Journal of Ethnopharmacology 164 (2015) 64–70

Contents lists available at ScienceDirect

Journal of Ethnopharmacology

journal homepage: www.elsevier.com/locate/jep

Ex vivo effects of ibogaine on the activity of antioxidative enzymes in human erythrocytes

Aleksandra Nikolić-Kokić [a], Zorana Oreščanin-Dušić [a], Ivan Spasojević [b], Marija Slavić [a], Ana Mijušković [a], Roman Paškulin [c,d,*], Čedo Miljević [e], Mihajlo B. Spasić [a], Duško P. Blagojević [a]

[a] Institute for biological research "Siniša Stanković", University of Belgrade, Despota Stefana 142, Belgrade, Serbia
[b] Institute for Multidisciplinary Research, University of Belgrade, Belgrade, Serbia
[c] OMI Institute, Trnovska 8, Ljubljana, Slovenia
[d] Institute of Pathophysiology, University of Ljubljana, Vrazov trg 2, Ljubljana, Slovenia
[e] Institute of Mental Health, School of Medicine, University of Belgrade, Belgrade, Serbia

ARTICLE INFO

Article history:
Received 26 August 2014
Received in revised form
13 January 2015
Accepted 27 January 2015
Available online 7 February 2015

Keywords:
Adaptogen
Aging
Alkaloids
Antioxidant
Metabolism
Stress

ABSTRACT

Ethnopharmacological relevance: Ibogaine is a naturally occurring alkaloid with psychotropic and metabotropic effects, derived from the bark of the root of the West African *Tabernanthe iboga* plant. The tribes of Kongo basin have been using iboga as a stimulant, for medicinal purposes, and in rite of passage ceremonies, for centuries. Besides, it has been found that this drug has anti-addictive effects.

Aim of the study: Previous studies have demonstrated that ibogaine changed the quantity of ATP and energy related enzymes as well as the activity of antioxidant enzymes in cells thus altering redox equilibrium in a time manner. In this work, the mechanism of its action was further studied by measuring the effects of ibogaine in human erythrocytes in vitro on ATP liberation, membrane fluidity and antioxidant enzymes activity.

Materials and methods: Heparinized human blood samples were incubated with ibogaine (10 and 20 μM) at 37°C for 1 h. Blood plasma was separated by centrifugation and the levels of ATP and uric acid were measured 10 min after the addition of ibogaine using standard kits. The activity of copper–zinc superoxide dismutase (SOD1), catalase (CAT), glutathione peroxidase (GSH-Px) and glutathione reductase (GR) were measured in erythrocytes after incubation period. The stability of SOD1 activity was further tested through in vitro incubation with H_2O_2 and scanning of its electrophoretic profiles. Membrane fluidity was determined using an electron paramagnetic resonance spin-labelling method.

Results: Results showed that ibogaine treatment of erythrocytes in vitro increased ATP concentration in the blood plasma without changes in neither erythrocytes membrane fluidity nor uric acid concentration. Ibogaine also increased SOD1 activity in erythrocytes at both doses applied here. Treatment with 20 μM also elevated GR activity after in vitro incubation at 37 °C. Electrophoretic profiles revealed that incubation with ibogaine mitigates H_2O_2 mediated suppression of SOD1 activity.

Conclusion: Some of the effects of ibogaine seem to be mediated through its influence on energy metabolism, redox active processes and the effects of discrete fluctuations of individual reactive oxygen species on different levels of enzyme activities. Overall, ibogaine acts as a pro-antioxidant by increasing activity of antioxidative enzymes and as an adaptagene in oxidative distress.

1. Introduction

Ibogaine is a naturally occurring alkaloid with psychotropic and metabotropic effects, derived from the bark of the root of the West African *Tabernanthe iboga* plant. Iboga (*Tabernanthe radicis* cortex) has been traditionally used in tribes of the Congo basin in Central Africa as a psychoactive sacrament used in the ceremony of initiation into adulthood. It induces trance and is considered to reveal one's purpose of life and his role in a society (Fernandez, 1982). In smaller

Abbreviations: 12-DS, 12-doxyl stearate; 5-DS, 5-doxyl stearate; ATP, adenosine triphosphate; AD, antioxidative defense; CO_2, carbon dioxide; CAT, catalase; SOD1, copper–zinc superoxide dismutase; EPR, electron paramagnetic resonance; GSH-Px, glutathione peroxidase; GR, glutathione reductase; Hb, hemoglobin; H_2O_2, hydrogen peroxide; NADPH, nicotinamide adenine dinucleotide phosphate; NO, nitric oxide; NBT, nitro blue tetrazolium; ROS, reactive oxygen species; RBC, red blood cells; TCA, tricarboxylic acid; UA, uric acid

* Correspondence to: OMI Institute, Trnovska 8, 1000 Ljubljana, Slovenia. Tel.: +386 41 351 531; fax: +386 1 5497235.

http://dx.doi.org/10.1016/j.jep.2015.01.037
0378-8741/

doses it is appreciated due to its stimulant and aphrodisiac properties or as a tonic during convalescence after diseases (Naranjo, 1969; Schultes, 1970). Hunters use it to promote vigilance on long, tiring marches, on lengthy canoe voyages, and on difficult night watches while stalking pray (Fernandez, 1982; Schultes et al., 2001). Youthfulness and longevity are claimed effects of low daily iboga supplementation (personal communication with Fang tribe – Paškulin, Gabon 1997).

More recently, based on human case reports and experimental data from animal studies it has been found that this drug has anti-addictive effects. The administration of ibogaine reduced self-administration of cocaine, morphine, heroin, alcohol, and reduced nicotine preference (Alper, 2001). Doses up to 1 mg/L (3,22 μM) correspond to moderate stimulant effect, raising the dose brings psychoactive range and approaching 4 mg/L (12,89 μM) relates to the anti-addictive properties, while above are the traditional initiation doses (Fernandez and Fernandez, 2001; Mash et al., 2000). Although ibogaine possess affinity for different types of receptors, its effects appear not to be mediated via any single type of receptor (Alper, 2001). Paškulin et al. (2006) showed the stimulating influence of ibogaine on rat brain energy metabolism. The results pointed out the elevation of the enzymes of glycolysis and tricarboxylic acid (TCA) cycle and subsequent higher metabolic turnover. Separate experiment on yeast *Saccharomyces cerevisiae* showed that induction of energy metabolism-related enzymes was not mediated via receptor bindings and it is not linked to cell differentiation or organization in tissue. Yeast incubation with ibogaine at doses from 1 to 20 mg/L (approximately 3 to 60 μM) is followed by a fall in cellular ATP level and an increase in CO_2 production in the first hour of exposure to ibogaine in a dose dependant manner, suggesting that ibogain promotes ATP consumption (Paškulin et al., 2010, 2012). A transient and reversible fall in ATP pool happens immediately (10 min) after addition of ibogaine. Consequently increased cellular respiration is associated by the production of reactive oxygen species (ROS) and, surprisingly, significant drop in the total oxidative load. After 5 h of exposure, energy metabolism related enzymes (glyceraldehyde-3-phosphate dehydrogenase, phosphoglycerate kinase, enolase and alcohol dehydrogenase) were induced, as well as SOD1 protein suggesting increased energy production and antioxidant cellular protection (Paškulin et al., 2010). Since ibogaine did not show any significant in vitro antioxidant properties per se (Paškulin et al., 2012), the results indicate its stimulating influence on physiological oxidative stress defense system in a pro-antioxidant manner. It seems that ibogaine triggers remodeling of the housekeeping metabolism and under the initial energy cost it results in increased metabolic efficacy, enhanced free energy availability and sustainability.

Since the ATP depletion affects the cellular mitochondrial metabolism, here we used erythrocytes as a model system that is not dependant on mitochondrial energetics. Furthermore, it was shown that erythrocytes deliberated ATP from membrane bound ATP pools (Chua et al., 2012) after different kind of stimuli (Ellsworth et al., 2009). Therefore, we measured the concentration of ATP in blood plasma after ibogaine treatment and its influence on membrane fluidity. Because ATP in the blood plasma is dephosphorylated (Coade and Pearson, 1989) and further decomposed by adenosine deaminase to hypoxanthine (Plagemann et al., 1985) and finally uric acid (UA), here we also measured the content of UA.

In physiological settings, erythrocytes show a self-sustaining activity of antioxidative defense (AD) enzymes, such as: copper–zinc superoxide dismutase (SOD1, EC 1.11.16), catalase (CAT, EC 1.11.1.6), glutathione peroxidase (GSHPx, EC 1.11.1.9) and glutathione reductase (GR, EC 1.6.4.2). Their coordinate actions protect the erythrocyte's bio-macromolecules from free radical-mediated damage. Since there is no de novo synthesis of AD enzymes in mature erythrocytes, their defense capacity is limited (Nikolić-Kokić et al., 2010). According to previous results that ibogaine treatment led to elevated anti-oxidant defense (Paškulin et al., 2006, 2010), the aim of our study was also to explore in vitro effects of ibogaine on the activity of the key antioxidant enzymes: SOD1, CAT, GSHPx and GR in the model of erythrocytes where de novo enzyme synthesis is absent.

2. Materials and methods

2.1. Material

Ibogaine HCL was donated by Sacrament of Transition, Maribor, Slovenia. Ibogaine was used in our series of experiments since it is directly related to the iboga plant as its principal alkaloid. Besides, majority of the literature concerns this pure form.

2.2. Subjects and blood sampling

Fresh blood was obtained from 13 normal weights, non-smoking, male volunteers (ages 23–39). They were healthy and free of clinical evidence of any chronic illness. All study participants provided written informed consent and the study protocol was reviewed and approved by principal institution's ethical review boards. After 12–14 h overnight fast, blood samples were obtained by venipuncture and pooled in tubes containing heparin (1 g/L).

2.3. Experimental procedures

Fresh whole blood aliquots were incubated in vitro for 1 h at 37 °C without (control) or with ibogaine (doses of 10 and 20 μM according to Paškulin et al., 2010). ATP and uric acid concentrations in blood plasma were measaured after only 10 min of incubation. After 1 h erythrocytes and plasma were immediately separated by centrifugation (10 min, 5000 rpm, 4 °C).

Aliquots of three-times washed erythrocytes with saline (0.9% w/w) were centrifuged and separated erythrocytes were hemolysed in ice-cold distilled water. Antioxidant defense enzyme activities were measured in lysate. For SOD1 activity determination hemoglobin was removed by the method of Tsuchihashi (1923). Hemoglobin was estimated by the method of Drabkin and Austin (1935).

2.4. ATP assay

ATP was measured in human plasma samples as suggested by Gorman et al. (2007). Whole blood was separated in 3 aliquots: control (1) and treated with 10 (2) and 20 μM (3) of ibogaine. Samples were centrifuged at 5000 g/10 min/4 °C to extract plasma. ATP concentration was measured using an ATP bioluminescence assay kit. Luciferin–luciferase agent (100 μL) was added to samples (100 μL), and ATP-dependent luminescence was measured with a luminometer (CHAMELEON™V, Hidex, Turku, Finland). The standard curve was obtained by serial dilutions of 2 nM ATP stock solution. ATP concentrations were expressed as pmol/ml of blood plasma.

2.5. Uric acid determination

Uric acid (UA) concentration in human plasma samples was determined using commercially available enzymatic colorimetric assay according to the manufacturer's instructions (Randox-analyzer Monarch, Milan, Italy). The reference values for UA are 142–339 μmol/L for females and 202–416 μmol/L for males.

2.6. Indication of membrane fluidity

Fresh blood was obtained from four healthy volunteers (ages 23–39) using tubes containing 0.072 mL of 7.5% K_3EDTA as the anticoagulant per 3 mL of blood (Vacuette EDTA, Greiner Bio-One,

Austria). Spin-labeling of the erythrocytes (erythrocyte membranes) was performed as described by Ajdžanović et al. (2011). After ibogaine incubation blood was washed three times with an isotonic Phosphate Buffer Solution (PBS; NaCl 8.8 g/L, Na_2HPO_4 1.2 g/L, NaH_2PO_4 0.43 g/L, pH was adjusted to 7.4 with 1 M HCl) by centrifugation at 3500 g/10 min/4 °C. The hematocrit in fresh blood was 40%, and the same hematocrit was adjusted in all samples before incubation. An ethanol solutions of fatty acid spin-probes 5-DS (2-(3-carboxypentyl)-2-tridecyl-4,4-dimethyloxazolidine-3-oxyl; Molecular Probes, Junction City, OR, USA) and 12-doxyl stearate were supplemented on the walls of tubes. After the ethanol evaporated, sample was added and gently mixed. The amount of 5-DS and 12-doxyl stearate was added to the erythrocytes to obtain the optimal spin-label/membrane-lipid ratio of approximately 1:100. EPR spectra were recorded using a Varian E104-A EPR spectrometer operating at X-band (9.1 GHz) and adjusted to the following settings: modulation amplitude, 2 G; modulation frequency, 100 kHz; microwave power, 10 mW; scan range, 100 G; and scan time, 4 min; time constant 0.25 s. The temperature was controlled at 20 °C during the measurements. Spectra were recorded and analyzed using EW software (Scientific Software). The order parameter (*S*), calculated as shown previously (Ajdžanović et al., 2011; see Fig. 1), was used as an indication of membrane fluidity. The data are presented as the mean ± standard deviation of at least three separate experiments. Significances of differences were calculated using Student's *t*-test. Means were considered significantly different at $p < 0.05$.

2.7. Measurement of the activity of antioxidant enzymes

The activity of SOD1 in erythrocytes was assayed using SOD inhibition of epinephrine autooxidation to adrenochrome mediated by superoxide anion radical at pH 10.2 (Misra and Fridovich, 1972) and expressed in U/g Hb. CAT activity was measured by the procedure of Beutler (1982) and expressed in U/g Hb. The activity of GSH-Px was determined using t-butyl hydroperoxide as a substrate (Paglia and Valentine, 1967) and expressed in μmol NADPH/min/g Hb. Glutathione reductase (GR) activity was assayed as reported by Glatzle et al. (1974) and expressed in μmol NADPH/min/g Hb.

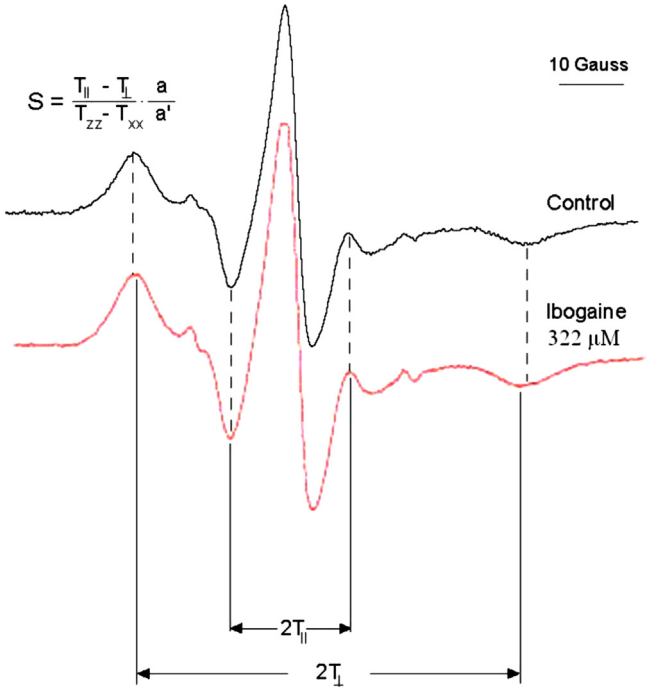

Fig. 1. Characteristic EPR spectra of erythrocytes labeled with 5-DS without or with ibogaine (322 μM i.e. 100 mg/L). *S*, order parameter; 2 $T_{\parallel}$, outer hyperfine splitting; 2 $T_{\perp}$, inner hyperfine splitting; *a*, isotropic hyperfine coupling constant in crystal [$a=1/3(T_{xx}+T_{yy}+T_{zz})$]; *a'*, isotropic hyperfine coupling constant in membrane [$a'=1/3(T_{\parallel}+2T_{\perp})$]; *Txx*, *Tyy*, *Tzz*, hyperfine constants (for 5-DS they were taken to be $T_{xx}=T_{yy}=6.1$ G, $T_{zz}=32.4$ G; for 12-DS $T_{xx}=6.26$ G $T_{yy}=5.81$ G, $T_{zz}=33.46$ G). Dashed lines marking parameters in control spectra, are shown in order to stress out differences between controls and treated samples. Two narrow lines originate from the DSs in the solution (arrows) (Ajdzanovic et al., 2011).

2.8. Electrophoresis

Native polyacrylamide gel electrophoresis (PAGE) was performed according to Laemmli (1970), using 12% acrylamide. Just before PAGE, erythrocytes or Thushihashi erythrocytes were incubated for 1 h at 37 °C under gentle stirring in the absence (controls) and presence of 1) H_2O_2 (final concentration of 5 mM), 2) ibogaine (10 and 20 μM), and 3) H_2O_2 and ibogaine (respective doses). Incubated SOD containing samples were diluted to the activity of 2 U/ml with buffer (12% glycerol, 0.5 mM Tris–HCl, 0.2 M EDTA, pH 6.8), before loading 50 μl well. SOD bands were visualized using the activity staining procedure described by Beauchamp and Fridovich (1971), using the reduction of nitro blue tetrazolium (NBT) with superoxide produced by photochemical reduction of riboflavin with TEMED.

2.9. Statistic analyses

Sample assays were determined in triplicate and presented as mean ± standard deviation (SD). Statistical significance was established by protocols described in Hinkle et al. (2002). Differences among parameters were assessed by Repeated Measures analysis of variance (ANOVA) followed by Partial Correlation analysis and Analysis of Variance of Contrast Variables. In all statistical analyses, *p* values less than 0.05 were deemed significant.

3. Results

Earlier reports showed that ibogaine effects on ATP in cells is fast and occurs in minutes (Paškulin et al., 2010). Therefore, we measured the concentration of ATP in blood plasma after 10 min of incubation. An increase of ATP in the blood plasma after 10 min of incubation was observed (Table 1). The effect was dose dependant. On the other hand, there were no changes in UA concentration in the blood plasma (Table 1).

Table 1
ATP and uric acid concentration in the blood plasma after the treatment of the whole blood with ibogaine in vitro. Results are expressed as mean ± SD ($n=6$). Statistical significance was tested by one way ANOVA and post-hoc Tukey's HSD test between all tested samples. Different letters in superscript represents statistically significant differences between samples (a vs. b, $p < 0.05$; a vs. c, $p < 0.001$; b vs. c, $p < 0.01$).

SamSamples	Controls	Ibogaine 10 μM	Ibogaine 20 μM	ANOVA
ATP (pM)	59.8 ± 22.2^a	86 ± 19.6^b	127 ± 30^c	$p < 0.001$
UA (μM)	318 ± 74	305 ± 68	300 ± 86	N.S.

Table 2
The effect of ibogaine on membrane fluidity using 12-DS. Order parameter (*S*) is inversely proportional to membrane fluidity. Results are expressed as mean ± SD (n=4). Statistical significance was tested by one way ANOVA and post-hoc Tukey's HSD test between all tested samples. There are no statistically significant differences between samples.

Samples	Controls	Ibogaine 10 μM	Ibogaine 20 μM
12-doxyl stearate (*S*)	48.2 ± 12.1	40.8 ± 4.8	39.7 ± 4.9

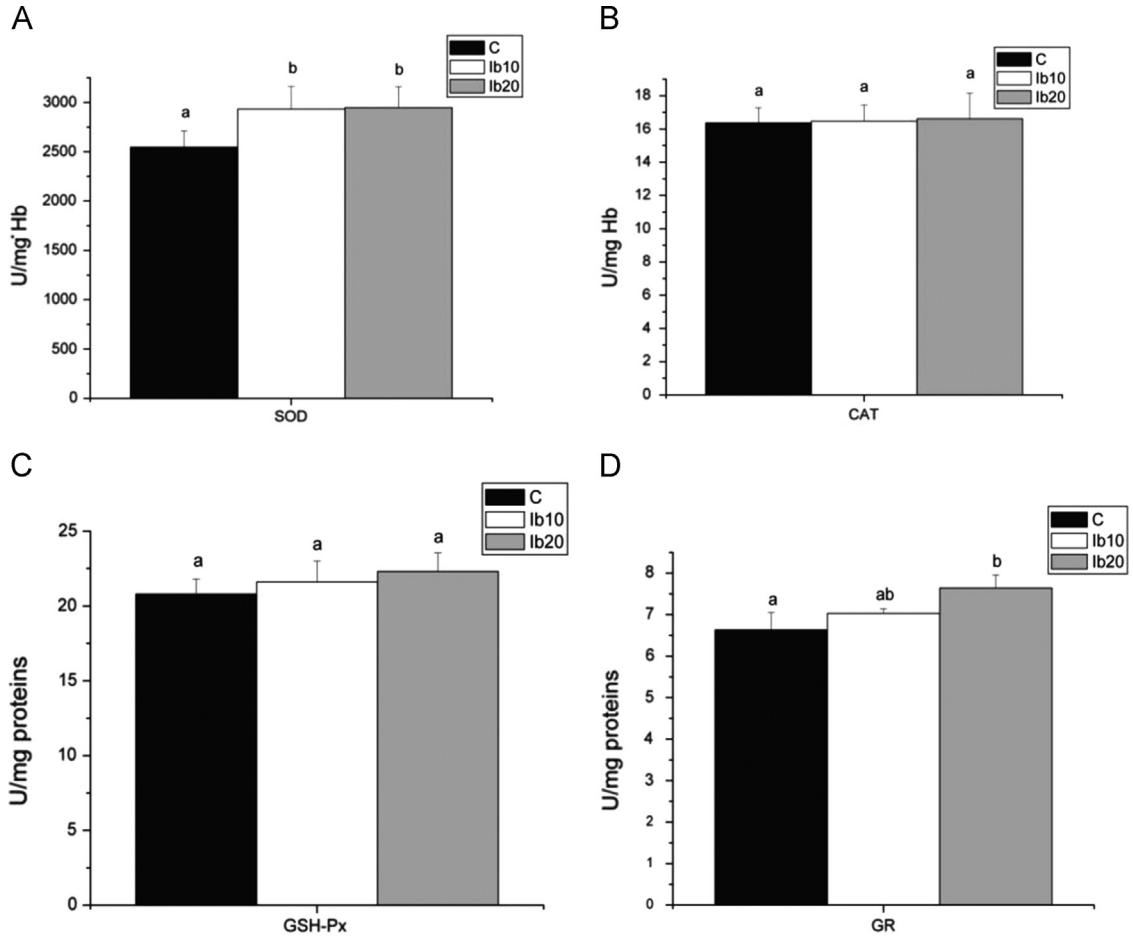

Fig. 2. Antioxidant enzymes activity in erythrocytes of control blood samples incubated in vitro with ibogaine (10 and 20 μM final concentration) for 1 h at 37 °C. Results are expressed as mean ± SE. Significance between groups was tested by Repeated Measures analysis of variance (ANOVA) and Univariate Tests of Hypotheses for Within Subject Effects. Different letter at superscript means statistical significance ($p < 0.05$) between groups.

Table 3
SOD activity in erythrocytes of control blood samples incubated in vitro with ibogaine (10 and 20 μM final concentration) for 1 h. Results are expressed as mean ± SE. Significance between groups was tested by Repeated Measures analysis of variance (ANOVA) and Univariate Tests of Hypotheses for Within Subject Effects. Different letters in superscript represents statistically significant differences between samples (a vs. b, $p < 0.001$; a vs. ab, non-significant).

	Controls (C)	C+ibogaine (10 μM)	C+ibogaine (20 μM)	ANOVA
SOD	2545 ± 155^{a}	2932 ± 218^{b}	2947 ± 203^{b}	$p < 0.001$
CAT	16.4 ± 1	16.5 ± 0.9	16.6 ± 1.5	NS
GSH-Px	21 ± 1	21.2 ± 1.2	22.3 ± 1.3	NS
GR	6.6 ± 0.4^{a}	7 ± 0.3^{ab}	7.6 ± 0.3^{b}	$P < 0.01$

To test ibogain ability to affect membrane fluidity, characteristic EPR spectra of erythrocytes labeled with 5-DS and 12-doxyl stearate without or with ibogaine were recorded (Fig. 1, Table 2). According to *S* order parameter (order parameter (*S*) is inversely proportional to membrane fluidity), ibogaine at doses of 10 and 20 μM did not influence membrane fluidity (Table 2). At dose of 32.2 μM (10 mg/L) ibogaine slightly elevated membrane fluidity, but without statistical significance (data not shown). However, *S* order parameter was significantly lower ($p < 0.05$) in erythrocytes membrane incubated with ibogaine at dose of 322 μM (100 mg/L) which means that ibogaine increases fluidity, but at doses rather high than therapeutic (Fig. 1).

Results showed that blood incubation for 1 h at 37 °C with ibogaine with both doses 10 and 20 μM in vitro elevated SOD activity in erythrocytes and GR activity, but statistically significant only at dose of 20 μM (Fig. 2, Table 3). The elevation of SOD activity was recorded in every individual samples and ranged from 9% to 40% for 10 μM dose and from 4% up to the 59% for 20 μM dose (Fig. 3; the norm of the reaction for every sample is shown) with high and significant coefficient of regression ($r = 0.876$, $p < 0.01$ and $r = 0.766$, $p < 0.05$, respectively). The norm of reaction for each GR activity sample (Fig. 4) shows the increase of GR activity at 20 μM treatment ranged from 8% to 39%. However, there were no statistical significant changes in CAT and GSHPx activities after ibogaine incubation with both doses. Furthermore, individual differences in measured samples were inconsistent, without any significant correlation (the norms of reaction for individual samples were not shown).

Electrophoretic profiles of SOD activity from RBC and partially purified erythrocytes by a Tsuchihashi method showed visible ibogain effects on SOD per se (Fig. 5, line 3 vs. 5, and line 7 vs. 9). Tsuchihashi method removes hemoglobin and a large quantity of proteins from erythrocytes and remaining part is purified SOD. On the other hand, the results showed that blood in vitro incubation with H_2O_2 decreased SOD activity in both RBC controls and partially purified cells with the Tsuchihashi method (Fig. 5, line 1 vs. 2, line 3 vs. 4, and line 7 vs. 8). Incubation of both treated RBC (controls) and partially purified with the Tsuchihashi method with ibogaine in vitro increased SOD resistance to hydrogen peroxide inhibition (line 4 vs. 6; line 8 vs. 10). Also, the results indicate that the Tsuchihashi method significantly decreased SOD resistance comparing to the controls (line 6 vs. 10).

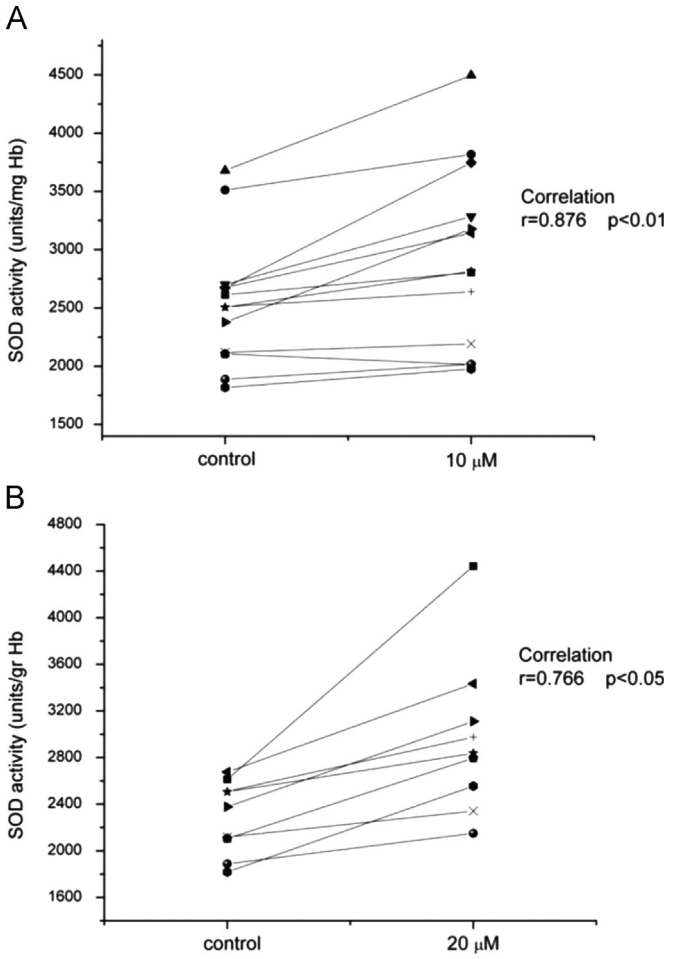

Fig. 3. SOD activity in individual blood samples after in vitro incubation with ibogaine A) 10 μM and B) 20 μM for 1 h at 37 °C. Correlation analysis showed significant positive correlation between activities before and after in vitro incubation with ibogaine 10 μM ($r=0.876$; $p<0.01$), and 20 μM ($r=0.766$; $p<0.05$).

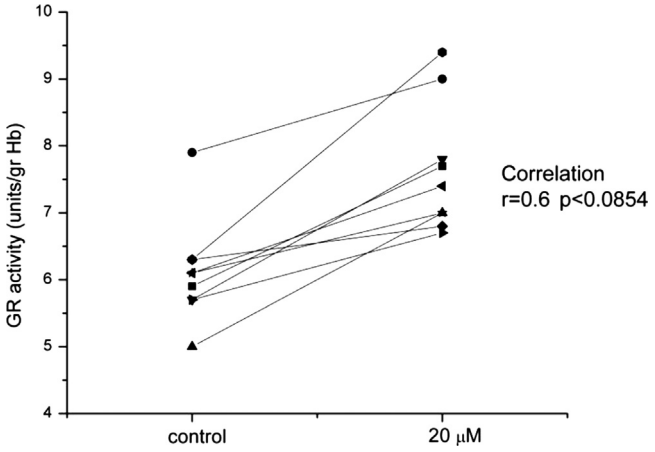

Fig. 4. GR activity in individual blood samples before and after in vitro incubation with ibogaine (20 μM) for 1 h at 37 °C. Correlation analysis showed positive correlation between activities before and after in vitro incubation with ibogaine 20 μM ($r=0.6$; $p<0.085$).

4. Discussion

Previous results already indicated that a part of the effects of ibogaine were mediated via energy and antioxidative metabolism. The main effect of ibogaine action appears to be an increase of ATP consumption, ATP release from cellular pool and subsequent induction

Fig. 5. SOD1 electrophoresis gels (light bands indicate SOD1 activity): 1. RBC Controls; 2 RBC Controls+H_2O_2 (5 mM); 3. RBC Controls in vitro incubation – 1 h; 4. RBC Controls+H_2O_2 (5 mM) in vitro incubation – 1 h; 5. RBC Controls+ibogaine (10 μM) in vitro incubation – 1 h; 6. RBC Controls+ibogaine (10 μM)+H_2O_2 (5 mM) in vitro incubation – 1 h; 7. Tsuchihashi method SOD in vitro incubation – 1 h; 8. Tsuchihashi method SOD+H_2O_2 (5 mM) in vitro incubation – 1 h; 9. Tsuchihashi method SOD+ibogaine (10 μM) in vitro incubation – 1 h; 10. Tsuchihashi method SOD+ibogaine (10 μM)+H_2O_2 (5 mM) in vitro incubation – 1 h.

of its synthesis (Paškulin et al., 2006, 2010, 2012). The exact mechanism of such effect is still unknown, but the effects were rather fast and ATP consumption occured in minutes after ibogaine application on baker yeast cells. Herein, we measured the concentration of ATP in blood plasma after 10 min of incubation with ibogaine and recorded an increase of ATP in the blood plasma. This increase happened without changes in UA concentration suggesting that elevated ATP levels in plasma were not followed by increased ATP decomposition in our experimental model. Erythrocytes have several membrane bound ATP pools (Chua et al., 2012). The releasing of ATP from erythrocytes to extracellular space is one of the postulated mechanisms of signal transduction adapting oxygen supply with local tissue oxygen demand within the erythrocyte and microvessels (Ellsworth et al., 2009). Controlled ATP release occurs in response to both physiological and pharmacological stimuli, hypoxia and hypercapnia (Bergfeld and Forrester, 1992), reductions in oxygen tension (González-Alonso et al., 2002), and even mechanical deformation (Sprague et al., 2001) and the amount of ATP released is influenced by the magnitude of the stimulus. However, since whole blood was incubated with ibogaine, other blood cell types could also produce pleiotropic substances and induce or modulate ibogaine effects, which could be mitochondrial mediated.

Our results suggest that ATP release that is provoked by ibogaine in concentrations applied in this and previous experiments (10 and 20 μM) is not related to changes in erythrocytes membrane fluidity. Ibogaine as a lipophilic molecule freely enters the cell (Paškulin et al., 2012), so doses applied in our experiment could influence membrane ATP pools, but not through changes in membrane fluidity. Our results show that ibogain influences membrane fluidity only in very high, clinically irrelevant doses (322 μM i.e. 100 mg/L).

ATP release and consumption trigger its rapid resynthesis in erythrocytes by increased glycolysis and pentose phosphate pathway (PPP) generating also more NADPH. On the other hand, elevated lactate led to elevated dissociation of oxygen from hemoglobin providing more oxidative environment. ATP release also happens when hemoglobin intensively dissolves oxygen, and feedback mechanism for ATP release attenuation seems to be mediated by elevation of NO (Olearczyk et al., 2004). NO modulates glycolysis in erythrocytes by nitrosoglutathione (GSNO) fastening reestablishment of ATP pool and thus providing more NADPH (Galli et al., 2002). Elevated level of NADPH in erythrocytes is favorable cell environment for the elevation of GR activity (Deponte, 2013). Elevated GR activity found in our experiment seems to be compensatory to increased NADPH providing conservation of functional thiol groups and the physiological attempt to preserve intracellular redox homeostasis. Our results showed that GR activity in erythrocytes is higher after incubation of blood with ibogaine, but this increase is statisticaly significant only when 20 μM dose was applied suggesting concentration related effect. These suggest that ibogaine also provocates sequences of different cellular events that lead to changed redox potential of cells.

Our results demonstrated that treatment with ibogaine (both 10 and 20 μM), even in erythrocytes where mitochondrial apparatus and nucleus with protein synthesis system are absent, elevated SOD1 activity. The elevation is not so high in the order of magnitude, but is highly significant ($p < 0.001$), and very correlated between individual samples ($r = 0.876$, $p < 0.01$), approved by correlation analysis. Furthermore, the analysis of SOD1 activity on electrophoretic profiles showed that ibogain treatment stabilize SOD1 molecule against H_2O_2 mediated inhibition of SOD1 activity and the disruption of its structure (no presence of extra bands). It is already known that the exposure of SOD1 to micromolar concentration of H_2O_2 lead to inhibition of SOD1 activity, partial sequestration of the molecule and the formation of an extra band upon electrophoresis SOD1 profile (Hodgson and Fridovich, 1975; Mavelli et al., 1983). This effect is postulated as feedback mechanism of the control of SOD1 activity by its product (Bray et al., 1974). In our work, SOD1 from blood incubated with ibogaine ex vivo showed (according to electrophoretic profile) stabile both the structure and the activity upon exposure to H_2O_2. There are data on serotonin and dopamine transporters where ibogaine as a pharmacochaperon binds directly to the transporter in an inward-open conformation and stabilize the molecule (Bulling et al., 2012). To show possible direct effects of ibogaine to sole SOD1 molecules, SOD1 was separated from non-treated blood (according to the Tsuchihashi method) and practically isolated. Isolated SOD1 molecules were incubated with ibogaine in vitro at the same dose and incubation time as RBC. Isolated SOD1 molecules showed, again, elevated resistance to H_2O_2 according to electrophoretic profile. It seems that ibogaine somehow stabilize SOD1 structure and retain its activity. The mechanism of this action is unknown. It can be attributed to direct effect on SOD1 molecule, but also to indirect physiological influence. There is data that physiologicaly active redox molecules can increase SOD activity (Sun et al., 2012). Increased SOD1 activity removes faster superoxide in cells decreasing possibility of further oxidative process and ROS generation, but generates hydrogen peroxide (H_2O_2) that is decomposed by CAT and GPx. On the other hand, superoxide produces with NO toxic peroxynitrite (Koppenol et al., 1992), and thus, (1) proceeds to subsequent cellular toxicity and (2) diminishes NO physiological activity. Superoxide, H_2O_2, and peroxynitrite are known inhibitors of glyceraldehyde-3-phosphate dehydrogenase (Hyslop et al., 1988; Souza and Radi, 1998). Elevated SOD1 activity (and increased superoxide removal) can be attributes to the stabilization of the feedback mechanism and the conservation of physiological NO/ATP mediated signal transduction mechanism in erythrocytes.

Our results showed that transient and fast ibogaine mediated energy depletion led to later increase of antioxidant defense, especially SOD1. The antioxidant defense is closely related to energy metabolism, since energy production and respiration are source for ROS. While free radicals are undesired side product of energy production, their elimination at the same time consumes energy by utilization of reduced molecules that would otherwise serve as an energy source. Besides and even more, oxidative demage of biomolecules demands their repair i.e. replacement, which is even higher energy expenditure. So, pro-antioxidant economizing on this level liberates some energy, which is used in above mentioned indications for traditional use – stimulant, renewal of exhausted tissues after disease, detoxication, or mental shifts on psychonautic expeditions.

Taken together, our results prove that important part of the effects of ibogaine is mediated through its influence on energy metabolism, redox active processes and the effects of discrete fluctuations of individual reactive oxygen species on different levels of enzyme activities. Overall, ibogaine acts as a pro-antioxidant by increasing activity of antioxidative enzymes and as an adaptogene in oxidative distress.

Acknowledgments

This work was supported by a grant from the Ministry of Science and Technological Development of the Republic of Serbia, Project no: 173014 “Molecular mechanisms of redox signaling in homeostasis: adaptation and pathology”, University of Belgrade.

References

Ajdžanović, V., Spasojević, I., Sošić-Jurjević, B., Filipović, B., Trifunović, S., Sekulić, M., Milošević, V., 2011. The negative effect of soy extract on erythrocyte membrane fluidity: an electron paramagnetic resonance study. Journal of Membrane Biology 239 (3), 131–135.

Alper, K.R., 2001. Ibogaine: A Review. Academic Press, San Diego, California.

Beauchamp, C., Fridovich, I., 1971. Superoxide dismutase: improved assays and an assay applicable to acrylamide gels. Analytical Biochemistry 44, 276–287.

Bergfeld, G.R., Forrester, T., 1992. Release of ATP from human erythrocytes in response to a brief period of hypoxia and hypercapnia. Cardiovascular Research 26, 40–47.

Beutler, E., 1982. Red Cell Metabolism – a Manuel of Biochemical Methods, third ed. Grune and Stratton, New York, NY.

Bray, R.C., Cockle, S.A., Fielden, E.M., Roberts, P.B., Rotilio, G., Calabrese, L., 1974. Reduction and inactivation of superoxide dismutase by hydrogen peroxide. Biochemical Journal 139 (1), 43–48.

Bulling, S., Schicker, K., Zhang, Y.W., Steinkellner, T., Stockner, T., Gruber, C.W., Boehm, S., Freissmuth, M., Rudnick, G., Sitte, H.H., Sandtner, W., 2012. The mechanistic basis for noncompetitive ibogaine inhibition of serotonin and dopamine transporters. The Journal of Biological Chemistry 287 (22), 18524–18534.

Chua, H., Puchulu-Campanellaa, E., Galanb, J.A., Taob, W.A., Lowa, P.S., Hoffman, J.F., 2012. Identification of cytoskeletal elements enclosing the ATP pools that fuel human red blood cell membrane cation pumps. Proceedings of the National Academy of Science 109 (31), 12794–12799.

Coade, S.B., Pearson, J.D., 1989. Metabolism of adenine nucleotides in human blood. Circulation Research 65, 531–537.

Deponte, M., 2013. Glutathione catalysis and the reaction mechanisms of glutathione-dependent enzymes. Biochimica et Biophysica Acta 1830, 3217–3266.

Drabkin, D.L., Austin, J.H., 1935. Spectrophotometric studies. II. Preparations from washed blood cells: nitric oxide hemoglobin and sulfhemoglobin. The Journal of Biological Chemistry 112, 51–55.

Ellsworth, M.L., Ellis, C.G., Goldman, D., Stephenson, A.H., Dietrich, H.H., Sprague, R.S., 2009. Erythrocytes: oxygen sensors and modulators of vascular tone. Physiology 24, 107–116.

Fernandez, J.W., 1982. Bwiti: an ethnography of the religious imagination in Africa. Princeton University Press, Princeton.

Fernandez, J.W., Fernandez, R.L., 2001. “Returning to the path”: the use of iboga[ine] in an equatorial African ritual context and the binding of time, space, and social relationships. The Alkaloids Chemistry and Biology 56, 235–247.

Galli, F., Rossi, R., Di Simplicio, P., Floridi, A., Canestrari, F., 2002. Protein thiols and glutathione influence the nitric oxide-dependent regulation of the red blood cell metabolism. Nitric Oxide: Biology and Chemistry 6 (2), 186–199.

Glatzle, D., Vuilleumie, J.P., Weber, F., Decker, K., 1974. Glutathione reductase test with whole blood a convenient procedure for the assessment of the riboflavin status in humans. Experientia 30, 665–668.

Gorman, M.W., Feigl, E.O., Buffington, C.W., 2007. Human plasma ATP concentration. Clinical Chemistry 53 (2), 318–325.

González-Alonso, J., Olsen, D.B., Saltin, B., 2002. Erythrocyte and the regulation of human skeletal muscle blood flow and oxygen delivery: role of circulating ATP. Circulation Research. 91, 1046–1055.

Hinkle, E.D., Wiersma, W., Jurs, G.S., 2002. Applied Statistics for Behavioral Sciences. Houghton Mifflin Company, Boston (fifth ed.).

Hodgson, E.K., Fridovich, I., 1975. The interaction of bovine erythrocyte superoxide dismutase with hydrogen peroxide: chemiluminescence and peroxidation. Biochemistry. 14 (24), 5299–5303.

Hyslop, P.A., Hinshaw, D.B., Halsey, W.A., Schraufstatter, I.U., Sauerheber, R.D., Spragg, R.G., Jackson, J.H., Cochrane, C.G., 1988. Journal of Biological Chemistry 263, 1665–1675.

Koppenol, W.H., Moreno, J.J., Pryor, W.A., Ischiropoulos, H., Beckman, J.S., 1992. Peroxynitrite, a cloaked oxidant formed by nitric oxide and superoxide. Chemical Research in Toxicology 5, 834–842.

Laemmli, U.K., 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 7, 680–685.

Mash, D.C., Kovera, C.A., Pablo, J., Tyndale, R., Ervin, F.D., Williams, I.C., Singleton, E.G., Mayor, M., 2000. Ibogaine: complex pharmacokinetics, concerns for safety, and preliminary efficacy measures. Annals of the New York Academy for Sciences 914, 394–401.

Mavelli, I., Ciriolo, M.R., Rotilio, G., 1983. Multiple electrophoretic variants of Cu, Zn superoxide dismutase as expression of the enzyme aging. Effects of H_2O_2, ascorbate and metal ions. Biochemical and Biophysical Research Communications 117 (3), 677–681.

Misra, H.P., Fridovich, I., 1972. The role of superoxide anion in the autoxidation of epinephrine and a simple assay for superoxide dismutase. The Journal of Biological Chemistry 247 (10), 3170–3175.

Naranjo, C., 1969. Psychotherapeutic possibilities of new fantasy-enhancing drugs. Clinical Toxicology 2, 209–224.
Nikolić-Kokić, A., Blagojević, D., Spasić, M.B., 2010. Complexity of free radical metabolism in human erythrocytes. Journal of Medical Biochemistry 29, 189–195.
Olearczyk, J.J., Ellsworth, M.L., Stephenson, A.H., Lonigro, A.J., Sprague, R.S., 2004. Nitric oxide inhibits ATP release from erythrocytes. Journal of Pharmacology and Experimental Therapeutics 309, 1079–1084.
Paglia, D.E., Valentine, W.N., 1967. Studies on the quantitative and qualitative characterization of erythrocyte glutathione peroxidase. Journal of Laboratory and Clinical Medicine 70, 74–77.
Paškulin, R., Jamnik, P., Danevčič, T., Koželj, G., Krašovec, R., Krstić-Milosević, D., Blagojević, D., Štrukelj, B., 2012. Metabolic plasticity and the energy economizing effect of ibogaine, the principal alkaloid of *Tabernanthe iboga*. Journal of Ethnopharmacology 143, 319–324.
Paškulin, R., Jamnik, P., Obermajer, N., Slavić, M., Štrukelj, B., 2010. Induction of energy metabolism related enzymes in yeast Saccharomyces cerevisiae exposed to ibogaine is adaptation to acute decrease in ATP energy pool. European Journal of Pharmacology 627, 131–135.
Paškulin, R., Jamnik, P., Živin, M., Raspor, P., Štrukelj, B., 2006. Ibogaine affects brain energy metabolism. European Journal of Pharmacology 552 (1-3), 11.
Plagemann, P.G.W., Wohlhueter, R.M., Kraupp, M., 1985. Adenine nucleotide metabolism and nucleoside transport in human erythrocytes under ATP depletion conditions. Biochimica et Biophysica Acta 817, 51–60.
Schultes, R.E., 1970. The plant kingdom and hallucinogens. Bulletin on Narcotics 22, 25–51.
Schultes, R.E., Hofmann, A., Ratsch, C., 2001. Plants of the gods: their sacred, healing and hallucinogenic powers. Healing Art Press, Rochester.
Souza, J.M., Radi, R., 1998. Glyceraldehyde-3-phosphate dehydrogenase inactivation by peroxynitrite. Archives of Biochemistry and Biophysics 360, 187–194.
Sprague, R.S., Ellsworth, M.L., Stephenson, A.H., Lonigro, A.J., 2001. Participation of cAMP in a signaltransduction pathway relating erythrocyte deformation to ATP release. American Journal of Physiology: Cell Physiology 281, C1158–C1164.
Sun, W.H., Liu, F., Chen, Y., Zhu, Y.C., 2012. Hydrogen sulfide decreases the levels of ROS by inhibiting mitochondrial complex IV and increasing SOD activities in cardiomyocytes under ischemia/reperfusion. Biochemical and Biophysical Research Communications 421, 164–169.
Tsuchihashi, M., 1923. Zur Kenntnis der Blutkatalase. Biochemistry. 140, 65–74.