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MODULATORY EFFECTS OF CHRONIC ETHANOL INGESTION ON HEPATOTOXICITY-RELATED PARAMETERS IN MALE RATS

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ABSTRACT

5 g of ethanol (20% w/v) per kg body weight was administered to ten rats weighing between 166 – 170 grams daily for 28 days along side with normal feeds and water ad libitum. The administration of ethanol was done orally using oral catheter. A control group of ten rats was set up for a proper experimental evaluation. Analysis at the end of the administration showed that ethanol in a dose of 5g/kg body weight significantly modulates ($P < 0.05$) the activities of selected liver enzymes: alanine amino transferase (ALT), aspartate amino transferase (AST), gamma glutamyl transferase (GGT) and alkaline phosphatase (ALP) as well as those of selected antioxidant enzymes: catalase (CAT) and superoxide dismutase (SOD).

Keywords: Rats, Ethanol, Oral administration, Catheter, Liver, Hepatotoxicity, Antioxidant enzyme

INTRODUCTION

Alanine amino transferase (ALT), aspartate amino transferase (AST), gamma glutamyl transferase (GGT) and alkaline phosphatase (ALP) are physiological and drug metabolizing enzymes located mainly in the liver and are often used as markers of liver functions and soft tissue damage (Lum and Gamino, 1972; Marsano *et al.*, 2003). They play very important roles in the functional integrity of the liver. Structural damage of the liver probably through oxidative stress results in the leakage of these enzymes from the cytoplasm into the blood stream (Vermaulen *et al.*, 1992). High hepatic activity of these enzymes indicates a healthy state of the liver, whereas, high plasma enzyme activity characterizes a disease condition of the liver. A healthy physiological condition is strongly underlined by a balance between generated oxidants (ROS) and the body's antioxidant systems. Oxidative stress is the disturbance in the balance between generated oxidants (oxygen metabolites) and antioxidants in favor of the oxidants, and is characterized with the degradation of cell membranes and biomolecules such as lipids and nucleic acids (De Leve *et al.*, 1996). Cellular defenses against ROS-induced damage involve antioxidant enzymes such as superoxide dismutase (SOD) and catalase (CAT) (Aruoma, 1998; Wu and Cederbaum, 2003). SOD catalyzes the dismutation of superoxide anion radicals into hydrogen peroxide (H_2O_2) while CAT catalyzes the conversion of hydrogen peroxide into water and oxygen. Thus, these enzymes are

important in protecting the cells against ROS-induced damage (Fridovich, 1997).

Ethanol is the active chemical component contained in alcoholic beverages and drinks. It is a clear and colorless liquid with a characteristic agreeable taste and smell. It is contained in varying concentrations in products like beer; wine, gin, brandy, whiskey and liquors, and is well known for its intoxicating effect on its consumers. It is primarily metabolized in the liver (Maher, 1997) and absorbed into the blood stream in the gastrointestinal tract (GIT) (Bode and Bode, 1997). Ethanol breakdown generates potentially dangerous by-products such as acetaldehyde and highly reactive molecules called free radicals (ROS), which are capable of attacking cell membranes and biomolecules (Lindros, 1995; Balkan *et al.*, 2001). A lot of scientific research findings have implicated alcohol as a major player in variety of disease conditions via excessive generation of free radicals or reactive oxygen species (oxidative stress).

The focus of this work was therefore; to evaluate the effects of ethanol on the activities of the selected liver and antioxidant enzymes in rats with a view to either or not substantiate the implication of alcohol in variety of oxidative-stress linked pathological conditions as earlier reported.

MATERIALS AND METHODS

Chemicals: Chemicals and reagents of pure analytical (ANALAR) grade were selectively used and necessary precautions were strictly followed.

They include: Tris (hydroxymethyl) aminoethane (Sigma Chemical Company, USA), potassium chloride, Tris-HCl, Sucrose, NaCl (BDH England), $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ (Hopkins and Williams, England), sodium potassium tartrate (BDH, England), potassium iodide (BDH, England), Stock Bovine Serum Albumin (BSA), Alanine transaminase kit (Randox Laboratories, USA) Gamma glutamyl transferase kit (Biolabo, France), aspartic acid (BDH Chemicals Limited, England), α -ketoglutaric acid, Na_2HPO_4 (Hopkins and Williams Limited, England), dihydrogen potassium phosphate (Hopkins and Williams Limited, England), 2,4 dinitrophenyl hydrazine (Hopkins and Williams Limited, England) sodium hydroxide pellet (BDH Chemical Limited, England, epinephrine (Sigma Chemical Company, London), $\text{Na}_2\text{CO}_3 \cdot 10\text{H}_2\text{O}$, NaHCO_3 , H_2O_2 , potassium heptaoxidochromate (vi), $\text{K}_2\text{Cr}_2\text{O}_7$, glacial acetic acid, $\text{NaH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$, $\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$ (BDH Chemical Limited, England) and absolute ethanol.

Preparation and Administration of Ethanol: Five grams per kilogram body weight of 20 % (w/v) ethanol solution was used as chronic dose in this experiment. 20 g absolute ethanol was dissolved in distilled water and made up to 100 ml. 4.25 ml of the solution was daily administered for four weeks to each tested rat treatments.

Animal Treatment: Twenty male rats of the Wister strain were used for the experiment. They were purchased from the Institute for Advance Medical Research and Training (IMRAT), University College Hospital (UCH) Ibadan. The animals were kept in well-ventilated cages under suitable conditions of temperature and humidity. They were fed on normal laboratory rat feeds (Bendel Feeds and Flour Mill Limited, Edo State) and with water supplied ad libitum. The rats were grown to a weight range of 166 g – 170 g before used. The animals were divided into two groups containing 10 rats each. Group A (Control) rats were fed with normal laboratory chow and with water for 28 days. Group B (Ethanol Treatment) rats were administered ethanol orally at dosage of 5 grams per kilogram body weight per day for 28 days.

Tissue Preparation for Biochemical Analysis: The animals (control and ethanol-treated) were fasted for 12 hours and sacrificed by cervical dislocation without anesthesia. The liver was quickly removed, washed in ice-cold 1.15 % KCl solution, blotted and weighed. It was then homogenized in four volumes of the homogenizing buffer (ice-cold Tris-HCl buffer, 0.1 M, pH 7.4), using a Potter Elvehjem type homogenizer. The resulting

homogenate in each case was centrifuged at 10,000 rpm for 30 minutes in a Beckman L5-50B ultra centrifuge with a 220.78 V02 rotor at 4 °C. Further centrifugation was done at 105,000 rpm for 1 hour with a type 35 fixed angle rotor in the same ultracentrifuge. The resultant supernatant was collected and used for different biochemical analysis. Storage was done below 4 °C to maintain enzyme activity.

Estimation of ALT Activity: The activity of the enzyme alanine amino transferase (ALT) formerly known as glutamate pyruvate transaminase (GPT) was measured as described by Reitman and Frankel (1957), using alanine transaminase kit along with freshly prepared 0.4 M sodium hydroxide solution. Pyruvate is a reaction product of transamination reaction catalyzed by ALT. The pyruvate produced reacts with 2, 4-dinitrophenyl hydrazine to produce hydrazone on the addition of sodium hydroxide. The ALT activity was measured by monitoring the concentration of pyruvate hydrazone formed. The optical density of the color developed was measured after 5 minutes at 546 nm. ALT activity was obtained from the standard curve.

Estimation of AST Activity: The estimation of the activity of aspartate amino transferase (AST) formerly known as glutathione-oxaloacetate transaminase (GOT) was according to the method of Reitman and Frankel (1957). Oxalacetate is a reaction product of the transamination reaction catalysed by aspartate aminotransferase. The unstable oxalacetate produced was then quantitatively decarboxylated to pyruvate which was complex with 2, 4 dinitrophenyl hydrazine to produce an intensely colored pyruvate hydrazone on the addition of 0.4M sodium hydroxide solutions. AST was estimated by monitoring the concentration of pyruvate hydrazone formed. The optical density of the color developed was measured after 5 minutes at 545 nm. AST activity was obtained from the standard curve.

Estimation of GGT Activity: The enzyme, gamma glutamyl transferase activity was estimated as described by Szasz and Persyn (1974) using gamma-glutamyl transferase kit from Biolabo, France. GGT catalyses the transfer glutamyl group from L-Glutamyl-p-nitroanilide to glycylglycine with the formation of L-G glutamylglycylglycine and p-nitroaniline. 50 μl of sample was added to 1ml of the working reagent in a test tube. Absorbance was read after 30 seconds and at the same time the stop watch was started. Absorbance was read again after exactly 1, 2 and 3 minutes.

Table 1: Effects of ethanol on plasma activity (IU/mg protein) of liver enzymes

Group	Plasma ALT	Plasma AST	Plasma GGT	Plasma ALP
A (Control)	14.01 ± 1.01	23.51 ± 1.28	28.05 ± 1.72	18.66 ± 0.38
Ethanol Treatment	26.32 ± 0.19	48.39 ± 1.20	51.12 ± 1.01	33.28 ± 1.15

Values are expressed as mean ± S.D for ten animals per group

From the reading, the change in absorbance per minute ($\Delta A/\text{min}$) was calculated. GGT activity was calculated from the equation: GGT activity (IU/L) = $\Delta A/\text{min} \times 2121$.

Estimation of ALP Activity: ALP activity was measured by monitoring the concentration of p-nitrophenol formed when ALP reacts with p-nitrophenyl phosphate. 0.2 ml of each sample was added to 0.5ml of the ALP reagent (buffer and substrate) and mixed. The initial absorbance reading was taken at 450nm and timer was started, subsequent readings were taken at 1 minute interval for 3 minute. The enzyme activity was then estimated using the formulae: ALP activity (iu/l) = $\Delta A/\text{minute} \times 2760$.

Estimation of Superoxide Dismutase (SOD) Activity: The level of total SOD activity in liver homogenate was estimated by the method of Misra and Fridovich (1972). This method is based on the ability of superoxide dismutase to inhibit the auto-oxidation of adrenaline at pH 10.2. Superoxide anion generated by the xanthine oxidase reaction is known to be responsible for the oxidation of adrenaline to adrenochrome. The amount of adrenochrome produced per superoxide anion present increased with increasing pH (Valerino and McCormack, 1971) and also with increasing concentration of adrenaline. An aliquot of 0.2 ml of diluted tissue supernatant was added to 2.5 ml of 0.05 carbonate buffer (pH 10.2) to equilibrate in the spectrophotometer and the reaction was started by the addition of 0.3 ml of freshly prepared 0.3 mM epinephrine to the mixture which was quickly mixed by inversion. The reference cuvet contained 2.5 ml of carbonate buffer, 0.3 ml epinephrine and 0.2 ml of water and it serves as blank. Increase in absorbance at 480 nm was monitored every 30 seconds for 150 seconds. Increase in Absorbance per minute = $A_3 - A_0/1.5$, where A_0 = absorbance after 30 seconds and A_3 = absorbance after 150 seconds. % Inhibition = $100 - (100 \times \text{increase in absorbance for substrate}) / \text{Increase in absorbance for blank}$. One unit of SOD activity is given as the amount of SOD necessary to cause 50 % inhibition of the oxidation of epinephrine.

Estimation of Catalase Activity: The method of Sinha (1971) was used in the estimation of catalase activity. This method is based on the reduction of dichromate in acetic acid to chromic acetate when heated in the presence of hydrogen peroxide (H_2O_2) with the formation of perchromic acid as an unstable intermediate. The formed chromic acetate is then measured calorimetrically at 570 nm to 610 nm. 1 ml of the supernatant fraction of the liver homogenate was mixed with 19 ml distilled water to give a 1.20 dilution. The assay mixture contained 4ml of the H_2O_2 solution (800 μMoles) and 5 ml of phosphate buffer pH 7.0 in a 10ml flat bottom flask. 1ml of well diluted sample was rapidly mixed with the reaction mixture by a gentle swirling motion at room temperature. 1ml portion of the reaction mixture was withdrawn and blown into 2ml dichromate/acetic acid reagent at 60 seconds interval. The monomolecular velocity constant, K for the decomposition of H_2O_2 by catalase was determined by using the equation a first-order reaction. $K = 1/t \log S_0/S$, where S = initial concentration of H_2O_2 at 1 minute interval and S_0 = initial concentration of H_2O_2 , and t = time. The values of K were plotted against time in minutes and the velocity constant of catalase, K_0 at 0 minute was determined by extrapolation. The catalase content of enzyme preparation was expressed in terms of catalase feiahigkeit or 'kat f' as $\text{Kat f} = \text{K}/\text{mg protein/ml}$.

Data Analysis: The statistical significance of difference between groups was analyzed using the one-way analysis of variance (ANOVA) and Duncan's Multiple Range Test (DMRT). Statistical tests were done using the version 11 of SPSS package. Comparisons made were among the treated groups and with the control group.

RESULTS AND DISCUSSION

The results of this study showed that treatment with ethanol in a dose of 5gkg^{-1} body weight caused a significant increase ($P < 0.05$) in the plasma levels of ALT, AST, GGT and ALP by 87.87%, 105.82%, 82.24% and 78.34% (Table 1) respectively with a concomitant reduction in their hepatic concentrations thus: ALT by 36.27%, AST by 47.73%, GGT by 41.34% ALP by 35.17% (Figure 1).

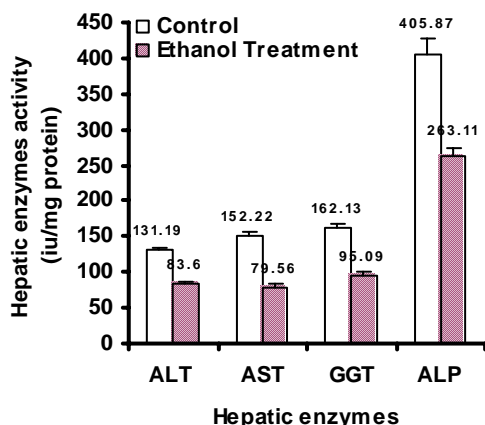


Figure 1: Effect of ethanol on hepatic activity (iu/mg protein) of liver enzymes

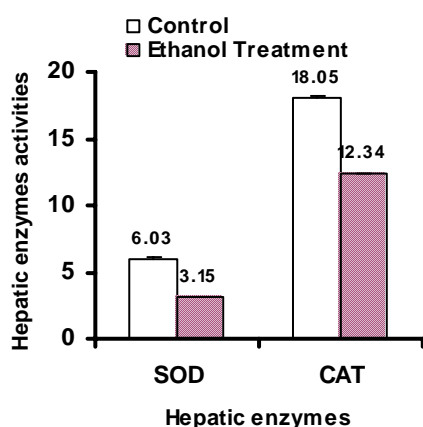


Figure 2: Effects of ethanol on SOD and CAT hepatic activities

The respective rise and decrease in the plasma and hepatic levels of these enzymes may be attributed to damaged structural integrity of the liver, which results in the leakage of these enzymes from the cytosol into the blood stream. This observation agreed with the report of Vermaulen *et al.* (1992) that ALT, AST, GGT and ALP are normally located in the cytoplasm and released into the circulation after cellular damage. The result is also consistent with the findings of Uzun *et al.* (2005) who reported a significant increase in the activity of plasma ALT and AST following ethanol administration.

Relative to the control group, treatment with ethanol markedly reduced the activities of hepatic SOD and CAT by 47.76% and 31.63% respectively (Figure 2). The reduction in the activities of these antioxidant enzymes may be due to the inhibition of their synthesis by some reactive molecules generated during ethanol metabolism, which occurs primarily in the liver. It could also be as a result of oxidation of the enzymatic proteins by the generated reactive oxygen species.

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