substrates and monitoring the increase in absorbance at 340 nm [29]. XOD activity was determined by monitoring uric acid production in the presence of xanthine as a substrate, according to Terada et al. [30].

Measurement of hemoglobin concentration

Hemoglobin concentration in erythrocytes was measured by the method of Drabkin [31].

Protein determination

The concentrations of proteins in liver samples were measured by the method of Lowry using bovine serum albumin as a standard [32].

Determination of the amounts of consumed food and water

At the end of the experiment, the amount of consumed food and water for each animal was measured and expressed as g (for food) or mL (for water) per h per kg b.w. The total volume of water consumed during the experiment was calculated as a sum of the volume that the animals have drank freely and the volume applied by gavage.

Determination of blood glucose concentration

Glucose concentration was measured in the whole blood immediately after decapitation using a Blood Glucose Test Strip TOUCH-IN® Micro, ApexBio (Taiwan).

Determination of urine parameters

Bilirubin, urobilinogen and urine specific gravity were determined using commercial strips, Uriscan urine test strips, YD Diagnostics (Yongin, Republic of Korea).

Data analysis and statistical procedures

Statistical analyses were performed according to the protocols described by Hinkle et al. [33]. The presence of glycogen in hepatocytes, the activities of antioxidant enzymes, XOD, GST, the concentrations of glucose, TBARS, the concentrations of total and non-protein free -SH groups and the amount of consumed food and water were compared by two-way ANOVA (ibogaine dose and time after treatment as factors), followed by Tukey's HSD *post hoc* test. The statistical significance of the distribution of the intensity of PAS staining was calculated by the Kolmogorov-Smirnov χ^2-test. The significance level was $p < 0.05$, with values expressed as the mean±standard error of the mean (SEM).

RESULTS

Concentration of ibogaine and noribogaine in blood plasma

Six hours after administration of 1 mg/kg b.w., the plasma of only two animals out of six contained ibogaine measurable above the detection limit (0.9±0.2 ng/mL), while noribogaine was measured in all six plasma samples of L6 animals (6.0±2.2 ng/mL). The samples from the L24 group were under the limit of detection of either ibogaine or noribogaine in blood plasma at 24 h post-administration. In the H6 group, ibogaine was measured in five out of six animals (2.2±1.5 ng/mL), and noribogaine was measured in all six animals (70.5±7.2 ng/mL). In the H24 group, ibogaine was above the limit of detection in the blood plasma of two out of six animals (0.9±0.2 ng/mL), along with noribogaine, which was detected in all six animals (5.7±3.2 ng/mL).

Presence of glycogen and color intensity of PAS staining in the liver

Histological analysis of the left lobe of livers by H&E staining (Fig. 1) revealed a normal liver morphology without any histopathological changes in all animals. Both control and treated H&E-stained livers presented the hexagonal structure of the classic lobule, without signs of necrosis, portal infiltration of lymphocytes and plasma cells, or of hyperplasia of Kupffer cells. PAS staining (Fig. 2) revealed a different proportion of glycogen-positive cells, as well as intensity of PAS staining (i.e. the amount of glycogen) of cells in the experimental groups. Analysis for the presence of glycogen in hepatocytes (Fig. 3) showed that the number of glycogen-positive cells per 100 cells was decreased in groups treated with 1 and 20 mg/kg b.w. of ibogaine (two-way ANOVA, effect of dose, p<0.001). Treatment with 1 mg/kg b.w. of ibogaine caused a decrease in
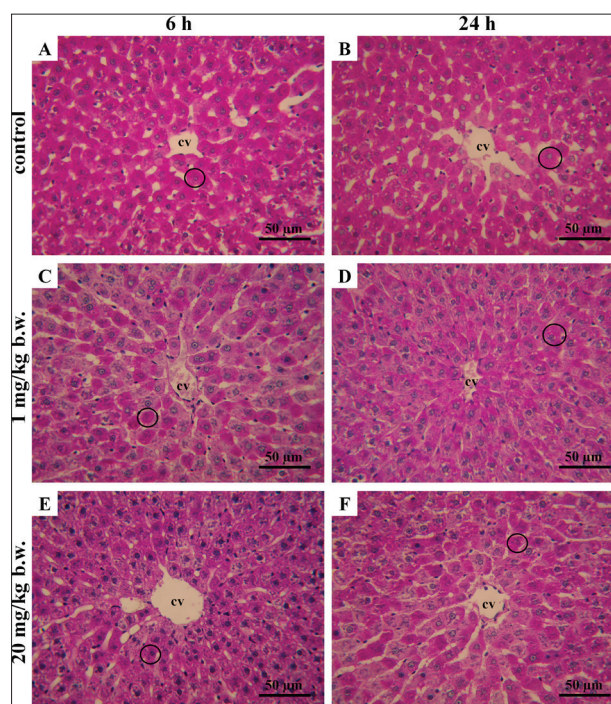

Fig. 2. Micrographs of rat liver after PAS-staining. Differences in the numbers of glycogen-positive cells and staining intensity of glycogen are shown in representative fields. Group C (control groups): animals treated with dH_2O, decapitated after 6 h (C6) – **A** and after 24 h (C24) – **B**; group L: animals treated with ibogaine 1 mg/kg b.w., decapitated after 6 h (L6) – **C**, and after 24 h (L24) – **D**; group H: animals treated with ibogaine 20 mg/kg b.w., decapitated after 6 h (H6) – **E**, and after 24 h (H24) – **F**. A-F: PAS staining, magnification: 400x. cv – central vein. Example of a cell that contains glycogen are in circle.

Fig. 1. Micrographs of rat liver after H&E-staining. Histomorphological examination showed regular, hexagonal, lobular liver structure without histopathological changes. Group C (control groups): animals treated with dH_2O, decapitated after 6 h (C6) – **A**, and after 24 h (C24) – **B**. Group L: animals treated with ibogaine 1 mg/kg b.w., decapitated after 6 h (L6) – **C**, and after 24 h (L24) – **D**; group H: animals treated with ibogaine 20 mg/kg b.w., decapitated after 6 h (H6) – **E**, and after 24 h (H24) – **F**. A-F: H&E staining, magnification: 200x. cv – central vein, pt – portal triads.
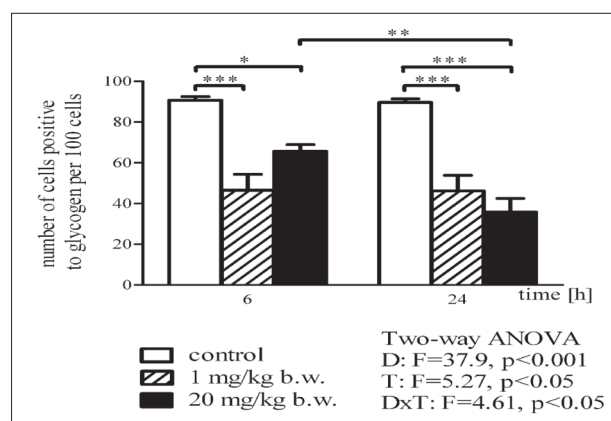

Fig. 3. Presence of glycogen in hepatocytes (quantitative analysis). The presence of glycogen was expressed as the number of cells that contain glycogen per 100 total cells; body weight (b.w.). Results are expressed as the mean±SEM. Statistical significance was calculated by two-way ANOVA (dose (D) and time (T) as factors; F and p values are presented) and *post hoc* Tukey's HSD test. *** – p<0.001, ** – p<0.01, * – p<0.05.
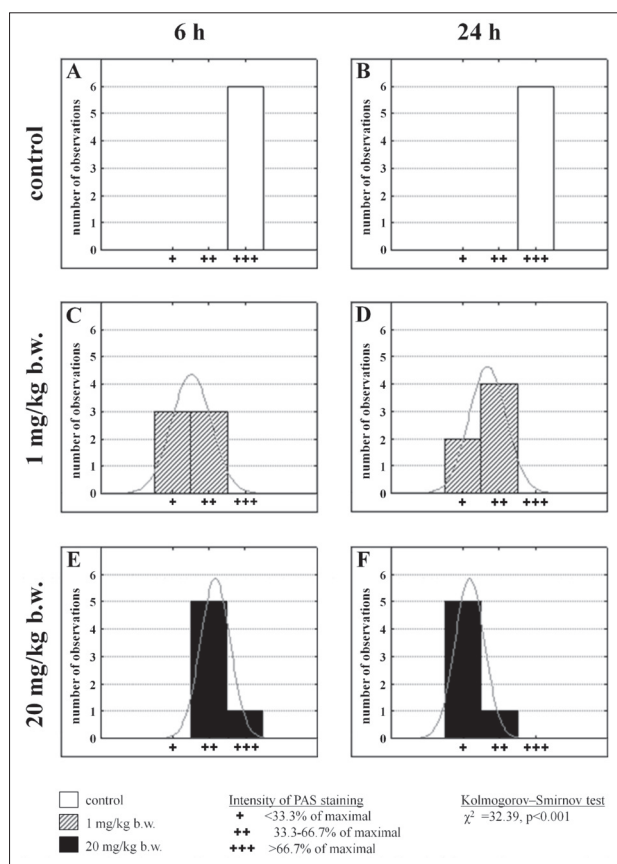
Fig. 4. Intensity of PAS staining, i.e. amount of glycogen (semiquantitative analysis). Each sample is classified in one of the three classes: + amount of glycogen <33.3% of the maximum; ++ amount of glycogen 33.3-66.7% of the maximum; +++ amount of glycogen >66.7% of the maximum. Group C (control groups): animals treated with dH_2O, decapitated after 6 h (C6) – **A**, and after 24 h (C24) – **B**; group L: animals treated with ibogaine 1 mg/kg b.w., decapitated after 6 h (L6) – **C**, and after 24 h (L24) – **D**; group H: animals treated with ibogaine 20 mg/kg b.w., decapitated after 6 h (H6) – **E**, and after 24 h (H24) – **F**; body weight (b.w.). Statistical significance was calculated by the Kolmogorov-Smirnov chi-squared test; χ^2 and p values are presented.

Fig. 5. Concentration of TBARS (**A**) and activity of xanthine oxidase (XOD) in liver (**B**); body weight (b.w.), nonsignificant (ns). Results are expressed as the mean±SEM. Statistical significance was calculated by two-way ANOVA (dose (D) and time (T) as factors; F values are presented, p<0.05) and *post hoc* compared by Tukey's HSD test; * represents Tukey's HSD test significant difference p<0.05.

the proportion of glycogen-positive cells after 6 h, which remained at the same level after 24 h. On the other hand, treatment with 20 mg/kg b.w. of ibogaine also caused a decrease after 6 h, followed by an even greater decrease after 24 h (two-way ANOVA, effect of time, p<0.05; interaction of dose and time, p<0.05; Tukey's HSD test, H6 vs. H24, p<0.01). Analysis of intensity of the PAS-staining, i.e. the amount of glycogen (semiquantitative analysis) (Fig. 4) showed significant differences among the groups (Kolmogorov-Smirnov chi-squared test, p<0.001). In both control groups, all samples were classified as +++. Treatment with 1
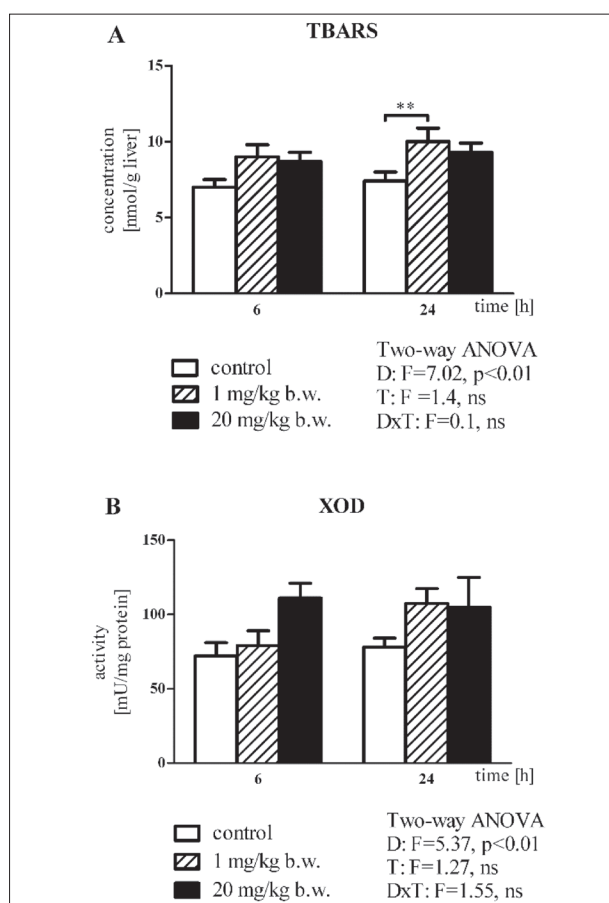
mg/kg b.w. of ibogaine caused a decrease in staining intensity of glycogen after 6 h (three specimens were classified as + and three as ++), whereas after 24 h, the staining intensity of glycogen was slightly greater (two samples were classified as + and four as ++). However, treatment with 20 mg/kg b.w. of ibogaine also caused a mild decrease after 6 h (five specimens were classified as ++ and one as +++), followed by a greater decrease after 24 h (five specimens were classified as + and one as ++).

Concentration of TBARS and -SH groups and enzyme activities

While there were no observable changes in the concentrations of total and non-protein free -SH groups (Table 1) in the liver, the concentration of TBARS was elevated in the L24 group (Fig. 5A). Two-way ANOVA revealed that XOD was higher in the livers of rats that received 20 mg/kg b.w. compared to the control, but the *post hoc* Tukey's HSD test did not show any statistical significance among individual groups (Fig. 5B). There were no differences in the activities of antioxidant enzymes and GST in rat liver after ibogaine treatment, regardless of the dose used (Table 1). The same was found in erythrocytes, where the activity of antioxidant enzymes was similar in treated animals compared to controls (Table 2). In erythrocytes, there was no difference in XOD activity among groups.

Amounts of consumed food and water, blood glucose concentration and urine parameters

There were no significant differences in the consumption of water and food. No difference in blood glucose concentration was observed after treatment with ibogaine, regardless of dose and time of observation (Table 3). There were no discernible changes in the bilirubin and urobilinogen levels or urine specific gravity in any of the treated groups when compared to the controls (data not shown).

Table 1. Activities of antioxidant enzymes (SOD1, SOD2, CAT, GSH-Px, GR), GST, XOD, concentration of TBARS, total and non-protein free -SH groups in the liver.

group	time [h]	TBARS [nmol/g liver]	total free –SH groups [µmol –SH/L]	non-protein free –SH groups [nmol–SH/L]	SOD1 [U/mg protein]	SOD2 [U/mg protein]	CAT [µmol H_2O_2/ min/ mg protein]	GSH-Px [nmol NADPH/ min/mg protein]	GR [nmol NADPH/ min/mg protein]	GST [nmol GSH/ min/ mg protein]	XOD [nmol uric acid/min/ mg protein]
C	6	7.35±0.77	0.83±0.06	0.10±0.03	23.97±0.86	2.89±0.16	130.38±1.66	135.66±6.78	89.04±1.52	637.05±64.69	0.076±0.009
	24	7.01±0.97	0.80±0.03	0.12±0.03	20.89±3.36	2.87±0.43	122.93±11.59	126.72±10.43	85.43±6.9	727.73±62.66	0.069±0.011
L	6	9.62±1.31	0.80±0.03	0.13±0.01	22.67±2.19	3.09±0.24	122.93±10.81	153.78±9.15	95.20±4.83	716.25±47.76	0.079±0.006
	24	10.04±0.86*	0.84±0.05	0.13±0.02	23.39±2.73	2.24±0.25	169.32±1.84	136.40±8.75	81.95±4.77	629.80±29.21	0.107±0.009
H	6	7.95±1.22	0.84±0.03	0.12±0.03	21.12±2.78	2.32±0.10	160.48±26.41	148.62±7.68	91.48±3.61	744.73±26.41	0.114±0.007
	24	9.34±0.59	0.81±0.04	0.12±0.01	22.84±1.81	2.49±0.19	137.28±9.95	142.43±13.95	85.22±6.10	679.32±71.00	0.105±0.016
two-way ANOVA	dose: F=5.37; p<0.05	ns	ns	ns	ns	ns	ns	ns	ns	dose: F=7.02; p<0.01

The control group (C) was treated with dH_2O; groups treated with ibogaine were as follows: 1 mg/kg b.w. (L), 20 mg/kg b.w. (H).
Values are expressed as the mean±SEM. Results were compared by two-way ANOVA, dose and time as factors, p<0.05 and *post hoc* compared by Tukey's HSD test;
* represents Tukey's HSD test significant difference compared to C24, p<0.05.
Nonsignificant – ns.

Table 2. Activities of antioxidant enzymes (SOD1, CAT, GSH-Px, GR), and XOD in erythrocytes. There were no significant changes between groups.

group	time [h]	SOD1 [U/g Hb]	CAT [mmol H_2O_2/ min/g Hb]	GSH-Px [µmol NADPH/ min/g Hb]	GR [µmol NADPH/ min/g Hb]	XOD [nmol uric acid/min/g Hb]
C	6	20.06±2.27	539.11±88.06	73.84±7.28	37.62±8.99	3.79±0.69
	24	25.17±3.46	541.91±100.4	97.30±12.24	57.88±20.10	3.71±0.73
L	6	26.95±1.21	525.60±23.94	79.93±5.29	45.19±3.08	4.37±0.56
	24	26.36±3.61	488.72±51.66	72.77±10.94	41.30±6.74	4.15±0.71
H	6	26.57±1.38	403.04±16.18	74.84±3.46	32.13±9.54	4.47±0.78
	24	23.77±4.56	442.46±54.31	57.81±8.41	20.16±6.30	4.10±0.97
two-way ANOVA		ns	ns	ns	ns	ns

The control group (C) was treated with dH_2O; groups were treated with ibogaine were as follows: 1 mg/kg b.w. (L), 20 mg/kg b.w. (H).
Values are expressed as the mean±SEM. Results were compared by two-way ANOVA, dose and time as factors, p<0.05 and *post hoc* compared by Tukey's HSD test.
Hemoglobin – Hb; nonsignificant - ns.

Table 3. Amount of consumed food and water, and blood glucose concentration. There were no significant changes between groups.

group	time [h]	food [g/h/kg b.w.]	water [mL/h/kg b.w.]	glucose [mmol/L]
C	6	2.98±0.26	4.15±0.59	6.53±0.40
	24	2.68±0.38	4.60±0.48	6.47±0.41
L	6	3.15±0.20	4.77±0.36	6.05±0.31
	24	3.07±0.21	4.56±0.29	5.75±0.27
H	6	3.13±0.16	4.91±0.41	5.75±0.27
	24	3.01±0.13	4.50±0.37	6.14±0.54
two-way ANOVA		ns	ns	ns

The control group (C) was treated with dH_2O; groups were treated with ibogaine were as follows: 1 mg/kg b.w. (L), 20 mg/kg b.w. (H).
Values are expressed as the mean±SEM. Results were compared by two-way ANOVA, dose and time as factors, $p<0.05$ and *post hoc* compared by Tukey's HSD test.
Nonsignificant – ns.

DISCUSSION

In spite of the existing knowledge about the molecular mechanisms of ibogaine action and its effects on ROS and redox homeostasis, there is little published research about its various systemic *in vivo* oxidant/antioxidant effects. It has been shown that ibogaine can act as a pro/antioxidant, promoting increased antioxidant activity in a dose- and time-dependent manner in different model systems, including yeast [11] and human erythrocytes [13]. However, our results presented here show that liver and erythrocyte antioxidant enzyme activities after administration of ibogaine p.o. at doses of 1 and 20 mg/kg b.w. at 6 and 24 h post-application did not significantly differ from those in control rats. However, TBARS was elevated after application of 1 mg/kg b.w., suggesting a mild prooxidant and systemic effect that was insufficient to promote an increase in activities of antioxidant enzymes to a measurable level. Our results also showed that ibogaine caused a depletion of glycogen in the liver, suggesting extensive utilization of glucose. In animals that received the low dose, ibogaine caused a depletion that was partially compensated at 24 h. On the other hand, in the liver of animals that received 20 mg/kg ibogaine, the depletion in liver glycogen was more profound at 6 h post-ibogaine administration, and even greater after 24 h. It would appear that ibogaine increased overall tissue energy consumption, which was both time- and dose-dependent, confirming earlier results that showed ibogaine-mediated deple-

tion of cellular energy stores were followed by ATP re-synthesis and elevated ROS production, as well as restitution of energy homeostasis [11]. The degrees of change in these processes *in vivo* can be tissue-specific; however, if we take into account that there were no changes in blood glucose concentration, we can assume that ibogaine-associated tissue energy depletion was compensated from blood glucose that was maintained at a steady state via liver glycogen utilization. It has been reported that ibogaine itself can induce elevation of local cerebral glucose consumption [34], but at a higher dose and after a different route of application (40 mg/kg, i.p.). Thus, it would appear that the effect of ibogaine on glucose metabolism is tissue-, dose- and route-of-application-dependent.

No histopathological changes were observed in the liver after administration of either dose of ibogaine; however, we detected glycogen depletion. Urine analysis showed that the metabolic function of the liver was preserved. However, the higher dose of ibogaine induced a slight elevation in XOD that suggests a faster turnover of ATP and adenosine. The xanthine oxidase reaction can cause tissue injury in hypoxic organs due to increased superoxide, as well as enhanced adenine nucleotide breakdown that occurs during oxygen deprivation. Faster turnover implies an elevated synthesis of uric acid that possesses significant antioxidant properties so that ibogaine application can be considered as pro/antioxidative. Uric acid itself is a strong antioxidant and its elevation was found as a side effect of XOD prooxidant action during ischemia-reperfusion and hypoxia when intensive ATP depletion occurs [35-37]. Since no change in XOD activity was observed in the blood, it seems that the faster purine turnover was not systemic but only characteristic for the liver.

Measurements of ibogaine and noribogaine concentrations in blood plasma revealed a pharmacokinetics that was generally consistent with earlier publications. In our experiment with animals treated p.o. (dose 1 mg/kg b.w.), noribogaine was detected after 6 h, while the concentration of ibogaine was low, close to the threshold of detectability by the employed method. After 24 h, neither ibogaine nor noribogaine were detected in the plasma of rats treated with the low-dose, suggesting efficient metabolism of ibogaine and/or possible sequestration into adipose tissue and the brain. It was

shown that ibogaine largely accumulates in adipose tissue and to some extent in the brain [38]. Ibogaine and noribogaine were detected in blood plasma after 6 h in the group treated with 20 mg/kg b.w. At 24 h following the 20 mg/kg b.w. of ibogaine, the plasma noribogaine concentration was 20 times higher than that of ibogaine. This is in accordance with the known ibogaine metabolism and pharmacokinetics [39]. Ibogaine is subject to a substantial "first pass" effect [38] and there is a significant difference in ibogaine metabolism even among humans [40,41]. The maximal concentration of ibogaine in blood or plasma and the time needed for ibogaine to reach its maximal concentration in the plasma are dependent on animal sex, dose and type of application (i.v., subcutaneous (s.c.), i.p. or p.o.) [20]. After i.v. application of 10 mg/kg to rats, the maximal concentration in whole blood (18.2 μg/mL) was attained already after 1 min [20], while after i.p. application of 40 mg/kg to rats, ibogaine reached the maximal concentration in whole blood (3.9 μg/mL) after 6 min [20]. In our experiment where ibogaine application was p.o., its incorporation was slower. Furthermore, after i.v. application ibogaine bypasses the first pass of liver-mediated detoxification. Hough et al. [38] revealed that in female rats, ibogaine (40 mg/kg) administered s.c. achieved a higher concentration in blood plasma after 1 h and 12 h when compared to its i.p. route of administration. After p.o. application of 5 mg/kg, the maximal concentration of ibogaine in the plasma was 10 ng/g (about 10 ng/mL) [19], but the time when ibogaine peaked was not reported. Having in mind that ibogaine peaks in blood plasma within 1-2 h after p.o. application in humans [18], the ibogaine concentration measured in our experiment after 6 and 24 h for both doses seems reasonable for this route of application. Moreover, it is clear that the maximal concentration attained in the blood or plasma after p.o. application is much lower compared to other types of application. Based on the pharmacokinetics described by Jeffcoat [19] and other authors [7,18,20,38,39], it is not possible to extrapolate precisely the blood plasma ibogaine and noribogaine concentrations from different graphs due to variations in animal models and treatments. Furthermore, Shrep et al. [8] suggested additional safety factors need to be applied to animal data prior to considering an appropriate and safe initial dose for humans.

Ibogaine is metabolized in the liver by the cytochrome P-450-dependent system and different classes of GST that eliminate a broad spectrum of xenobiotics and final metabolic products [42]. Liver GSTs are inducible enzymes that follow liver xenobiotic metabolism. However, our results showed no changes in GST activity, suggesting that ibogaine could be metabolized before the time points when GST activity was measured or when the treatment with ibogaine did not significantly affect the liver microsomal system. This assumption is supported by the fact that there were no changes in the concentration of GSH, as concluded based on the measurement of non-protein free -SH groups, which is a cofactor utilized during GST activity.

In our experiment, ibogaine did not affect food and water consumption. This is in agreement with the results of Rezvani et al. [43], who showed that ibogaine treatment (i.p., p.o.) with doses of 10, 30, and 60 mg/kg had no significant effect on food and water consumption.

In spite of the changes in liver glycogen, there were no changes in the activities of the studied antioxidant enzymes in either the liver or erythrocytes. At the doses used in our study, it seems that ibogaine did not provoke a significant antioxidative response either in the liver or at the systemic level. However, since ibogaine effects are rapid, it is possible that compensatory antioxidative activities by antioxidative enzymes were carried out soon after ibogaine administration, much earlier than at 6 or 24 h. Previous results from *in vitro* studies on yeast [11,12], *ex vivo* data obtained in human erythrocytes [13] and isolated rat uterus [15] showed that antioxidant enzymes responded to an ibogaine challenge by changing their activities. Furthermore, previous results on yeast showed that 5 h is the time period necessary for ROS production to return to the normal cellular level after application of ibogaine at doses ranging from 1 to 20 mg/L [11]. Additionally, the liver has a greater antioxidant capacity compared to other tissues [44,45], and it seems that the applied doses were not sufficient to create a persistent antioxidant response at the level of antioxidant enzyme activities after 6 or 24 h.

Our results also suggest that ibogaine at the applied doses did not disturb liver redox equilibrium since the levels of -SH groups and GR activity were similar in treated and control groups at both ibogaine doses and time points. However, there was a slight elevation of

TBARS in the treated group after 24 h, implying that cell membranes were oxidatively attacked and damaged, yielding lipid peroxide byproducts. Ibogaine is a lipophilic molecule. There are membrane lipid rafts that store ATP [46]. In our earlier work [13], we showed that ibogaine addition to fresh whole blood (10 and 20 μM) releases ATP from erythrocytes within 1 h of incubation. The release of ATP from erythrocytes is accompanied by increases in SOD1 and GR activities. However, our results showed that there were no changes in erythrocyte SOD1 and GR after 6 h or 24 h after the application of either dose. The much higher concentrations of ibogaine (3.1 μg/mL and 6.2 μg/mL) applied in our earlier work on erythrocytes *ex vivo* [13], which were several times higher than the concentrations of ibogaine and noribogaine measured in blood plasma herein, could be the reason for the apparent absence of antioxidant effects of ibogaine on erythrocytes.

CONCLUSION

Our results confirm ibogaine-associated changes in tissue energy and its dose- and time-dependent redox-related actions. No overt pathological signs of damage to hepatic cells and tissue were detected. However, due to the different rates of rat and human metabolism, a linear extrapolation of results (and applied doses) from rat to human should be avoided. Since our research is based on doses used by humans, additional safety factors need to be applied to the animal data before recommending a safe initial dose for humans.

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