Vol.64: e21200773, 2021
https://doi.org/10.1590/1678-4324-2021200773
ISSN 1678-4324 Online Edition

BABT
BRAZILIAN ARCHIVES OF BIOLOGY AND TECHNOLOGY

Article - Biological and Applied Sciences

Ex Vivo Effect of Ibogaine on the Transcriptional Level of Antioxidant Defense Related Genes in Honey Bee (*Apis mellifera*, L.) Midgut

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Editor-in-Chief: Alexandre Rasi Aoki
Associate Editor: Najeh Maissar Khalil

Received: 2020.12.08; Accepted: 2021.07.21.

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HIGHLIGHTS

- The ibogain action has not been completely clarified.
- Honey bee midgut is suitable model system for testing the antioxidative mechanisms.
- The *ex vivo* ibogaine treatment induced an up-regulation of *Sod1* gene.
- Ibogaine shows pro-antioxidant action.

Abstract: The aim of the present study was to analyze the mechanisms of ibogaine action by measuring its *ex vivo* effects on antioxidant defense in the honey bee (*Apis mellifera*, L.) midgut. The transcriptional levels of selected genes: Cu/Zn dependent and Mn dependent superoxide dismutases (*Sod1* and *Sod2*, respectively), catalase (*Cat*) and transcription factor Nrf2 (*Nrf2*) were determined. The applicability of midgut tissue, which expected to have well developed antioxidant protection system, for this type of analysis was confirmed by testing cell viability and response to paraquat, an effective inducer of oxidative stress, *ex vivo*. Incubation for 2 h with paraquat (10 µg/mL) induced a significant increase in expression of *Sod1* and *Cat* genes. The results of ibogaine treatment showed that exposure to 5 µg/mL and 10 µg/mL of ibogaine for 2 h induced significant increase in expression of *Sod1* gene. On the other hand, ibogaine did not lead to a significant increase of *Sod2*, *Cat* and transcription factor *Nrf2* genes expression in honey bee midgut *ex vivo*. Our results confirmed positive effect of ibogaine on the antioxidant protective system and its pro-antioxidant action.

Keywords: Ibogaine; Paraquat; Insect midgut; Superoxide dismutase; Catalase.

INTRODUCTION

Ibogaine is a naturally occurring indole alkaloid, derived from the bark of the root of the West African *Tabernanthe iboga* plant, with psychotropic and metabotropic effects which influences many processes in the body. In West Central Africa, low dosages of its extracts have been employed by indigenous people against fatigue, hunger and thirst. Higher dosages of this indole alkaloid are used by the tribes of Kongo for different ritual purposes during religious ceremonies. Beside traditional use (its stimulant effect), in the last four decades, the "urban" use of iboga root bark, iboga extract or pure ibogaine is on the rise, as promising anti-addiction therapy against opiates, stimulants, alcohol, nicotine and pharmacological drugs [1-3], owing to its anti-depressive, anti-epileptic and stimulant properties. However, the use of this alkaloid in medicine is questionable, as there was a period with alarming reports with life-threatening complications and sudden death cases following ibogaine application [4].

Considering the assumptions about the medicinal effect of ibogaine, numerous studies have been done to clarify the mechanism of its action. The induction of energy related enzymes in rat brain as a consequence of ibogaine administration was shown [5]. Afterword, similar results of ibogaine's influence on energy metabolism cluster as well as Cu/Zn dependent superoxide dismutase (SOD1) enzymes in yeast *Sacharomyces cerevisiae* were demonstrated, while the changes in ATP pool showed its transient reduction in dose dependent manner [6]. Transient oxidative energy metabolism acceleration was directly confirmed by increased CO_2 production in yeast after ibogaine exposure [3] followed by intense higher production of reactive oxygen species (ROS). However, reduction in oxidative load was reported suggesting its influence on the enzymes of the antioxidative defense system, especially SOD1, in a pro-antioxidant manner and its indirect influence on oxidative stress reduction [3]. The association of ibogaine with redox status in the cell was confirmed with experiment on human erythrocytes [7], where induction of SOD1 was found.

The aim of the present study was to analyze the mechanisms of ibogaine actions, by measuring *ex vivo* effects of ibogaine on antioxidant defense in the honey bee (*Apis mellifera*, L.) midgut. The transcriptional levels of selected genes: Cu/Zn dependent and Mn dependent superoxide dismutases (*Sod1* and *Sod2*, respectively), catalase (*Cat*) and transcription factor Nrf2 (*Nrf2*) were analyzed. The midgut tissue was chosen because it was expected to have well developed antioxidant protection system. Applicability of midgut tissue for this type of analysis was confirmed by testing cell viability and response to paraquat - oxidative stress inducer, *ex vivo*.

MATERIAL AND METHODS

Honey bee midgut tissue isolation

In the present study honey bee midgut cells in primary tissue culture (*ex vivo*) were analyzed. All honey bee workers (*A. mellifera*, L.) were collected in summer 2019. from the beehive kept in the backyard of the Department of Biology and Ecology, Faculty of Sciences, Novi Sad. Honey bee workers were cold anesthetized by placing the cups on ice, and transferred one at a time onto a glass Petri dish filled with melted paraffin wax, colored by Sudan black, where the isolation was performed under a light magnifying glass. Using fine forceps, the insect head was first cut off and the abdomen was pulled from the posterior end until the midgut detaches. The isolated midguts were then rinsed and collected in standard insect saline (135 mM NaCl, 5 mM KCl, 10 mM $MgCl_2$, 1.6 mM $CaCl_2$, 65 mM Tris–HCl buffer at pH 7) [8]. Thereafter, the midgut samples were cultivated in the 1.5 mL Eppendorf tubes containing DMEM/F12 medium (Sigma-Aldrich) (90 µL DMEM/F12+10 µL insect saline) enriched by 100 IU/mL penicillin and 100 µg/mL streptomycin, shaking at 27°C for 2 h, 4 h and overnight.

Viability testing

The viability of midgut cells in primary culture cultivated in 100 µL medium (90 µL DMEM/F12+10 µL insect saline) for 2 h, 4 h and overnight, was determined by colorimetric assay using MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide). The viability of midgut cells treated with paraquat and ibogaine (separately) for 2 h was also determined. After cultivation, 10 µL of MTT (5 mg/mL) solution was added and the incubation was continued at 37°C for 1 h. The medium was discarded, 100 µL acidic isopropanol (0.04 M HCl in isopropanol) was added and left on the room temperature for 5 min. The absorbance was determined on 540 nm and 690 nm (referent). The results were expressed as percentage of absorbance of freshly isolated tissue. The analyses were done in triplicate.

Paraquat treatment

Oxidative stress was induced by paraquat, a common, broad-spectrum herbicide which was shown to cause intense oxidative stress in honey bees [9]. The midgut tissue samples were treated by paraquat (90 µL DMEM/F12+10 µL paraquat, 0.01 mg/mL, final conc.) during 1 h and 2 h shaking at 27ºC. After the treatment the medium was discarded and samples were frozen at -70ºC until analysis. The midgut cultivated in DMEM/F12 medium for 2 h was used as control. The analyses were done in triplicate.

Ibogaine treatment

In this study the midgut tissue samples were treated by two different concentrations of ibogaine [7] - 10 µg/mL (90 µL DMEM/F12+10 µL ibogaine 100 µg/mL) and 5 µg/mL (90 µL DMEM/F12+10 µL ibogaine 50 µg/mL) for 2 h shaking at 27ºC. After the treatment the medium was discarded and samples were frozen at -70ºC until analysis. The midgut cultivated in DMEM/F12 medium for 2 h was used as a control sample. The analyses were done in triplicate.

Total RNA extraction, cDNA synthesis and qPCR

Total RNA was isolated from frozen midgut samples using TRIreagent (Sigma-Aldrich), following manufacturer protocol. The concentration and purity of RNA samples were estimated with BioSpec-nano spectrophotometer (Shimatzu, Kyoto, Japan), while integrity of total RNA was verified on 1% agarose gel. Synthesis of cDNA was carried out using the QuantiTect Reverse Transcription Kit (Qiagen, Valencia, CA, USA) according to manufacturer's protocol starting with 1 µg of total RNA and obtained solutions were stored at −20°C.

Relative gene expression was determined in midgut samples treated with paraquat and ibogaine, compared to control cultivated in DMEM/F12 medium. The expression of four genes of antioxidative system was measured, using two reference genes, with gene specific primers (Table 1). Primers for transcription factor Nrf2 were designed using the NCBI PrimerBlast (http://www.ncbi.nlm.nih.gov/tools/primer-blast). Quantitative PCR on the cDNA products was carried out in 96-well plates using MasterCycler RealPlex4 (Eppendorf) in final volume of 14 µL. Reaction included 7 µL of 2X Power SYBR® Green PCR Master Mix (Applied Biosystems), 200 nM of each primer and 50 ng cDNA. Each sample was run as a duplicate. Amplification program consisted in an initial preincubation step at 95°C (10 min) and 40 cycles of 95°C (15 s) and 60°C (1 min). Melting curves were recorded to confirm amplification of a single gene product.

Table 1. Primer sequences used for determination of relative gene expression by qPCR

Gene	Forward primer	Reverse primer
Actin, *Act* (reference) [10]	ATGCCAACACTGTCCTTTCTGG	GACCCACCAATCCATACGGA
Ribosomal protein, *Rps5* (reference) [10]	AATTATTTGGTCGCTGGAATTG	TAACGTCCAGCAGAATGTGGTA
Cu, Zn- superoxide dismutase, *Sod1* [11]	AGCAGATGCAAGTGGTGTTG	GAGCACCAGCATTTCCTGTAG
Mn-superoxide dismutase, *Sod2* [12]	GTCGCCAAAGGTGATGTCAATAC	CGTCTGGTTTACCGCCATTTG
Catalase, *Cat* [11]	GGCGGCTGAATTAAGTGCTA	TTGCGTTGTGTTGGAGTCAT
Transcription factor, *Nrf2*	ATCTCTCCCAGGAAGGTGTGCT	GCGACAAGCGCCAAGTACCTC

Data analysis

The relative gene expression was calculated using REST 2009 (Relative Expression Software Tool) (Qiagen, Hilden, Germany), where relative up- or downregulations were calculated and tested for statistical significance by the integrated Bootstrap randomization test (2,000 iterations) for $P<0.05$ significance level.

RESULTS

Viability testing

The modified MTT assay, on honey bee workers midgut tissues, cultivated in DMEM/F12 medium for 2 h, 4 h and overnight, was used to determine the viability of midgut tissue cells in primary culture in comparison with the freshly isolated tissues. The percentage of survival was high after 2 h and 4 h of cultivation, nearly

100%, while overnight cultivation reduced the survival approximately to 10%. A high survival rate, nearly 90%, was observed after 2 h cultivation with paraquat or ibogaine.

Paraquat treatment

The expression of some antioxidant-related genes in the midgut samples treated by paraquat for 1 h and 2 h, is shown on Figure 1. The *ex vivo* treatment by paraquat (10µg/mL) for 2 h induced significant increase in the expression of *Sod1* and *Cat* genes compared to non-treated tissues while did not induce significant change in the expression of transcription factor *Nrf2* gene (Figure 1).

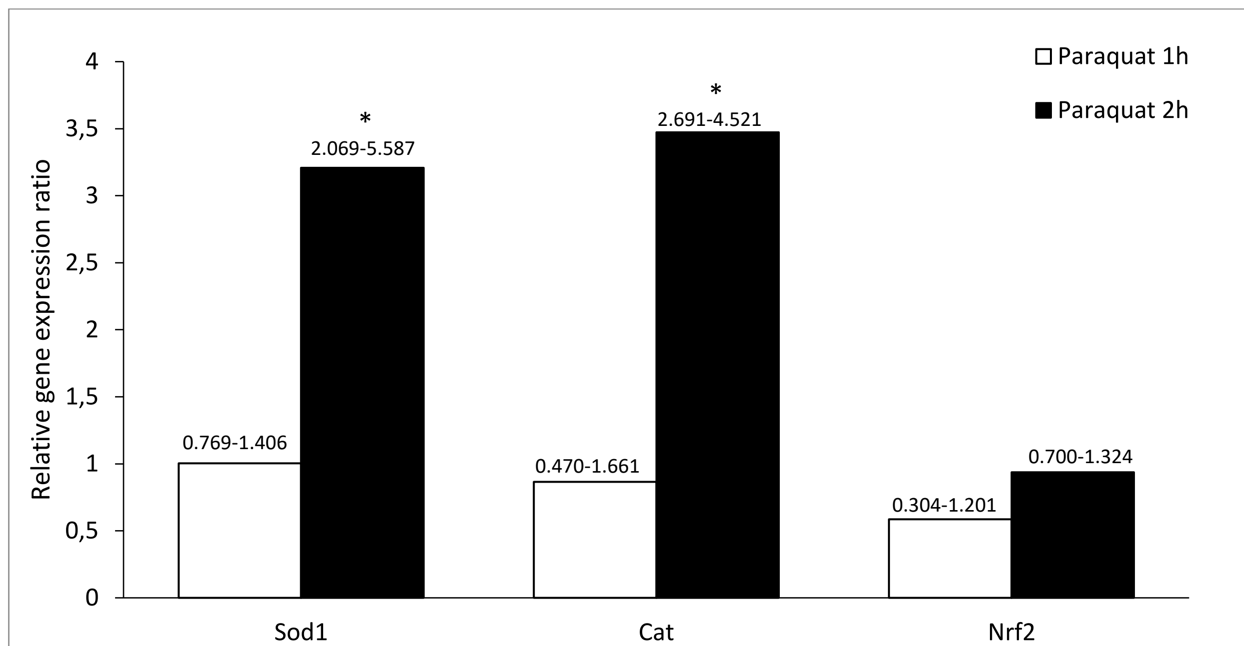

Figure 1. The relative expression of the antioxidant-related genes: Cu/Zn dependent superoxide dismutase (*Sod1*), catalase (*Cat*) and transcription factor (*Nrf2*) in the midgut samples treated by paraquat (10µg/mL) for 1 h and 2 h compared to non-treated tissues (the relative expression=1). The statistically significant difference ($P \leq 0.05$) compared to the control group is indicated with asterisk (*). The range for the standard error for 68% C.I. (confidence interval) is presented above the bars.

Ibogaine treatment

The expression of some antioxidant-related genes in the midgut samples treated *ex vivo* by two different concentrations of ibogaine (5 µg/mL and 10 µg/mL) for 2 h, is shown on Figure 2. Both ibogain concentrations induced a significant increase in the expression of *Sod1* gene compared to non-treated midgut tissues (Figure 2). The expression of other analyzed genes did not change significantly even after the treatment with higher concentration of ibogaine, for 2 h.

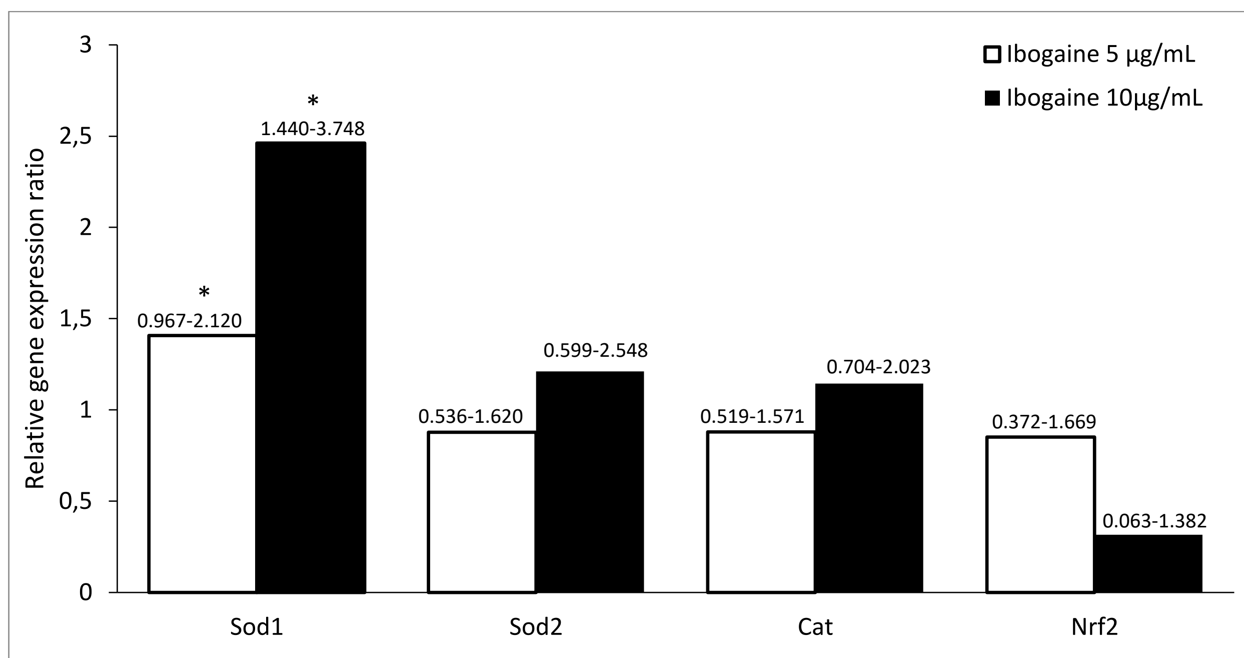

Figure 2. The relative expression of the antioxidant-related genes: Cu/Zn and Mn dependent superoxide dismutases (*Sod1* and *Sod2* respectively), catalase (*Cat*) and transcription factor (*Nrf2*) in the midgut samples treated by two different concentrations of ibogaine (5 µg/mL and 10 µg/mL) for 2 h compared to non-treated tissues (the relative expression=1). The statistically significant difference (P≤0.05) compared to the control group is indicated with asterisk (*). The range for the standard error for 68% C.I. (confidence interval) is presented above the bars.

DISCUSSION

Redox status is affected by balance between ROS generation and ROS elimination. The first line in antioxidative defense presents enzymes supeoxide dismutase and catalase that cooperatively dismutase superoxide radical and breakdown hydrogen peroxide. *Nrf2* is transcription factor that regulates the expression of *antioxidant* proteins that protect against oxidative damage [13]. In order to verify the applicability of the midgut cells in primary tissue culture, as model system, we tested cell viability and cultivated the tissue with paraquat the most widely used non-selective herbicide. Despite being considered non-toxic to slightly toxic to adult bees [14], paraquat has been proven to be effective inducer of oxidative stress in this species [9, 15]. Gut epithelial cells are of great importance for the insect health, involved in food digestion processes, but also as important component of innate immunity and local deference against microorganisms. Epithelial cells produce ROS to protect against ingested harmful pathogens [16], therefore, higher energy consumption and production is expected in midgut cell requiring developed antioxidant protection system [17]. In our experiment, incubation with paraquat (10µg/mL) for 2 h provoked reactions on the transcriptional level, a significant increase of the *Sod1* and *Cat* genes expression, as fundamentals mediators for ROS removal, while the expression of *Nrf2* gene did not change significantly. These results are in agreement with de Mattos and coauthors [15] who showed significant correlation of *Sod1* gene expression with the paraquat in honey bees, while *Cat* expression did not change in their study. The study about the effect of paraquat for 12 h in *Drosophila melanogaster* found its biphasic effect on the gene expression and enzyme activity of Sod1 and Cat with peak at 2.5 µM dose [18]. Our results confirmed paraquat as oxidative stress inducer, suggesting the honey bee midgut is suitable model system for testing the mechanism of various biological and chemical agents in terms of their impact on the free radical production and redox status generally.

As the goal of our study was to analyze the mechanisms of ibogaine action by measuring its *ex vivo* effects on antioxidant defense in the honey bee (*Apis mellifera*, L.) midgut, the transcriptional levels of selected genes: Cu/Zn dependent and Mn dependent superoxide dismutases (*Sod1* and *Sod2*, respectively), catalase (*Cat*) and transcription factor Nrf2 (*Nrf2*) were determined. The results showed that exposure to 5 µg/mL and 10 µg/mL ibogaine for 2 h induced an increase in expression of *Sod1* gene, which product is directly involved in antioxidant protection. It has been found that ibogaine greatly affects cellular energy, the existing redox state and the antioxidant capacity of the cell in dose- and time-dependent manner, by positively affecting the components of the antioxidant protective system and reducing oxidative stress. Our results are

in accordance with the result of Paškulin and coauthors [6], who observed the 2.2-fold induction of *Sod1* after the ibogaine treatment in yeast *S. cerevisiae*. Nikolić-Kokić and coauthors [7] measured the activity of SOD1, CAT, glutathione peroxidase (GSH-Px) and glutathione reductase (GR) activity in erythrocytes after 1 h of incubation with ibogaine. Applied doses in their study were the same as in our, and the results have shown that even in cells where there is no possibility of protein synthesis, ibogaine increased SOD1 activity by both doses, 10 and 20 μM, while treatment with 20 μM elevated GR activity as well. Furthermore, electrophoretic profiles revealed that incubation with ibogaine mitigates H_2O_2 mediated suppression of SOD1 activity [7]. Our results confirm ibogaine's impact on the antioxidant protective systems in pro-antioxidant manner [3] acting indirectly by regulation of the biosynthesis of antioxidant proteins [19, 20].

Comparison of mechanisms of action between paraquat and ibogaine showed that common mediator of cellular redox processes seems to be superoxide. But, paraquat itself is superoxide generating agent comparing to ibogaine that influence cellular energetic leads toward superoxide imbalance. Ibogaine glycogenolytic activity as well as elevation of SOD1 activity was also shown in different rat tissues [21, 22] suggesting some common cellular metabolic ibogaine mechanism(s) of action, but with different time scale and intensity that are species and tissue specific.

In our study, ibogaine did not lead to a statistically significant increase of *Sod2*, *Cat* and transcription factor *Nrf2* genes expression in honey bee midgut *ex vivo*. There is a possibility that short exposure time to ibogaine may result in changes only at the post-translational level.

CONCLUSION

Based on the results obtained in this study, it can be concluded that honey bee midgut used as model system could provide important information about ibogaine physiological and biochemical effects. Results showed the effect of ibogaine on redox status in cell by increasing the expression of the antioxidant enzyme *Sod1*.

Funding: This study was financially supported by the Ministry of Education, Science and Technological Development of the Republic of Serbia, Grants No. 451-03-68/2020-14/ 200125 and 451-03-68/2020-14/200007.
Acknowledgments: Authors are thankful to Prof. Dr. Silvana Andrić for valuable and constructive suggestions during the planning and development of this research work.
Conflicts of Interest: The authors declare that they have no competing interests.

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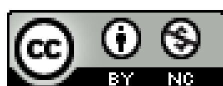

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