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AN INVESTIGATION ON THE HEPATOTOXIC EFFECTS OF GASOLINE ON MALE WISTER RATS

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ABSTRACT

The present study investigates the hepatotoxic effects of inhaled gasoline and gasoline contaminated diets on male wistar albino rats after four weeks of chronic exposure. Twelve (12) wistar albino rats were divided into three groups of four rats per group. Group One was established as the control group and was given normal rat feed and water, Group Two was subjected to gasoline inhalation, and Group Three was treated with gasoline contaminated feed. The LD_{50} value of gasoline was obtained as 128 mL kg^{-1}body weight of rats. The animals exhibited changes in behavioural pattern such as respiratory distress and sedation. The animals were sacrificed by cervical dislocation 24 hours after the final day of administration. Serum alanine amino transferase (ALT), aspartate aminotransferase (AST), and alkaline phosphate (ALP) levels, four weeks after feeding the rats with gasoline contaminated diets increased significantly ($p \leq 0.05$). Also, these parameters (ALT, AST, and ALP) increased significantly, four weeks after subjecting the rats to gasoline inhalation ($p \leq 0.05$). Feeding the rats with gasoline contaminated feed for four weeks also resulted in remarkable decrease in final body weights and percentage weight increase of test animals compared to control group. This significant decrease was also seen in the group subjected to gasoline inhalation for four weeks. Histological examination of the liver indicated that gasoline contaminated diets induced significant degenerative changes in the structural integrity of the hepatic cells. These degenerative changes were also observed in the rats subjected to gasoline inhalation. The results obtained in this study suggest that long term inhalation and ingestion of gasoline induced stress which impaired liver structural integrity and functions.

KEYWORDS: *Gasoline, Hepatotoxicity, Liver, Structural Integrity, Liver Functions, cytolethality, Aspartateaminotransferase (AST), Alanineaminotransferase (ALT) and Alkaline phosphatase (ALP.*

INTRODUCTION

Hepatotoxicity refers to liver dysfunction or liver damage that is associated with an overload of drugs or xenobiotics.[1] The chemicals that cause liver injury are called hepatotoxins or hepatotoxicants.[2] Hepatotoxicants are exogenous compounds of clinical relevance and may include overdoses of certain medicinal drugs, industrial chemicals, and natural chemicals like microcystins, herbal remedies and dietary supplements.[3] Certain drugs may cause liver injury when introduced even within the therapeutic ranges.[4] Hepatotoxicity may result not only from direct toxicity of the primary compound but also from a reactive metabolite or from an immunologically-mediated response affecting hepatocytes, biliary epithelial cells and/or liver vasculature.[5] The hepatotoxic response elicited by a chemical agent depends on the concentration of the toxicant, which may be parent compound or toxic metabolite, differential expression of enzymes and concentration gradient of cofactors in blood across the acinus.[6] Hepatotoxic response is expressed in the form of characteristic patterns of cytolethality in specific zones of the acinus.[7] Hepatotoxicity related symptoms may include jaundice or icterus; an appearance causing yellowing of the skin, eyes and mucous membranes due to high level of bilirubin in the extracellular fluid, pruritus, severe abdominal pain, nausea or vomiting, weakness, severe fatigue, continuous bleeding, skin rashes, generalized itching, swelling of the feet and/or legs, abnormal and rapid weight gain in a short period of time, dark urine and light coloured stool.[8]

Liver is the largest internal organ of the human body weighing approximately 1500 g, and is located in the upper right corner of the abdomen on top of the stomach, right kidney and intestines and beneath the diaphragm. The liver performs more than 500 vital metabolic functions.[9] It is involved in the synthesis of products like glucose derived from glycogenesis, plasma proteins, clotting factors and urea that are released into the bloodstream. It regulates blood levels of amino acids. Liver parenchyma serves as a storage organ for several products like glycogen, fat and fat soluble vitamins. It is also involved in the production of a substance called bile that is excreted to the intestinal tract. Bile aids in the removal of toxic substances and serves as a filter that separates out harmful substances from the bloodstream and excretes them.[4] An excess of chemicals hinders the production of bile thus leading to the body's

inability to flush out the chemicals through waste. Smooth endoplasmic reticulum of the liver is the principal 'metabolic clearing house' for both endogenous chemicals like cholesterol, steroid hormones, fatty acids and proteins, and exogenous substances like drugs and alcohol. The central role played by liver in the clearance and transformation of chemicals exposes it to toxic injury[4]. The use of gasoline in the industries and homes has rapidly increased in the recent times. In the course of usage, individuals are frequently exposed to pollutants from gasoline fuel in both outdoor and indoor environments. However, the major route of exposure is inhalation by workers during production and distribution of the fuel, and by the general public during refuelling at service stations.[10]

Gasoline, one of the fractionated products of crude oil, is widely used as fuels for automobiles and some electricity generating machines. Gasoline is known to be a very volatile liquid, with several organic and inorganic constituents. Gasoline vapours may be derived from direct evaporation of liquid gasoline.[11] These vapours, which are ubiquitous in the environment and constitute some components of petroleum pollutants in the air, are of great environmental and human health concerns. Exposures to these pollutants are common in the refineries, oil fields, refuelling stations, petrochemical industries, motor mechanical workshops, and traffic-congested areas. However, the population at greater risk of frequent exposure includes those occupationally exposed.[12] It has been reported that the oil drillers, refinery workers, petrochemical workers, refuel station attendants and motor mechanics suffer the greater risk of chronic exposures to petroleum pollutants.[13]

Aim: To examine if gasoline can cause an increase or decrease of liver enzyme markers in male wistar rats.

Objectives of the study

i. To determine the effect of gasoline vapour on the histology and enzymes of the liver.
ii. To determine the effect of gasoline contaminated food on the histology and enzymes of the liver.

MATERIALS AND METHODS

Materials

Gasoline, Laboratory materials (steel cages, test tubes, non-heparinised bottle, cotton wool, dissecting board, dissecting kit, hand gloves, masking tape, centrifuge), normal saline, distilled water, beakers, syringes.

Animals

Wistar Albino rats weighing between 180 and 250g were used in this experiment. The rats aged between 8 and 10 weeks were obtained from Bingham University Animal House.

The animals were kept in well ventilated stainless-steel cages at room temperature (24±2˚C) in hygienic condition under natural light and dark schedule and were fed on standard laboratory diet. Food and water were given *ad libitum*. The study was conducted after obtaining the institution's Animal Ethical Committee clearance.

Experimental design

The rats were divided based on their weights into three groups of four rats each. The LD_{50} value of petrol was obtained as 128mL/kg body weight of rats.

Group I (Control): No exposure to gasoline vapour or treatment with gasoline contaminated feed.

Group II (Inhalation): Experimental test group 1; Exposed to gasoline vapour only, for 6 hours per day, (9:00 am to 3:00 pm).

Group III (Feed): Experimental test group 2; Treated with gasoline contaminated feed daily.

Exposure to gasoline vapour

The animals in the test group 1 were exposed to gasoline vapour in an exposure chamber. The rats were placed inside the chamber with a 1000ml bottle filled with 500ml gasoline, and perforated at different parts of the top for easy diffusion of ungraded concentrations of the vapour generated from direct evaporation of liquid gasoline. The test animals were allowed to inhale the evaporating vapour in the chamber during the exposure period. An exposure period of 6 hours daily (9:00am to 3:00pm) was adopted for 4 weeks.

Treatment with gasoline contaminated feed

The animals in test group 2 were treated with gasoline contaminated feed. The LD_{50}value of gasoline, as calculated was multiplied by a factor of +2.5 to make up for loss of the volatile components of gasoline. Calculated volume of the gasoline was then mixed with measured amounts of animal feed and the mixture was compacted with water to form a compact mass. The feed was then moulded into small balls, air-dried and stored in a well-labelled sack to last for the duration of the test period.

Histological study

After the end of the period of administration, the rats were weighed and then sacrificed by cervical dislocation. The liver of each rat was excised through thoraco-abdominal incision. Histological investigation of the liver was done. The organ pieces (3-5 µm thick) were fixed in 10% formal saline for 72hrs. Samples were processed using an open automatic tissue processor through graded ethanol, cleared xylene, impregnated and embedded in paraffin wax. Thin sections were cut with rotary microtome, stained by haematoxylin and eosine technique, examined microscopically for histological changes.[14]

Biochemical study of serum

Biochemical tests are of immense value in diagnosis and monitoring of liver diseases, these tests are usually referred to as Liver Function Tests (LFT), and these tests are the most widely performed biochemical tests in the laboratory.[15] These Liver Function Tests are broadly classified as tests to detect hepatic injury and to assess hepatic function.[15] They include Alanine amino transferase (ALT), Aspartate amino transferase (AST), and Alkaline Phosphatase (ALP). Blood collected into non-heparinized tubes were centrifuged at 3000 rpm for 10 min. The serum separated was analysed to evaluate the liver enzymes [Aspartateaminotransferase (AST), Alanineaminotransferase (ALT) and Alkaline phosphatase (ALP), using the method of .[14]

Statistical Analysis

Results were expressed as the mean ± standard error of mean (SEM). Statistical analysis of data was carried out using Student's T-test.

RESULTS AND DISCUSSION

Histological Analysis

Histological profile of the rats' liver fed with normal rat feed (Fig 1) showed a hepatic lobule with a central vein (CV) surrounded by cords of hepatocytes separated by sinusoids (S). The individual hepatocytes showed vesicular nuclei with moderate amount of chromatin material, with kupffer cells lining the sinusoids. Histological profile of the rats' liver exposed to gasoline vapour (Fig 2) showed a hepatic lobule with a central vein (CV) surrounded by radiating cords of hepatocytes that are separated by sinusoids. There is an infiltration of the liver parenchyma by lymphocytes (lnf). The hepatocytes nuclei generally showed prominent nuclei with occasional enlargement and hyperchromasia indicative of reactive changes. Also, histological profile of rats' liver exposed to gasoline vapour (Fig 3) showed individual

hepatocytes showing nucleoli and cytoplasmic voculation indicative of tissue injury. There is infiltration of polymorphonuclear leucocytes (lnf) at the perilobular borders and to a lesser extent around the cords of hepatocytes. Histological section of the rats' liver exposed to gasoline vapour (Fig 4) also showed portal tract (pt) infiltration by polymorphonuclear leucocytes and on both sides of the portal tract are hepatocytes showing nuclei with prominent nucleoli, and slightly enlarged. There is slight cytoplasmic vacoulation. Histological section of rats' liver treated with gasoline contaminated feed (Fig 5) showed the portal tract (pt) with mild infiltration of lymphocytes (lnf), there is also a portion of liver parenchyma with nuclei showing chromatin granules with prominent nucleoli. Moderate amount of lymphocytes are also seen in liver parenchyma.

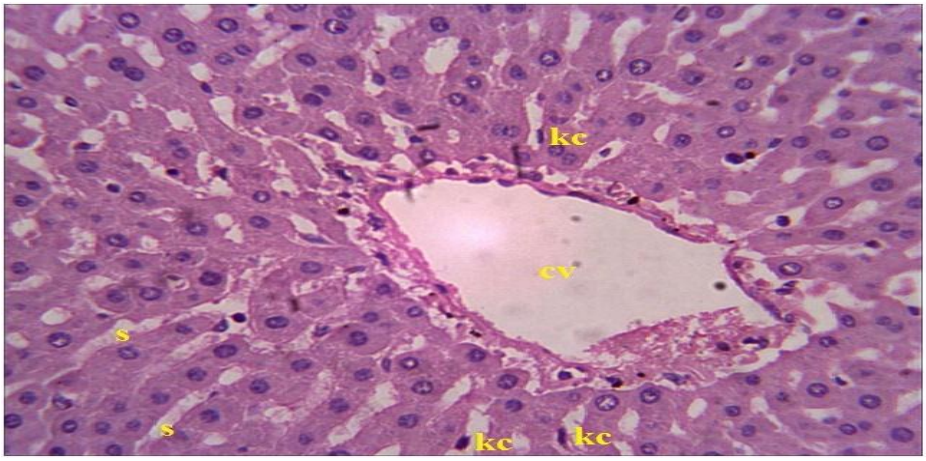


Fig 1: ***Photomicrograph of a cross-section of control (Group I) of rat liver given only normal feed showing CV = Central Vein surrounded by cords of hepatocytes separated by S = sinusoids. Lining the sinusoids are kc = kupffer cells. H&E x400***

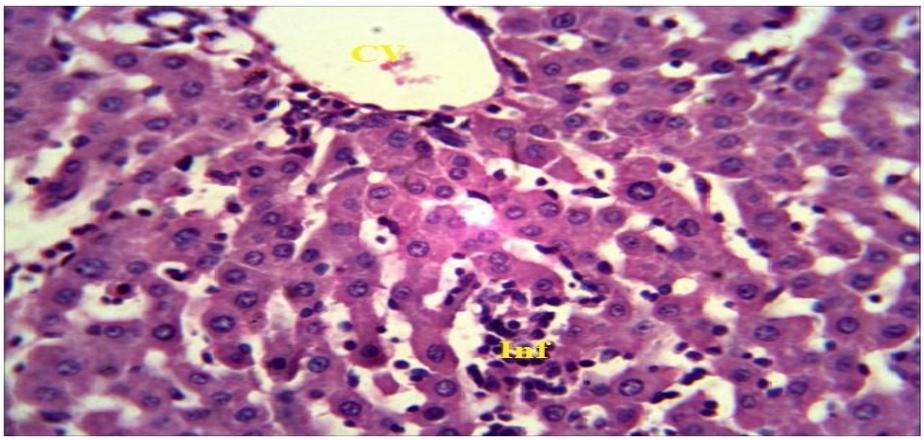


Fig 2*: photomicrograph of a cross section of Group 2 rat liver exposed to gasoline vapour showing CV = central vein surrounded by radiating cords of hepatocytes that are separated*

by sinusoids. lnf = lymphocytes. Hepatocytes nuclei generally show prominent nuclei with occasional enlargement and hyperchromasia indicative of reactive changes. H&E x400

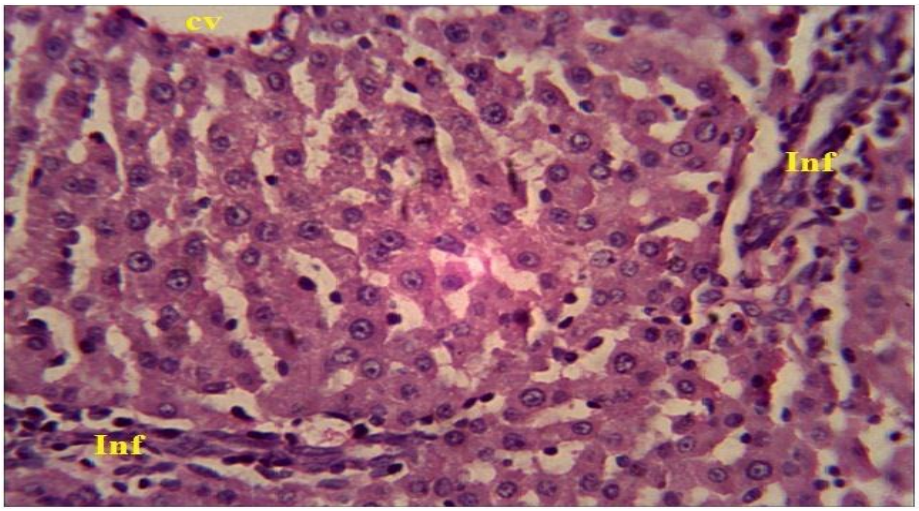


Fig 3: ***Photomicrograph of a cross section of Group 2 rat liver, exposed to gasoline vapour showing part of the cv = central vein from which cords of hepatocytes radiate. The individual hepatocytes show prominent nucleoli and cytoplasmicvacoulation, indicative of tissue injury. There is infiltration of polymorphonuclearlnf = leucocytes at the perilobular borders and to a lesser extend around the cords of hepatocytes. H&E x400***

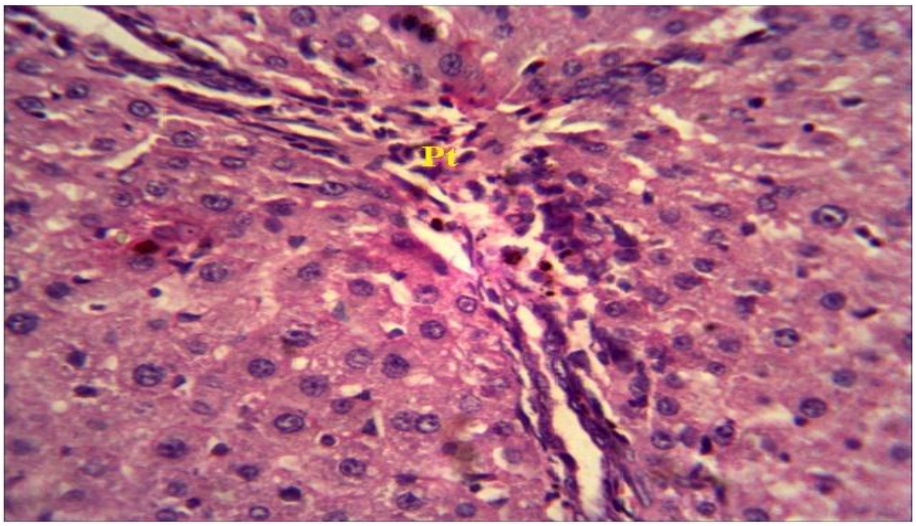


Fig 4: ***Photomicrograph of a cross-section of Group 2 rat liver exposed to gasoline vapour showing pt = portal tract infiltration by polymorphonuclear leucocytes and on both side of the portal tract are hepatocytes showing nuclei with prominent nucleoli and slightly enlarged. There is slight cytoplasmic vacoulation. H&E x400***

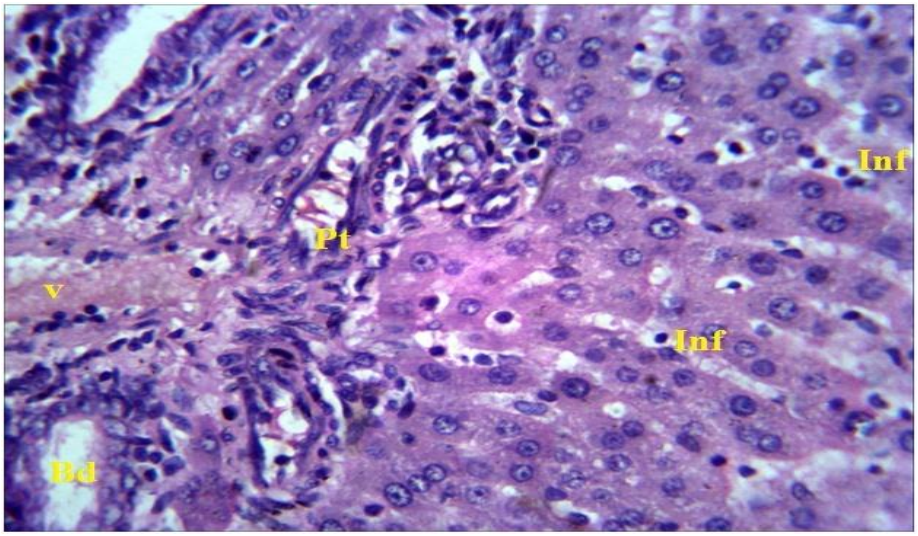


Fig 5: ***photomicrograph of a cross-section Group 3 rat liver treated with gasoline contaminated feeds showing the pt = portal tract with mild infiltration of lnf = lymphocytes, there is also a portion of liver parenchyma with nuclei showing chromatin granules with prominent nucleoli. Moderate amount of lymphocytes are also seen in the liver parenchyma. The bd = bile duct and v = vein are seen. H&E x400***

Histological procedures

Excised rats livers were fixed for 72 hours in organ bottles and were transferred to an open system of autonomic tissue processor for proper processing of the tissues as follows.

Fixation: Each organ (liver) was fixed in 10% formal saline. Fixation is the first step in this process and it is done to help preserve the cell and tissue component and make them exist in a life-like state and to prevent autolysis and decomposition by bacteria.

Dehydration: This was achieved using ascending grades of alcohol. The procedure was used to remove water constituents from the tissue: (70 %, 80%, 90%, and absolute 100%) in an hourly basis for each grade of alcohol.

Clearing: This was used to remove the constituents of alcohol from the tissue and was achieved by using the agent xylene and it was placed in two changes of xylene after 1 hour.

Infiltration: This is the immersion of tissue in molten paraffin wax. Molten paraffin wax melted at 70◦C was used to provide support for tissue by filling the holes created by clearing agents. It helps in proper embedding. This occurred in 2 changes of Paraffin Wax at 56◦C 1 hour each.

Embedding: After tissue processing, the tissue is then taken to the embedding machine using embedding mould greased with glycerol. The embedding mould is filled with molten paraffin wax, the tissue is picked with a forceps and oriented at the centre of the embedding mould, and then the cassette will be placed on it to allow it to solidify and then allowed to cool when placed in freezer. The solid blocks are then removed and trimmed to appropriate sizes.

Sectioning: The block of tissue is placed in the microtome and trimmed to expose the organ surface, after which the block is cooled on ice block to allow easy sectioning. The microtome is set to 3-5 micron and sectioned. Sections were picked with forceps and placed on a slide. 20% of alcohol was used to float section in water bath to allow the section to spread well and picked with a slide. The slide is labelled with a pencil according to the number on the block. After labelling of slide it is placed on hot plate for section to stick to the slide.

Staining: Haematoxylin and Eosin[16]

- Arranging dried slide in staining rack.
- De-wax in 2 changes of xylene for 10 minutes.
- Hydrate in descending grades of alcohol i.e from absolute to 70%.
- Rinse in water for a few minutes.
- Stain in haematoxylin (depending on the age of haematoxylin). This stains the nucleus blue.
- Rinse in water.
- Differentiate 1% acid alcohol.
- Blue in slow running tap water for 10 minutes.
- Counter-stain in eosin for few minutes, rinse in water.
- Dehydrate in ascending grades of alcohol i.e. in 70%, 80% and absolute.
- Dry in hot plate.
- Clear in xylene and mount using DPX mountant and cover slips (Dibutylphopshatexylene).
- Allow slides to dry in an airy place.

Biochemical procedures[17,18] Liver Function Test (LFT) ALT, AST & ALP kits were used.

For ALT, AST, and ALP, the materials used for the procedure include; Buffer, 2,4-dinitrophenylhydrazine, 0.4mol/L Sodium Hydroxide, Distilled water, De-ionized water. Procedure was carried out according to Reitman and Frankel's method.

BIOCHEMICAL ANALYSIS

The result of the effects of gasoline vapour and gasoline contaminated feed on serum biochemical parameters is presented in Table 1. There was significant (P≤0.05) increase in the levels of AST, ALP and ALT in Group 2 and 3.

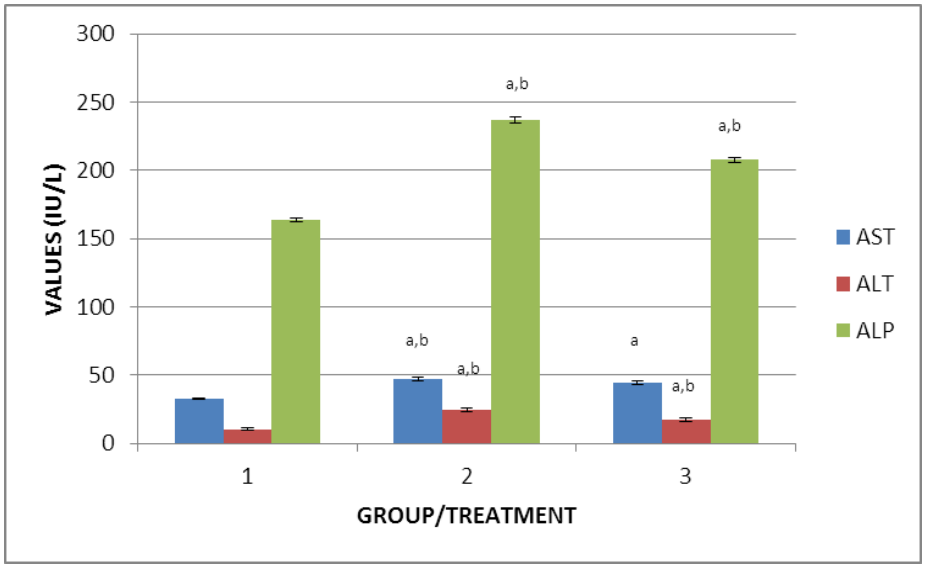
Table 1: Showing Biochemical Analysis of Alanine Aminotransferase (Alt), Aspartate Aminotransferase (Ast) and Alkaline Phosphatase (Alp)

GROUP	TREATMENT	AST (IU/L)	ALT (IU/L)	ALP (IU/L)
1	CONTROL	33.0 ± 0.41	10.5 ± 0.87	163.75 ± 1.75
2	INHALATION	47.0 ± 1.63[a,b]	24.5 ± 1.25[a,b]	237.25 ± 2.25[a,b]
3	FEED	44.5 ± 1.50[a]	17.25 ± 1.49[a,b]	207.75 ± 2.06[a,b]

[a]*signifies that p≤0.05 when compared with control, showing statistical significance.*

[b]*signifies that p≤0.05 when comparing inhalation with feed, showing statistical significance*

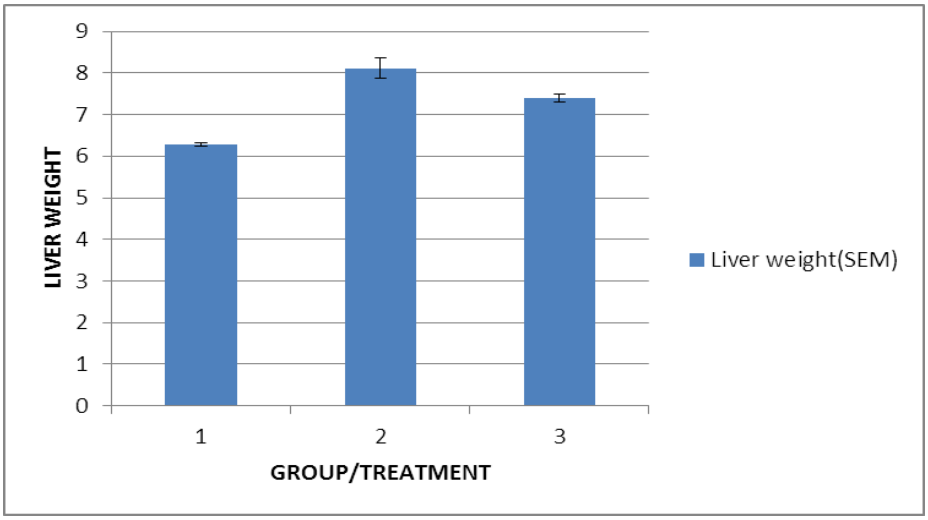
Values are Mean ± SEM



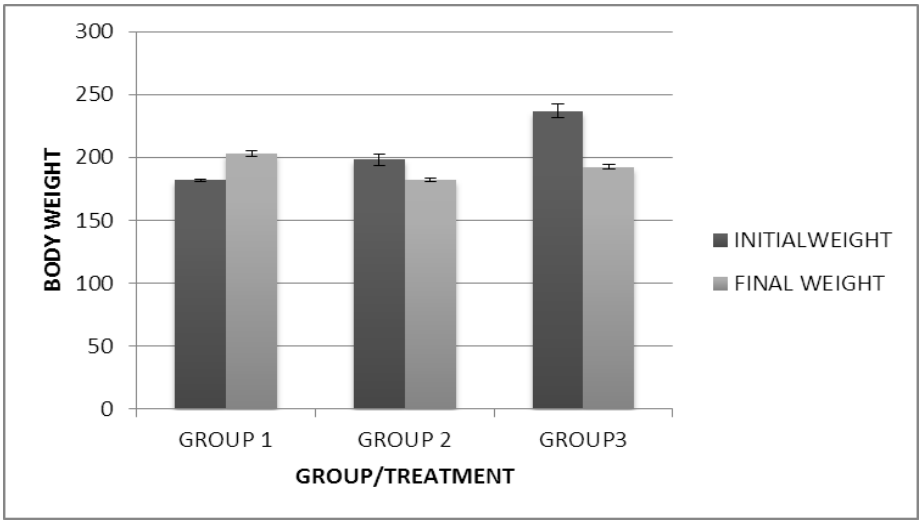
Graph 1: Showing Biochemical Parameters of Ast Alt and Alp Mean±Sem.

Table 2: Showing Animal Bodyweight/Liver Weight.

Group/Treatment	Initial body weight (MEAN±SEM)	Final body weight (MEAN±SEM)	Liver weight (MEAN±SEM)	Percentage mean body wt. increase (%)
GROUP 1 Control	181.75±1.181	203.00±2.380	6.28±0.054	+11.692
GROUP 2 Inhalation	198.25±4.534	182.00±1.080	8.11±0.249	-8.196
GROUP 3 Feed	237.00±5.400	192.50±2.101	7.39±0.096	-23.117



GRAPH 2: SHOWING LIVER WEIGHT MEAN ± SEM



GRAPH 3: SHOWING INITIAL AND FINAL BODY WEIGHT

DISCUSSION

As shown by the elevation in the levels of liver marker enzymes (ALT, AST, ALP) (Table 1), administration of gasoline resulted in a significant hepatic damage in Group 2 compared

to the control group (group 1). Also there was significant hepatic damage in group 3 compared to group 1, as well as group 2 when compared to group 3.

The result of the enzyme obtained shows elevated levels in the serum content of hepatic enzymes in rats administered gasoline through inhalation and feed contamination. In particular, the elevation of ALT is indicative of liver damage.[19,20] These enzymes are located in the cytoplasm and are emptied into the circulation once the cellular membrane is damaged.[19,21] High levels of AST indicates liver damage, such as that caused by viral hepatitis as well as cardiac infarction and muscle injury, ALT catalyzes the conversion of alanine to pyruvate and glutamate and is released in a similar manner. Therefore ALT is more specific to the liver, and thus a better parameter for detecting liver injury. Elevated levels of serum enzymes are indicative of cellular leakage and loss of functional integrity of cell membrane in liver. The result in this study is also in agreement with the findings of Sallie *et al*[22], which discovered that the rise in the enzyme AST is usually accompanied by an elevation in the levels of ALT, which plays a vital role in the conversion of amino acids to keto acids. This increase may be due to the abnormal dynamic properties of cellular membranes following exposure to hydrocarbon fractions present in kerosene and petrol. The changes in the cell membrane may have been as a result of the reactive free radical species from the metabolism of aliphatic and aromatic hydrocarbons which are the major constituents of petroleum products as well as other xenobitics.[23,24]

Alkaline phosphatase activity in the animals exposed to gasoline vapour and gasoline-contaminated diet were significantly higher ($p \leq 0.05$) when compared to the control animals. However, the remarkable increase in the level of ALP in the animals in group 2 and 3 may imply that damages occurred more in the liver cells of rats that inhaled gasoline than those fed with gasoline-contaminated feed, since the activity of this enzyme in the serum is reported to be increased in the liver damage.[25] Alkaline phosphatase is involved in the transport of metabolites across the cell membranes, protein synthesis, synthesis of certain enzymes, secretory activities and glycogen metabolism. However, the increase in this enzyme activity may not be unconnected with a disturbance in the transport of metabolites or alteration in the synthesis of certain enzymes as in other hepatotoxic conditions.[26]

Result observed in animals in Group 2 and 3 showed a marked decrease ($p \leq 0.05$) in the final body weight and percentage weight increase. Also, there was an increase in the relative liver

weight (hepatomegaly) and a reduction in the percentage weight increase in rats following exposure to gasoline vapours.

Metabolism of aliphatic and aromatic hydrocarbons, the major constituents of petroleum and petroleum-derivatives, as well as other xenobiotics generate a significant increase in the level of free radical species in various tissues. However, the decrease in overall body weight and organ weight may be an adaptive feature or a pathological response to the toxic compounds (hydrocarbon) present in the gasoline.

From the biochemical and histological analysis, it can be deduced that the elevated levels of these biochemical parameters are direct reflection of alterations in the hepatic structural integrity.

CONCLUSION

The findings in this study indicate that administration of rats with gasoline via inhalation or feed contamination caused a significant increase in hepato-specific serum enzymes. Thus, long term inhalation of gasoline and ingestion of gasoline contaminated products can cause severe impairment to the functions and structural integrity of the liver. Efforts should therefore be made to reduce the level of exposure to gasoline, especially with those that have occupational exposure. This could be either in the number of hours of exposure, masking the nose and mouth to reduce inhalation, or taking food and drinks far away from being contaminated with the product.

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