

YABA (METHAMPHETAMINE TABLET) ADMINISTRATION ALTERED CELLULAR ANTIOXIDANT STATUS AND DEVELOPED FIBROSIS IN LIVER OF RAT

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Abstract

The aim of our study was to evaluate the effect of methamphetamine contained Yaba tablet on rat liver, available in Bangladeshi underground market. Yaba tablet equivalent to 5 mg or 10 mg of methamphetamine /kg body weight was administered orally once daily in rat for 56 days. At the end of experiment, all rats were sacrificed; blood and tissue samples were analyzed for alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP), advanced oxidation protein product (AOPP), malondialdehyde (MDA), nitric oxide (NO), glutathione (GSH), catalase (CAT) and superoxide dismutase (SOD). Histopathological study of rat liver was done with Hematoxylin and Eosin, Picrosirius Red and Prussian Blue staining from all groups. Yaba powder administration significantly elevated the ALT, AST and ALP activities in plasma of rat. MDA and NO levels were elevated in plasma and tissues. However, AOPP and GSH level in plasma and liver tissue remain unchanged as compared to control rat. CAT and SOD enzyme activities were unaffected. Histological staining revealed that inflammatory cells were deposited alongside the veins in liver followed by collagen deposition after Yaba administration in rat as compared to control rat. The high dose of Yaba produced more significant changes in rat liver. The overall results suggested that long-term administration of Yaba tablet might develop oxidative stress and induce cellular inflammation and fibrosis in rat liver. Thus, hepatic dysfunction by Yaba tablet might manifest in human liver as well.

Keywords: *Yaba, oxidative stress, malondialdehyde, inflammation, fibrogenesis.*

Introduction

In Bangladesh, drug abuse has become a national crisis which is growing every day and appearing as a burden for society, economy and public health as well. People of different age groups (ranging from 18 to 30), different professions and different social status are involved in this dreadful habit. Recently, methamphetamine has become a popular drug of abuse among Bangladeshi drug addicts, in the name of Meth or Yaba [1]. This illicit drug enters into Bangladesh from various international drug traffic routes, including India and Myanmar. Methamphetamine causes significant health hazards from both short-term and long-term use. The mental disturbance observed in short-term use includes acute paranoia, anxiety, confusion etc. Prolonged use of methamphetamine develops depression, social isolation, mood disturbances and psychomotor dysfunction [2-4]. Different studies suggested that methamphetamine might cause brain degeneration after long-term use by damaging dopamine and serotonin axons. Left ventricular hypertrophy mediated by oxidative stress from methamphetamine abuse was described by a separate study [5]. This drug induces hyperthermia [6]. Amphetamine derivatives especially methamphetamine reported inducing rhabdomyolysis with the flow of massive toxins through kidney causing it to shut down. A blood test revealed a significant increase in creatinine and creatinine phosphokinase and a decrease in K, Ca & P suggesting renal tubule damage by repeated administration of methamphetamine [7]. Drug-induced hepatotoxicity comprises about 10% of all types of acute hepatitis [8]. However, in few studies, liver damage has been reported as remarkable symptom observed from long-term abuse of amphetamine derivatives [9]. However, relevant information and data on the toxic effect of amphetamine and its derivatives are not available in literature and poorly understood. Thus, the current investigation was undertaken to evaluate the long-term effect of Yaba on the liver in rat.

Methods

Chemicals and reagents

Yaba tablets used in the study were received from the Narcotics Control Department of Dhaka

Metropolitan Police, Bangladesh. Thiobarbituric acid (TBA), glutathione in reduced form (GSH) and trichloroacetic acid were purchased from Sigma Chemical Company (USA) and J.I. Baker (USA) respectively. Alanine aminotransferase (ALT), aspartate aminotransferase (AST) and alkaline phosphatase (ALP) assay kits were obtained from DCI diagnostics (Budapest, Hungary), 50,50-dithiobis-2-nitrobenzoate (Ellman's reagent) and sodium hydroxide purchased from Sigma (USA) and Merck (Germany) respectively. All other chemicals and reagents used in present study were of analytical grade.

Animal study and specimen collection

Male Long Evans rat, of 10-12 weeks old and 180 g – 200 g body weight were taken. Rats were supplied from the production area of the animal house of Pharmaceutical Sciences Department, NSU. Yaba tablets were suspended in distilled water and administered to rats through oral route for 56 days (once daily). Animals were divided into three groups (6 rats in each) such as-

Group I, served as control and given distilled water instead of drug;

Group II, received low dose, 5 mg of methamphetamine/kg of body weight and

Group III, received high dose, 10 mg of methamphetamine/kg of body weight.

All animals had free access to water and standard laboratory feed *ad libitum*. They were kept in ordinary cages with a 12 h light / dark cycles at temperature $25\pm 3^{\circ}\text{C}$. The protocol for the study was approved by The Ethical Committee of the Department of Pharmaceutical Sciences, NSU, Bangladesh for animal care and experimentation. We have monitored the body weight, water and food intake of all animals and recorded every day. Rats of three groups were sacrificed on the last day of eight weeks. Collected blood samples were centrifuged to separate the plasma and stored at -20°C until used for analysis. Liver, kidney, heart, spleen was also collected and weighed immediately. Halves of the organs were stored at -20°C until used for biochemical tests and halves were processed for histological study according to the established method.

Assessment of liver enzymes

Serum alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP), albumin and total protein levels, as well as the tissue histological assay are useful tools for the assessment of the functional integrity of liver. Rat plasma of three groups was assayed for liver transaminases (ALT and AST) and alkaline phosphatases (ALP). Chemical analysis was conducted by following the standard protocols of manufacturer provided with DCI diagnostics kits (Hungary).

Assessment of oxidative stress markers and antioxidant enzyme activity

Oxidative stress markers and antioxidant enzyme activity were measured in rat blood plasma and liver tissue. Liver tissue was homogenized in 10 times volume of ice cold phosphate buffer having pH 7.4 and centrifuged at 10000 rpm for 30 min at 4°C. The supernatant was collected and used for the determination of protein and enzyme contents.

Estimation of malondialdehyde (MDA), Nitric oxide and advanced oxidation protein products (AOPP)

We measured malondialdehyde (MDA) in plasma and liver tissue extract using thiobarbituric acid (TBA) as per method described by Niehaus and Samuelsson^[10]. Nitric oxide, in the form of nitrate, was measured following the method described by Tracey et al.^[11]. In this study, Griess-Illsovoy reagent was modified by replacing 5% 1-naphthylamine with naphthyl ethylene diamine dihydrochloride (0.1% w/v). A standard curve, expressed as nmol/ml, was prepared to measure plasma and liver tissue content of nitric oxide. The methods described by Witko-Sarsat et al.^[12] and Tiwari et al.^[13] were followed to detect AOPP level. AOPP concentrations were expressed as nmol/L as chloramine-T equivalents where the absorbance of chloramine-T was linear within the range of 0 to 100 nmol/L at 340 nm.

Measurement of glutathione (GSH), superoxide dismutase (SOD) and catalase (CAT) activity

The reduced form of glutathione in plasma and liver was estimated by the method previously described by Mitchell et al.^[14]. A yellow color mixture was immediately subjected to take

absorbance at 412 nm using a UV-Spectrophotometer. The calculated result was expressed in ng/mg protein. SOD was assayed in tissue homogenates by using previously described method^[15]. Auto-oxidation of epinephrine present in sample solution usually reduced to 50% with the action of one unit enzyme. Khan et al.^[16] reported the method which was used to test catalase activity. Changes in absorbance of the reaction mixture were determined at 240 nm. An absorbance change of 0.01 as units/min corresponds to one unit of CAT activity.

Histopathological observation

Liver tissues of all groups were prepared for histology study according to standard procedure. In short, tissues were fixed in neutral buffered formalin, treated with ethanol and xylene and then embedded in paraffin. Using microtome, tissue paraffin blocks were sectioned at 5µm. Tissue sections were then stained with Hematoxylin and Eosin, Picosirius Red and Prussian Blue separately for microscopic observation of the inflammatory cell invasion, deposition of collagen fiber and iron deposition respectively in the liver. Stained tissue sections were examined under a light microscope at 40X magnification.

Statistical analysis

The experimental results were evaluated by using the One-way ANOVA-test in Graph Pad Prism Software. The values have been expressed as mean±standard error of the mean (SEM). In all cases, statistical significance was considered $p < 0.05$.

Results

Physical & behavioral changes observed in rat during Yaba treatment

During the period of the experiment, rats of both dose groups were found restless with palpitation and increased thirst. 30% of the drug-treated rat of high dose group had skin lesion in different parts of the body. Rats of low and high dose group rapidly got the aged look with the loosening of skin. The body temperature of rats in low dose and high dose group increased by 7-8°C and persisted for the 1h app. after Yaba administration whereas body temperature remains unchanged in control rats.

Effects of Yaba on organ weight in rat

Rats treated with 5 mg of methamphetamine/kg of body weight were found to have no statistically significant change in their liver, heart, kidney and spleen weights as compared to control rats. 30% rats of this group had abscess in the liver. In another dose group where rats were treated with 10 mg of methamphetamine/kg of body weight showed insignificant changes of these vital organs when compared with controls.

Effects of Yaba on liver marker enzymes in rat

Yaba treatment induced transaminase ALT to increase significantly to 87.86 ± 14.00 in high dose group from 31.58 ± 2.87 U/L ($p < 0.01$, $N = 5-6$). AST increased significantly to 47.37 ± 6.19 in high dose group from 22.97 ± 1.82 U/L ($p < 0.01$, $n = 5-6$). Alkaline phosphatase (ALP) increased significantly to 165.32 ± 10.14 in low dose group from 79.81 ± 7.60 U/L ($p < 0.01$, $n = 5-6$). The increase in liver transaminases, ALT and AST, increased by 2.8-fold and 2-fold respectively by a high dose of Yaba and ALP by 2-fold by a low dose of Yaba as compared to control value. Change in ALT and AST concentrations in low dose groups and ALP concentration in high dose group was not statistically significant.

Effects of Yaba on oxidative stress in rat

Yaba treatment induced high concentration of lipid peroxidation product, MDA, which significantly increased to 50.58 ± 2.06 nmol/ml in high dose group from 36.49 ± 2.3 nmol/ml ($p < 0.05$, $n = 5-6$) in plasma and to 181.54 ± 11.96 nmol/ml in high dose group from 129.92 ± 15.61 nmol/ml ($p < 0.01$, $n = 5-6$) in liver homogenate. Yaba in high dose increased MDA by 1.4-fold both in rat plasma and liver tissue when compared with control value. However, low dose of Yaba couldn't induce a significant change in MDA concentration in plasma and liver tissue. In drug-treated rat liver tissue, nitrate level increased significantly in high dose group to 20.55 ± 1.01 nmol/ml from 17.43 ± 1.21 nmol/ml ($p < 0.05$, $n = 5-6$) which was 1.2-fold increase as compared to control value. In liver tissue of low dose group, nitrate level was not changed significantly as compared to control. In rat plasma, nitrate level was not significantly changed in any dose group as compared to control. No significant change in the value of AOPP level in plasma and liver tissue of

drug-treated groups found when compared with control.

Effects of Yaba on antioxidant enzyme system in rat

Treatment with 5 mg of methamphetamine/kg of body weight induced no statistically significant change in the activity level of antioxidant enzymes included GSH, CAT and SOD in plasma or liver tissue as compared to control. When the dose was increased to 10 mg of methamphetamine/kg of body weight, GSH, CAT and SOD antioxidant enzymes activity remain unchanged in plasma or liver tissue when compared with control.

Histological changes after Yaba treatment in rat liver

Inflammation was seen in stained tissue section of drug-treated rat liver. Massive invasion of inflammatory cells was found in the centrilobular part of liver section stained with Hematoxylin and Eosin in low dose Yaba treated rat which progressively increased in high dose treated rat. Liver fibrosis was evaluated histologically by visualizing the red color of collagen fibers deposition using Picrosirius Red stain in both dose groups. The collagen fibers were heavily deposited around portal tracts and central veins in Yaba treated group which was not seen in control rats. The extent of collagen fiber deposition was enormous in high dose group than that of low dose group. Iron deposition was not seen in the liver section of Yaba treated rats by Prussian Blue staining.

Discussion

Liver disease in methamphetamine abuser is evident besides its toxic effects on other vital organs of the body. To evaluate the chronic liver toxicity of methamphetamine, rats were administered Yaba tablet orally for eight weeks and investigated hepatic damage in rats. Our investigation revealed that chronic administration of Yaba induced oxidative stress, inflammation and fibrogenesis in rat liver. In the present study, a significant elevation in liver enzymes (ALT, AST and ALP) took place in plasma as compared to control indicated liver abnormality after Yaba treatment. During hepatic cell necrosis and membrane damage, the liver marker enzymes are

released into circulation and hence their concentration increased in plasma and serum. High level of AST indicates liver damage in viral hepatitis as well as cardiac infarction whereas ALT is more specific to the liver and thus a better parameter for detecting liver injury. In hepatobiliary tract disease and bone abnormality, alkaline phosphatases level was reported to be increased [17]. Increased production of ROS and resultant oxidative stress plays a vital role in the development of liver damage. The reactive species stimulates inflammatory responses through the activation of pro-inflammatory mediators. The episodes of inflammation eventually stimulate pro-fibrogenetic mediators to initiate hepatic fibrogenesis. Several *in vitro* studies have been reported on the significant increase in liver enzymes and ROS formation in rats which were subjected to methamphetamine-induced hepatotoxicity using isolated rat hepatocytes [18, 19]. Our investigation showed that Yaba treatment developed the production of thiobarbituric acid-related substances (TBARS), the lipid peroxidation product, as compared to control group significantly which indicates profound oxidative damage in the liver. Nitric oxide (NO), a mediator of systemic vasodilatation, has been reported to be increased in liver cirrhosis. Moreover, clinical studies showed that serum nitrite level in cirrhotic patients was significantly increased in comparison to that of control [20]. In our study, NO level of rat liver, increased significantly by a high dose of Yaba, directed to its contribution to liver cell damage. Previous studies reported that inhibition of nitric oxide synthase could protect against cellular damage suggests that NO plays a crucial role in methamphetamine induced cellular toxicities [21]. The antioxidant enzymes such as superoxide dismutase, glutathione peroxidase and catalase work collectively in human cells to protect from the toxic effect of reactive oxygen species. Therefore, when the body suffers from oxidative stress and pathology arises, the bodily defense system promotes the expression of these enzymes as a protective measure [22]. It is proven that increase in ROS production ultimately lowers these enzymes levels [23]. In contrast to this information, antioxidant enzymes were not depleted significantly in spite of evidence of increased lipid peroxidation after Yaba treatment. However, the mechanism is not still

clear. During the experimental period, body temperature of rats increased after Yaba administration by 6-8°C and persisted for one hour approximately. Laura et al. described that methamphetamine-induced persistent liver damage through hyperthermia which was characterized by increased plasma aspartate and alanine aminotransferase as well as ammonia [24]. Hyperthermia considered as destructive pro-oxidant factor. At high temperature glutathione peroxidase loses its activity. Histopathological assessment in liver tissues of Yaba treated rats revealed typical hepatotoxic effects same as described previously in the literature [25]. Inflammatory cells have been accumulated in the necrotized region and along the bile ducts and blood vessels in the liver of drug-treated rats. Inflammatory response involves a number of inflammatory cells. Identification test of those inflammatory cells was not performed in the present study. In the development of liver fibrosis, the contribution of infiltrating immune cells has recently been proven by studies besides the liver-resident Kupffer cells. Previous reports on mice suggested that in acute and chronic drug-induced liver injury monocytes infiltrates at the site of inflammation. During an inflammatory response, monocytic macrophages release their own cytokines which gives rise to chronic inflammation. Besides, hepatic stellate cells are activated, proliferated and differentiated into myofibroblasts which turn out collagen deposition in tissues [26, 27]. In our study, collagen fiber deposition varied in two different doses treated rat liver observed in Sirius Red staining. Progressive deposition of collagen fiber supported the inflammatory cell damage of rat liver treated with Yaba. Moreover, the possibility of iron-induced oxidative stress was not proven by Prussian blue staining of Yaba treated rat liver.

The liver was selected as a target organ of drug toxicity because of its major participation in drug metabolism and frequency of jaundice in many Yaba addicted people diagnosed in clinical practice as well. To our knowledge, a large number of clinical trials on a human has been conducted to assess liver toxicity by drugs but data regarding the role of oxidative stress in the development of liver disease is inadequate. However, the exact measurement of oxidative stress in order to correlate with disease status in patients is still needed [28]. Furthermore,

most of the investigations on drug-induced liver toxicity made on the basis of the retrospective study. Very few clinical trials carried on hepatotoxicity study of illicit and abused drugs included inadequate patient population. Several *in vitro* studies conducted using human cell line to simulate the pathogenesis developed in particular organ by drugs [29]. Lack of availability of pure methamphetamine reference standard, present research could not include the assessment of methamphetamine content in supplied Yaba tablets and a separate study using pure methamphetamine in order to compare the results with that of Yaba treatment where caffeine is included as an adjunct principle in these tablets. Treatment duration and doses were selected by keeping similarities with drug abuse pattern by people. Based on the investigation results of present study, the conclusion may be drawn in the way that evidence for inflammation and consequent fibrotic effect on rat liver were visible from long-term Yaba treatment. The possible mechanism of damage may be due to increased lipid peroxidation of hepatocytes by increased ROS and RNS which is followed by inflammatory response leading to deposition of collagen fiber by activated hepatic stellate cells. In spite of progressive fibrogenesis in drug-treated rat liver observed in the histologic study, the unchanged activity of antioxidant enzymes detected might suggest the specific stage and level of liver toxicity during the experimental eight weeks by Yaba tablet which requires further investigation.

Acknowledgments

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Figure 1. (A) Skin lesion during Yaba treatment (B) Abscess in rat liver after Yaba treatment.

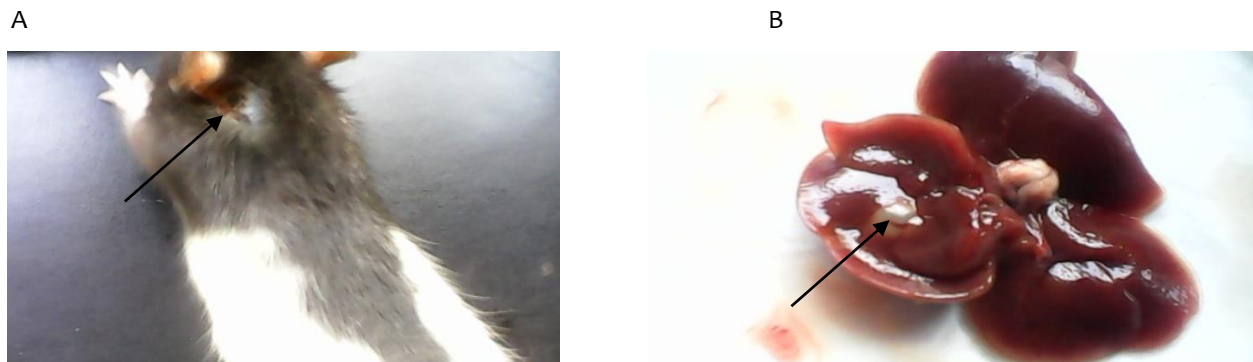


Figure 2. Effect of Yaba administration on liver transaminases (ALT, AST) and alkaline phosphatases (ALP) activities in rat. Data are presented as Mean \pm SEM, n=6. Statistical analysis was conducted by One way ANOVA followed by Bonferroni post hoc test. Statistical significance was considered as $p < 0.05$.

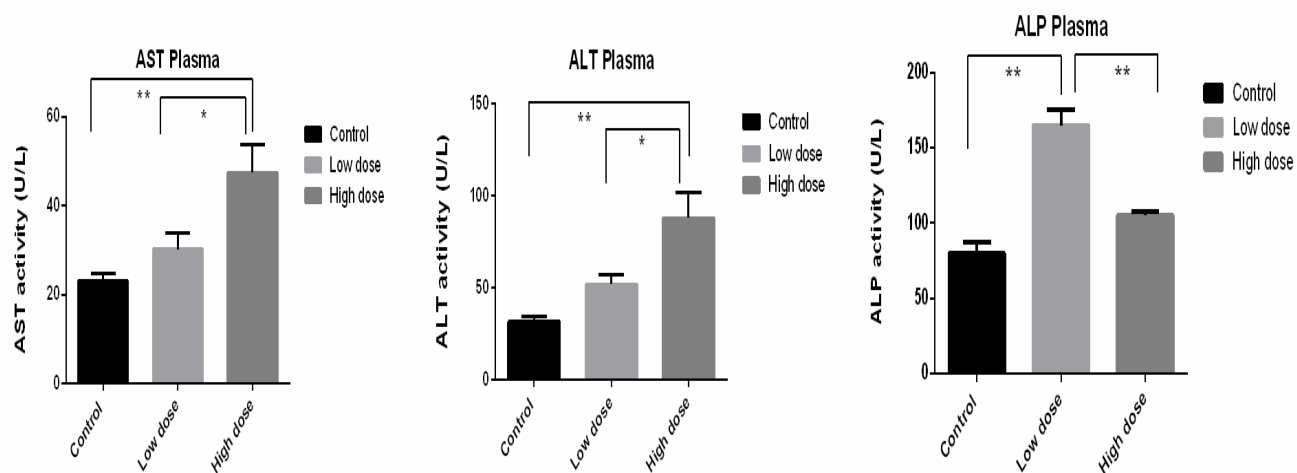


Figure 3. Effect of Yaba on malondialdehyde (MDA) level in plasma and liver in rat. Data are presented as Mean±SEM, n=6. Statistical analysis was conducted in One way ANOVA followed by Bonferroni post hoc test. Statistical significance was considered as $p < 0.05$.

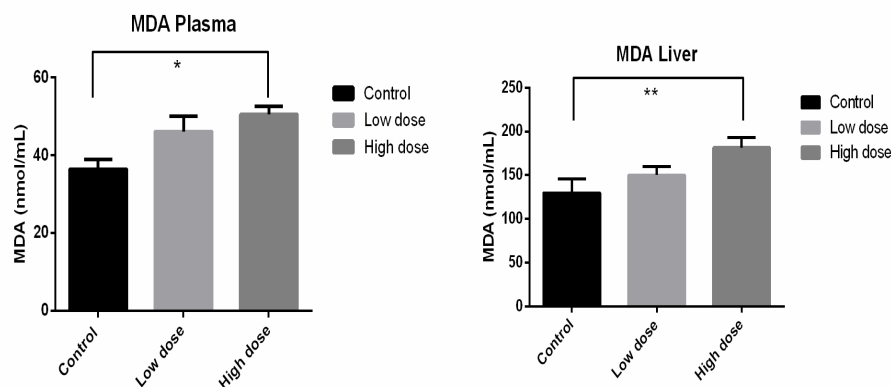


Figure 4. Effect of Yaba on nitric oxide (NO) level in plasma and liver in rat. Data are presented as Mean±SEM, n=6. Statistical analysis was conducted in One way ANOVA followed by Bonferroni post hoc test. Statistical significance was considered as $p < 0.05$.

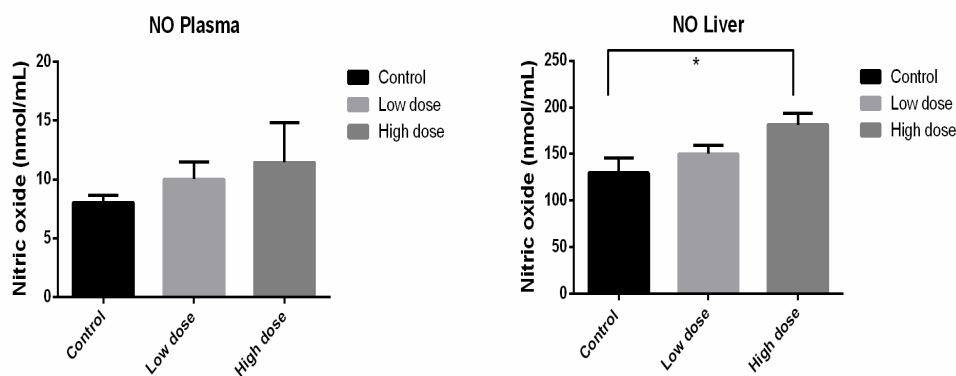


Figure 5. Effect of Yaba on advanced oxidation protein products (APOP) level in plasma and liver in rat. Data are presented as Mean±SEM, n=6. Statistical analysis was conducted in One way ANOVA followed by Bonferroni post hoc test. Statistical significance was considered as $p < 0.05$. No significant differences were noted among the group tested.

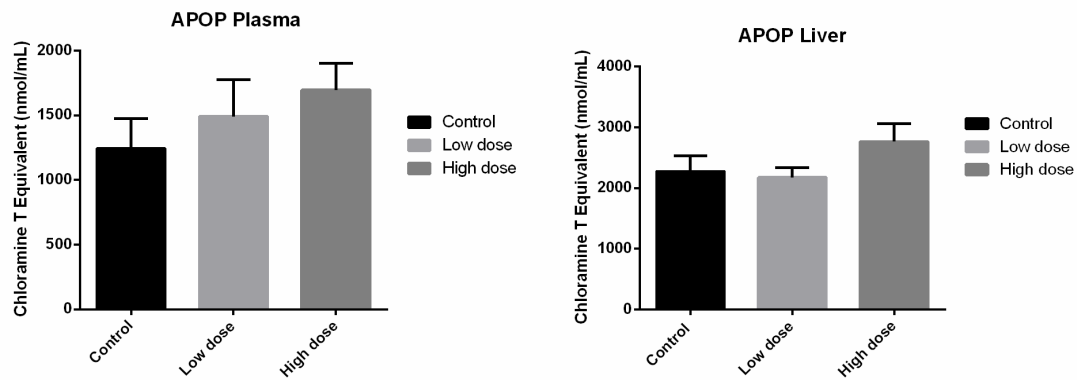


Figure 6. Effect of Yaba on glutathione (GSH) activity in plasma and liver in rat. Data are presented as Mean±SEM, n=6. Statistical analysis was conducted in One way ANOVA followed by Bonferroni post hoc test. Statistical significance was considered as $p < 0.05$. No significant differences were noted among the group tested.

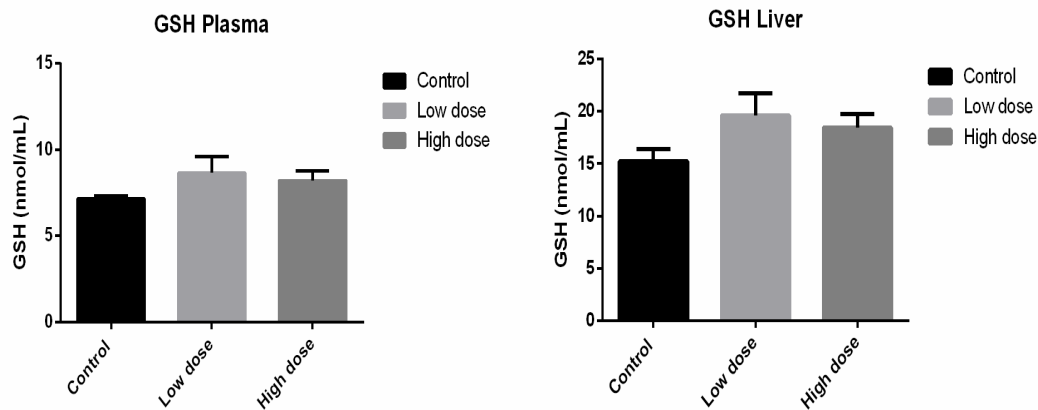


Figure 7. Effect of Yaba on catalase (CAT) activity in plasma and liver in rat. Data are presented as Mean±SEM, n=6. Statistical analysis was conducted in One way ANOVA followed by Bonferroni post hoc test. Statistical significance was considered as $p < 0.05$. No significant differences were noted among the group tested.

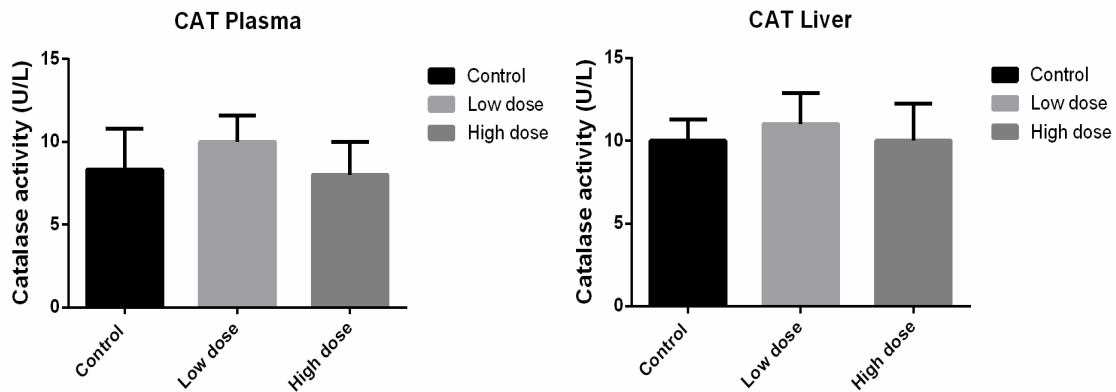


Figure 8. Effect of Yaba on superoxide dismutase (SOD) activity in the liver in rat. Data are presented as Mean±SEM, n=6. Statistical analysis was conducted in One way ANOVA followed by Bonferroni post hoc test. Statistical significance was considered as $p < 0.05$. No significant differences were noted among the group tested.

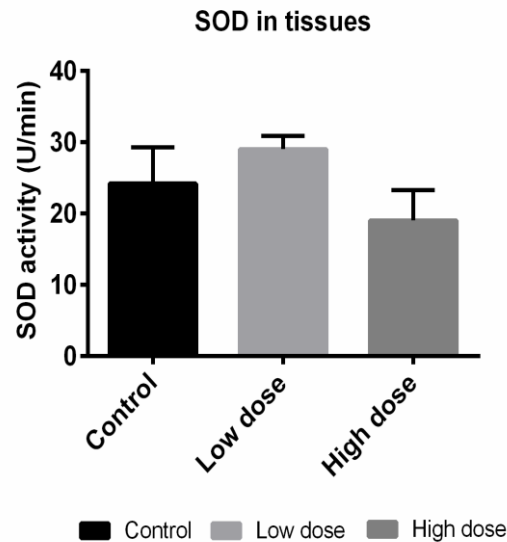


Figure 9. Invasion of inflammatory cells in the centrilobular part of rat liver after Yaba treatment. A. control group; B. low dose (5 mg of methamphetamine/kg of BW) group; C. high dose (10 mg of methamphetamine/kg of BW) group.

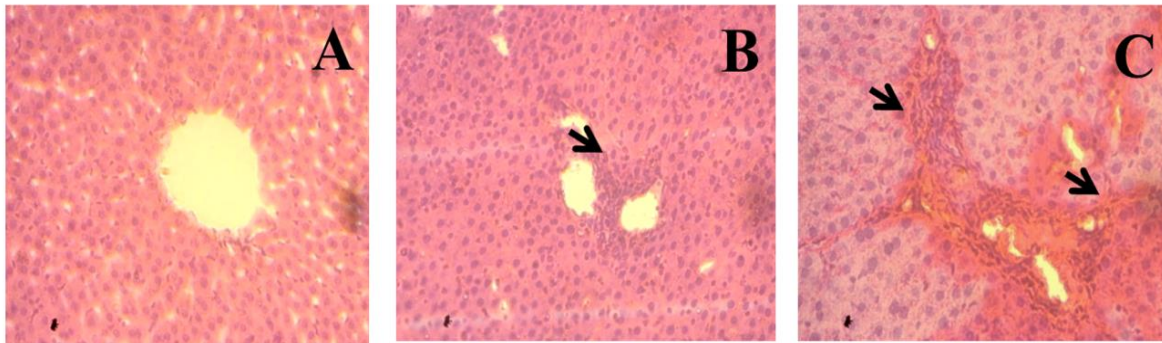


Figure 10. Deposition of collagen fiber in rat liver after Yaba treatment. A. control group; B. low dose (5 mg of methamphetamine/kg of BW) group; C. high dose (10 mg of methamphetamine/kg of BW) group

