Scientia Africana, Vol. 23 (No. 2), April, 2024. Pp41-46
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DOSE-DEPENDENT REDUCTION OF RAT COLON ANTIOXIDANT ENZYME ACTIVITIES AND INCREASED THIOBARBITURIC ACID REACTIVE SUBSTANCES (TBARS) BY ETHIDIUM BROMIDE

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Received: 09-03-2023
Accepted: 29-02-2024

https://dx.doi.org/10.4314/sa.v23i2.4

Journal Homepage: http://www.scientia-african.uniportjournal.info
Publisher: *Faculty of Science, University of Port Harcourt.*

ABSTRACT

The effect of exposure to varying doses of ethidium bromide on the activities of rat colon antioxidant enzymes has been examined. The rats were divided into eight (08) experimental groups of 5 rats each. Group I rats served as the control, not exposed to ethidium bromide (EthBr). Group II rats were administered 5 mg ethidium bromide kg^{-1}body weight (bd wt),Group III 10 mg ethidium bromide kg^{-1} body weight, Group IV 20 mg ethidium bromide kg^{-1} body weight, Group V 40 mg ethidium bromide kg^{-1}body weight, Group VI 60 mg ethidium bromide kg^{-1}body weight, Group VII 80 mg ethidium bromide kg^{-1} body weight, while rats in Group VIII were administered 100 mg ethidium bromide kg^{-1} body weight. Treatment was done once weekly via gavage for 24 weeks. At the end of the exposure period, each rat was anaesthetized by halothane inhalation. Colon sections were collected, homogenized and the antioxidant enzyme activities in the homogenate supernatants were determined. Relative to the control, colon catalase, superoxide dismutase and glutathione peroxidase showed evidence of significant ($p \leq 0.05$) decrease in activity in rats exposed to ≥ 60, ≥ 20 and ≥ 60 mgethidium bromide kg^{-1}body weight respectively. Compared to the control, thiobarbituric acid reactive substance (TBARS), as measured by malondialdehyde (MDA) levels were significantly ($p \leq 0.05$) increased in rats exposed to ≥ 20 mg ethidium bromide kg^{-1} bd wt. There was a strong and significant negative correlation between ethidium bromide dose and colon catalase ($r = -0.9823$; $p < 0.001$), superoxide dismutase ($r = -0.9107$; $p<0.001$) and glutathione peroxidase ($r = -0.9772$; $p < 0.001$) activities. There was a strong and positive correlation between ethidium bromide dose and colon MDA levels ($r = + 0.9808$; $p < 0.001$) but a strong negative correlation between colon catalase ($r = - 0.9455$; $p < 0.001$), superoxide dismutase ($r = - 0.8707$; $p < 0.001$) and glutathione peroxidase ($r = - 0.9623$; $p < 0.001$) and MDA level. Chronic oral exposure of albino rats to ethidium bromide at a dose above the nontoxic threshold of 10 mg kg^{-1} body weight significantly impaired the activities of colon antioxidant enzymes.

INTRODUCTION

Ethidium bromide (Figure 1) is a phenanthridinium derivative used in biochemical research laboratories for visualizing DNA fragments separated in agarose gel by electrophoresis (Saeidniaand Abdollahi, 2013). Besides the fact that it is a very sensitive indicator, another important

reason for its popularity among molecular biologists is its cost-effectiveness when compared to its alternatives (Walter *et al*., 2013). Ethidium bromide (EthBr) is considered unsafe. It's material safety data sheet (MSDS) projects it as a harmful agent if swallowed or inhaled. Even more worrisome is its capacity as a mutagen (Vardevanyan *et al.*, 2001) and possible carcinogenic and teratogenic potential (Ahmadimanesh *et al.*, 2013). Some persons in the scientific community have described the concern as baseless (Redfield, 2006) but others still believe that significant duration of chronic studies hasnot been done to be able to eliminate or implicate EthBr as a possible human carcinogen (Lowe, 2016). It is listed among agents likely involved in the aetiology of cancer among workers in biomedical research (Cordier *et al.*, 1995).

Ethidium bromide is a known inhibitor of mitochondrial DNA (mtDNA) replication and transcription (Vardevanyan *et al.*, 2001). It is reportedly capable of increasing mitochondrial DNA quantity and impairing oxidative phosphorylation as demonstrated in bovine fibroblast cells (Chiaratti *et al*., 2006). Perhaps as a function of dose, it is also capable of mtDNA depletion and enhancement (Warren *et al*., 2017; Von Wurmb-Schwark *et al*., 2016).

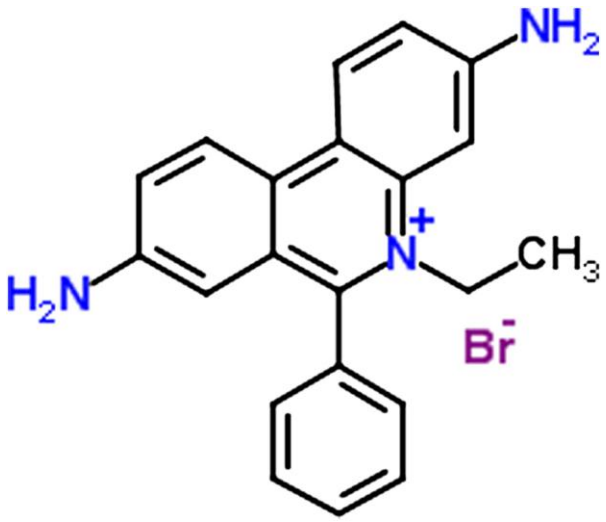

Figure 1: Chemical Structure of ethidium bromide

Mitochondrial dysfunction has been linked to reactive oxygen species (ROS) generation and attendant oxidative stress in cells. In particular, this has been demonstrated in cultured myoblasts (Min and Lee, 2019). It would appear that chemical agents that can induce loss of mitochondrial function can induce ROS production and this has been found to be so in cells where ethidium bromide induced mtDNA depletion. It is conceivable that EthBr-induced ROS production via mtDNA depletion may be due to a compromised antioxidant defense system and evidence in support of this idea has been found with cultured myoblasts (Min and Lee, 2019). However, the effects of EthBr on the antioxidant defense system in exposed animals remain largely unknown or undocumented. So studies on rat organs' susceptibility to EthBr carcinogenicity which were recently concluded (Akhabue, 2021) gave us the opportunity to investigate its effect on whole animal antioxidant enzyme activities if swallowed. Therefore, the purpose of this aspect of the study was to ascertain the effects of EthBr in rat colon antioxidant enzymes status due to chronic oral exposure.

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MATERIALS AND METHODS

Materials

Experimental animals

Results obtained from forty (40) healthy albino rats (Wistar strain) of both sexes were considered in this study. The rats were purchased from Department of the Biochemistry Animal Unit, Faculty of Life Sciences, University of Benin, Nigeria.

Chemicals

The chemicals used for this study include ethidium bromide (Lobachemie, India), hydrogen peroxide (30%) (Sigma H1009, USA), adrenaline (Fluka Chemika, Poole, England), pyrogallol (Kermel, China) and halothane (Piramel Healthcare Ltd, India). Others were routine laboratory chemicals and reagents. All were of analytical grade (Analar).

Methods

Treatment of animals

The forty rats were divided into eight experimental groups of 5 rats each. They were left for 14 days to acclimatize to the new location before the commencement of the study. They had free access to water and feed (Growers Mash, Bendel Feeds and Flower Mill Ltd (BFFM), Ewu, Edo State, Nigeria).

Group I rats served as the control and so had feed and water only, no EthBr. Group II rats were provided feed and water but administered EthBr solution, equivalent to 5 mg kg^{-1} bd wt. Groups III, IV, V, VI, VII and VIII rats were also provided with feed and water but were administered EthBr solution equivalent to 10, 20, 40, 60, 80 and 100 mg kg^{-1} bd wt respectively. Ethidium bromide solution was administered to each rat by gavage once weekly for 24 weeks.

Animal sacrifice, sample collection and preparation

At the end of the 24 weeks of treatment, each rat was anaesthesized by being put in halothane saturated chamber. While under anaesthesia the abdominal region was opened and the colon was excised.

Colon samples were homogenized in ice-cold physiological saline (1:4 w/v) to obtain a 20% homogenate. Each colon homogenate was centrifuged at 4000rpm for 10 minutes and the supernatant was stored at -20^0C until required for the biochemical assays.

Biochemical assays

Malondialdehyde (MDA) levels in the supernatant were estimated using 2 – thiobarbituric acid (Buege and Aust, 1978). Catalase (CAT), superoxide dismutase (SOD), and glutathione peroxidase (GPx) activities in the supernatants were determined using the methods of Cohen *et al*., (1970), Misra and Fridovich (1972) and Nyman (1959) respectively.

Statistical analysis

The data are presented as mean ± SEM. Analysis of variance (ANOVA) and LSD multiple range tests were done using SPSS computer software, version 21.0. Values were considered significant at $p \leq 0.05$. Correlation analyses were based on the approach described by Ogbeibu (2014).

RESULTS

Relative to the control, exposure of rats to EthBr at all the doses used in this study caused progressive elevation in colon homogenate supernatant MDA level (Table 1). The elevation was in a dose-dependent manner. However, significant ($p \leq 0.05$) elevations relative to the control were demonstrated only when the ethidium bromide dose was ≥ 20 mg kg^{-1} bd wt.

Table 1: Changes in colon catalase, glutathione peroxidase and superoxide dismutase activities and malondialdehyde levels in ethidium bromide exposed rats.

Group Number	Treatment	Catalase activity $Kmin^{-1}$ Mean ± SEM × 10^{-1} (n = 5)	Glutathione peroxidase activity units/mg tissue Mean ± SEM × 10^{-1} (n = 5)	Superoxide dismutase activity units/mg tissue Mean ± SEM × 10^{-2} (n = 5)	Malondialdehyde level mol/mg protein Mean ± SEM × 10^{-2} (n = 5)
I	Control	19.89 ± 0.00*	3.54 ± 0.07[a]	4.58 ± 0.00[a]	4.00 ± 0.40
II	5mg EthBr Kg^{-1}bd wt	19.88 ± 0.00[a]	3.52 ± 0.08 [a]	4.48 ± 0.00[a]	5.00 ± 0.40
III	10mg EthBr Kg^{-1}bd wt	19.87 ± 0.00[a]	3.39 ± 0.08 [a]	4.43 ± 0.00[a]	6.90 ± 0.70
IV	20mg EthBr Kg^{-1}bd wt	19.86 ± 0.00[a]	3.33 ± 0.09 [a]	4.41 ± 0.00[b]	8.10 ± 0.90[a]
V	40mg EthBr Kg^{-1}bd wt	19.85 ± 0.01[a]	3.27 ± 0.09 [a]	4.27 ± 0.00[b]	8.30 ± 1.10 [a]
VI	60mg EthBr Kg^{-1}bd wt	19.81 ± 0.02[b]	3.20 ± 0.08[b]	4.20 ± 0.00[b]	8.90 ± 1.30 [a,b]
VII	80mg EthBr Kg^{-1}bd wt	19.79 ± 0.02[b]	4.58 ± 0.00 [b]	3.95 ± 0.00[c]	9.80 ± 1.30 [a,b]
VIII	100mg EthBr Kg^{-1}bd wt	19.78 ± 0.03[b]	2.90 ± 0.19[c]	3.49 ± 0.00[d]	11.00 ± 1.80 [a]

*Values with different superscripts within the same column are significantly different from each other ($p \leq 0.05$).

Table 2: Relationship Between EthBr Doses, Colon Antioxidant Enzyme Activities and Malondialdehyde Levels.

Parameters	Pearson's Correlation Coefficient –"r"	*P* - value
EthBr Dose/Catalase	-0.9823	< 0.001
EthBr Dose/Glutathione peroxidase	-0.9772	< 0.001
EthBr Dose/Superoxide dismutase	-0.9107	< 0.001
EthBr Dose/ MDA level	+ 0.9808	< 0.001
MDA level/Catalase	- 0.9455	< 0.001
MDA level/Glutathione peroxidase	- 0.9623	< 0.001
MDA level/Superoxide dismutase	- 0.8707	< 0.001

When compared to the control there was a dose-dependent reduction in catalase activities present in colon homogenate supernatants. The reductions were statistically significantly ($p \leq 0.05$) different in the colon of rats exposed to 60, 80 and 100 mg EthBr kg^{-1} bd wt (Table 1). The same pattern of response was also observed in terms of SOD activities (Table 1) but a significant reduction in activity relative to the control was observed in rats exposed to ≥ 20 mg EthBr kg^{-1} bd wt. The most profound impairment in SOD activity occurred in rats exposed to 100 mg EthBr kg^{-1} bd wt.

The activity of GPx in colon homogenate supernatant was not markedly reduced by EthBr treatment. Significant impairment in GPx activity when compared to the control occurred only when rats were administered 60, 80 and 100 mg EthBr kg^{-1} bd wt. Again the most remarkable impairment was caused in rats exposed to 100 mg EthBr kg^{-1} bd wt.

The relationship between various colon antioxidant enzymes and malondialdehyde levels as well as that of the enzymes and MDA level with EthBr dose are presented in Table 2. There were negative but significant ($p< 0.001$) correlations between colon antioxidant enzymes activities and malondialdehyde level (CAT: r = - 0.9455; $p< 0.001$; SOD: r = - 0.8707; $p< 0.001$; and GPx: r = - 0.9623; $p< 0.001$). Relationships

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between colon antioxidant enzyme activities and EthBr doses were also strong and significant (CAT: r =-0.9823; $p< 0.001$; SOD: r = -0.9107; $p< 0.001$; GPx: r = -0.9772; $p< 0.001$). Ethidium bromide doses and colon MDA levels were also markedly strongly correlated (r = + 0.9808; $p< 0.001$).

DISCUSSION

The antioxidant enzymes investigated in this study had their activities significantly reduced in the colon of EthBr-exposed rats when the dose of EthBr administered reached a certain threshold. For CAT and GPx the threshold dose was 60 mg EthBr kg^{-1} bd wt but it was as low as 20 mg for SOD. Evidently, in exposed rats, SOD was more sensitive to EthBr-induced reduction in antioxidant enzyme activities by whatever mechanism.

The fact that the activities of these enzymes were reduced in EthBr-treated rat colons is in consonance with the findings of Ishihara *et al.*, (2016) who exposed cultured SHSY5Y cells to EthBr. They discovered that EthBr reduced SOD 1, CAT and gamma-glutamylcysteine synthetase (gGCS) activities although the treatment did not affect GPx 1 activity. Their finding on EthBr effect is interesting in view of the new finding in this report that somehow GPx is less sensitive to EthBr-induced reduction in activity.

Also in agreement with the general observation in this study is that the activities of antioxidant enzymes in rats are susceptible to EthBr inhibition as reported by Rhimluoye *et al*., (2019). They found that injection of EthBr to the hippocampus of male rats enhanced MDA level, and significantly reduced SOD and GPx activities.

In this study, the mechanism by which the activities of the antioxidant enzymes are reduced was not investigated. Whether it is due to enzyme inhibitor interaction or transcriptional inhibition remains to be established. However, the results of Ishihara *et al.*, (2016) and ~~that~~ of Rhimluoye *et al.*, (2019) suggest that EthBr effect was at the transcriptional level since mRNA of these enzymes were not adequately produced in EthBr-exposed rat hippocampus.

The levels of MDA in the report presented here and in the report of Rhimluoye *et al.*, (2019) were elevated consequent upon EthBr treatment. Elevated MDA levels suggest~~s~~ that ROS were induced but were not adequately dissipated by the antioxidant defense system. Low activities of SOD, GPx and CAT are evidence of a compromised oxidative stress defense system. The involvement or generation of oxidative stress as has been emphasized by others (Ishihara *et al.*, 2016; Rhimluoye *et al.*, 2019; Min and Lee, 2019) by whatever mechanism in EthBr-treated cultured cells and in rats implies uncontrolled/mismanaged ROS levels in the cells or affected organs of the rats. Reactive oxygen species can affect membrane polyunsaturated fatty acids leading to MDA production. However, ROS can damage proteins as well (Berkman and Ames, 2000) and this may be a likely explanation for the low antioxidant enzyme activities observed in this study of rats on chronic EthBr exposure. The chances are that mRNA of the enzymes were either not expressed significantly or not expressed at all due to EthBr impairment and as such the available ones would be incapacitated by an avalanche of EthBr-induced ROS production.

The conclusion from the present findings is that ethidium bromide can reduce antioxidant enzyme activities in animal tissues, which in the current investigation is the rat colon.

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