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CYTOTOXIC POTENTIALS OF THIOCYANATE ADMINISTRATION ON THE LIVER OF MALE WISTAR RATS (*RattusNorvegicus*)

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ABSTRACT

The use of thiocyanate as an anti-sickling drug is currently on the increase among sickle cell disease (SCD) patients. The continuous use of this substance without sufficient toxicity data does not guaranty continuously functional and healthy internal organs among the SCD patients that are susceptible to multi-organ failure such as hepatic failure. Hence this study was performed to elucidate the consequence(s)of thiocyanate administration on the liver of adult male wistar rats. Twenty adult male wistar rats with an average weight of 234.5g were used. The rats were grouped into four (A, B, C & D) with five animals in each group. Group A represented the control and was given only 1ml of distilled water daily while B,C,&D received 1ml of thiocyanate solution at doses of 10mg/Kg/day, 20mg/Kg/day, & 30mg/Kg/day for twenty-eight (28) days. The administration was carefully done with the use of an oral cannula. Thereafter, the rats were sacrificed via cervical dislocation. The rats were dissected and blood samples were immediately collected from the apex of the heart for the analysis of serum total bilirubin. A fraction of the liver was cut to prepare homogenates for biochemical enzymes (alanine aminotransferase-ALT, aspartate aminotransferase-AST) analysis. Thereafter the animals were wholly perfused with normal saline and then fixed with 4% paraformaldehyde. The fixed liver tissues were then taken for histological assessments. The slide sections (H&E and PAS stains) in the treated groups showed varying degrees (mild to severe disruption of hepatocellular morphology plus prominence and widening of sinusoids) of thiocyanate-induced liver damage. But hepatocellular appearance was normal in the control group. Biochemical assays of serum total bilirubin and tissue AST increased fairly with increasing dose although this was not significant. The increase in tissue level of ALT in group B was also not significant with respect to the control. But increase in ALT level was found to be significant in groups C&D when compared with the control. Therefore, this study can be used to infer that SCN use in sickle cell disease management regimen can cause hepatocellular damage in wistar rats.

Key Words: Thiocyanate,Biochemical enzymes,Hepatocellular morphology, Anti-sickling

INTRODUCTION

Thiocyanates (SCN) are a group of compounds formed from the combination of sulphur, carbon, and nitrogen. Thiocyanates are found in various foods and plants; they are produced basically from the reaction of free cyanide with sulphur. This reaction takes place within the environment (e.g. in industrial waste streams that contain cyanide) and in the human body after cyanide is swallowed or absorbed (ATSDR, 2006). Thiocyanate is the major product formed from cyanide that passes into the body as the body attempts to get rid itself of cyanide. Albeit thiocyanates are less harmful than cyanide in humans, they are known to affect the thyroid glands, reducing the ability of the gland to produce hormones that are necessary for the normal function of the body (ATSDR, 2006). Thiocyanate inhibits formation of (the abnormally shaped) sickle cells that contribute to obstruction of blood vessels. The concurrent intake of thiocyanate,

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eicosapentaenoic acid (EPA), and docosahexaenoic acid (DHA) can offer synergistic clinical benefit in sickle cell disease (SCD) than each agent taken singly (Okpala *et al.,* 2013).Thiocyanate has long been used to resolve cases of sickle cell crisis in-vitro and few preliminary clinical trials with a disappearance of pain and discomfort in the patients (Oyewole and Malomo, 2008). However, it has since been neglected only to be rediscovered many decades later (Oji, 1986). Thiocyanate in red blood cells is oxidised into cyanate by the haemoglobin which acts as a peroxidase (Chung and Wood, 1971).

The liver is one of the prime organs in the body that performs several different functions in the body. Although the liver's main function is the barrier-cleansing, it can also serve as a powerful filter, remove and destroy bacteria and neutralizing toxins which are produced by metabolic reactions. The liver could be adversely affected in cases of poisoning, drug overdose and poor nutrition. However, it rarely makes itself felt at the beginning of the disease because of its high functional reserve. Often times, when the symptoms manifest, it is obvious that the damage has been done and extensive. Among other functions of the liver is the production of a large amount of enzymes which when hepatocytes are destroyed they leak directly into the blood and serve as useful indicator of the degree of liver damage (Burtis*et al*., 2006). Therefore, liver cannot be overlooked in the use of thiocyanate as an anti-sickling drug.

MATERIALS AND METHODS

Ethics concerning experimental animals

The experimental protocol was approved by the University Ethical Review committee, University of Ilorin, Ilorin, Nigeria. The research was approved to be in compliance with the Institutional Animal Care and Use Committee (IACUC).

Experimental animals

Twenty (20) adult male Wistar rats with an average weight of 230.5g were obtained from the Faculty of Veterinary Medicine of the University of Ilorin and acclimatized in the animal house of the College of Health Sciences in the same university for two weeks. The animals were kept at normal room temperature. Pelletized rats feeds and water were made available*ad libitum*. The animals were grouped randomly into four, each comprising of five animals. Group A served as control while groups B, C, and D were given 10mg/Kg/day (therapeutic dose, TD), 20mg/Kg/day and 30mg/Kg/day (both test lethal dose) thiocyanate (SCN) respectively. Just before the start of administration, all the animals were weighed and records kept. The weighing was continued weekly throughout the period of the treatment.

Preparation of Thiocyanate solution

The Potassium thiocyanate (KSCN) administered is a product of Labtech chemicals and was bought from Labtrade(Nig.) Company, Ilorin, Nigeria. The salt pellet was measured carefully using sensitive weight scale with respect to the weight of the animals and dose specified for each test group. Thereafter, it was dissolved in distilled water and shaken vigorously to obtain a homogenous solution.

Group A (Control): Distilled water
Group B : 10mg/Kg/day
(Oyewole and Malomo, 2009)
Group C : 20mg/Kg/day
Group D : 30mg/Kg/day

Drug administration

Distilled water - The control group (A) was given 1.0ml of distilled water daily alongside the treated group;

Thiocyanate solution- The treated groups (B, C, & D) was given 1.0ml/day of the thiocyanate solution.

The thiocyanate solution was given through the oral cavity using an oral cannula daily for a period of twenty-eight (28) days (Oyewole*et al.,* 2009).

Animal sacrifice

On completion of the 28 days treatment period, all the experimental animals were sacrificed via cervical dislocation. The animals were pinned to a dissecting board after which an incision through the lineaalba and sternum was made to expose the thoraco-abdominal region. Part of the liver was cut for homogenate sample and the rest were properly fixed with 4% paraformaldehyde by the perfusion method.

Blood collection

Blood samples were drawn from the apex of the heart of the rats in every group using a 5ml syringe. The blood samples were then emptied into plain specimen bottles for onward biochemical assay of total bilirubin. This was done before the perfusion fixation.

Homogenate preparation

The liver samples obtained from each animal before fixation were weighed, dropped into separate plain sample bottles containing corresponding volume of ice cold 0.25M sucrose solution. Each of the sample was homogenized using mortar and pestle. The resulting homogenates were examined for the biochemical assay of *alanine aminotransferase (ALT)* and *aspartate aminotransferase (AST)*.

Histological and staining procedures

The liver tissues were properly fixed in 4% paraformaldehyde and processed duly. They were stained with haematoxylin and eosin (H&E) and periodic acid-Schiff (PAS) after which observations were made with a light Olympus microscope at a magnification of ×400.

Biochemical assay

Common and specific hepatocellular marker were analysed from the tissue homogenate prepared. Alanine aminotransferase (ALT) and aspartate aminotransferase (AST) was determined by the modified method of Reitman and Frankel which is based on the amount of pyruvate and oxaloacetate hydrazine produced in each case (Reitman and Frankel, 1957).

Similarly, the serum total bilirubin was determined as done routinely by the description of Burtis and Ashwood (Balisteri and Rej, 1994).

Statistical analysis

The animal weights and biochemistry data were analysed for mean, standard error of mean and significant difference by ANOVA using the SPSS version 22.0 for windows. Values of $P<0.05$ were considered to be statistically significant.

RESULTS

Gross/physical observations

All the animals showed no spontaneous adverse reaction to the different doses of SCN treatment. The animals were closely monitored over the first 24-hours post administration and subsequently throughout the course of treatment. Over the time, the animals were generally calm and relaxed with no obvious "agitations, hyperreflexia, tinnitus, miosis" lesion, discoloration or deformities noticed. Varying weight changes were also detected among the groups. For group A (control) there was a 15.4g gain in weight before sacrifice was made. The weight change in group B (10mg/Kg/day) was non-uniform though a little gain was finally observed. The weight changes in the test groups C (20mg/Kg/day), and D (30mg/Kg/day) were relatively progressive. Generally the changes in weight measured before and after SCN administration were not statistically significant across all the four experimental groups. This is illustrated in Table 1 and Figure 1 below:

Table 1: Comparison of weight of rats before and after the administration

Weight differences in the experimental animal groups			
	Final weight (g)	Initial weight (g)	Differences (g)
A (control)	311.6±61.88247	296.2±54.19373	15.4
B (10mg/Kg/day)	201.6±29.59459	181.2±19.74234	20.4
C (20mg/Kg/day)	218.4±27.57970	182.8±15.57408	35.6
D (30mg/Kg/day)	288.8±7.85875	261.6±31.99528	27.2

Values are mean± SEM of five animals.

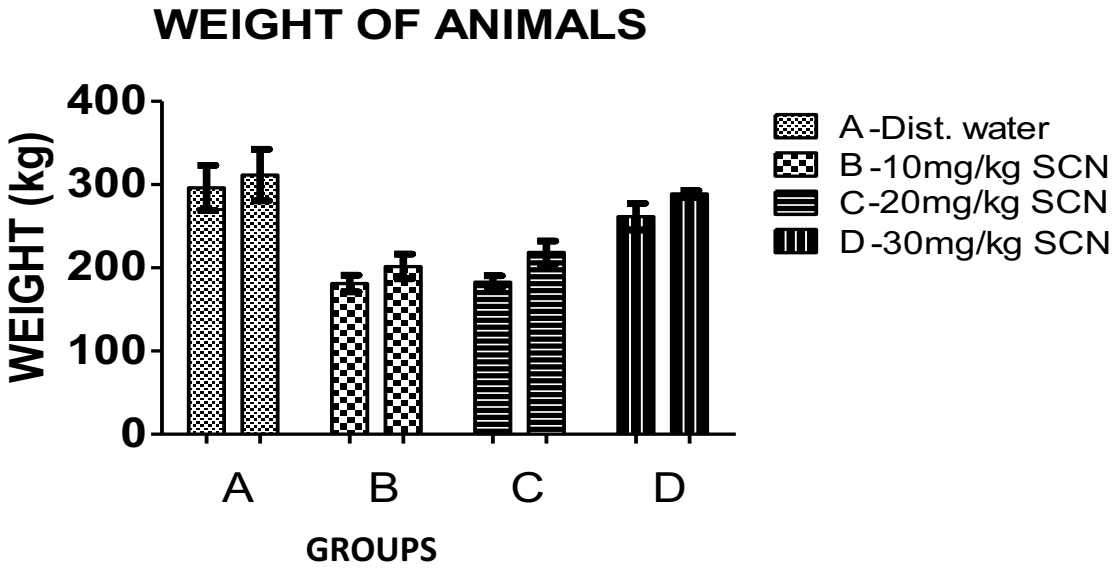

Figure 1: Bar chart showing the mean weight of experimental animals before and after administration.

Histological analysis

Experimental rats treated with 10mg/Kg/day SCN exhibited mild cellular hyperplasia, vascular congestion, and prominent sinusoids. Severe cellular hyperplasia with lysed red blood cell (RBC) filled central vein and disruption of sinusoidal pathway appear in the rats given 20mg/Kg/day SCN. The group which received 30mg/Kg/day displayed multi-foci cellular and fatty degeneration. Their hepatocytes were also compressed with ballooning sinusoid. But rats given distilled water showed normal hepatocytes with neither cell damage nor death in slide sections as shown below:

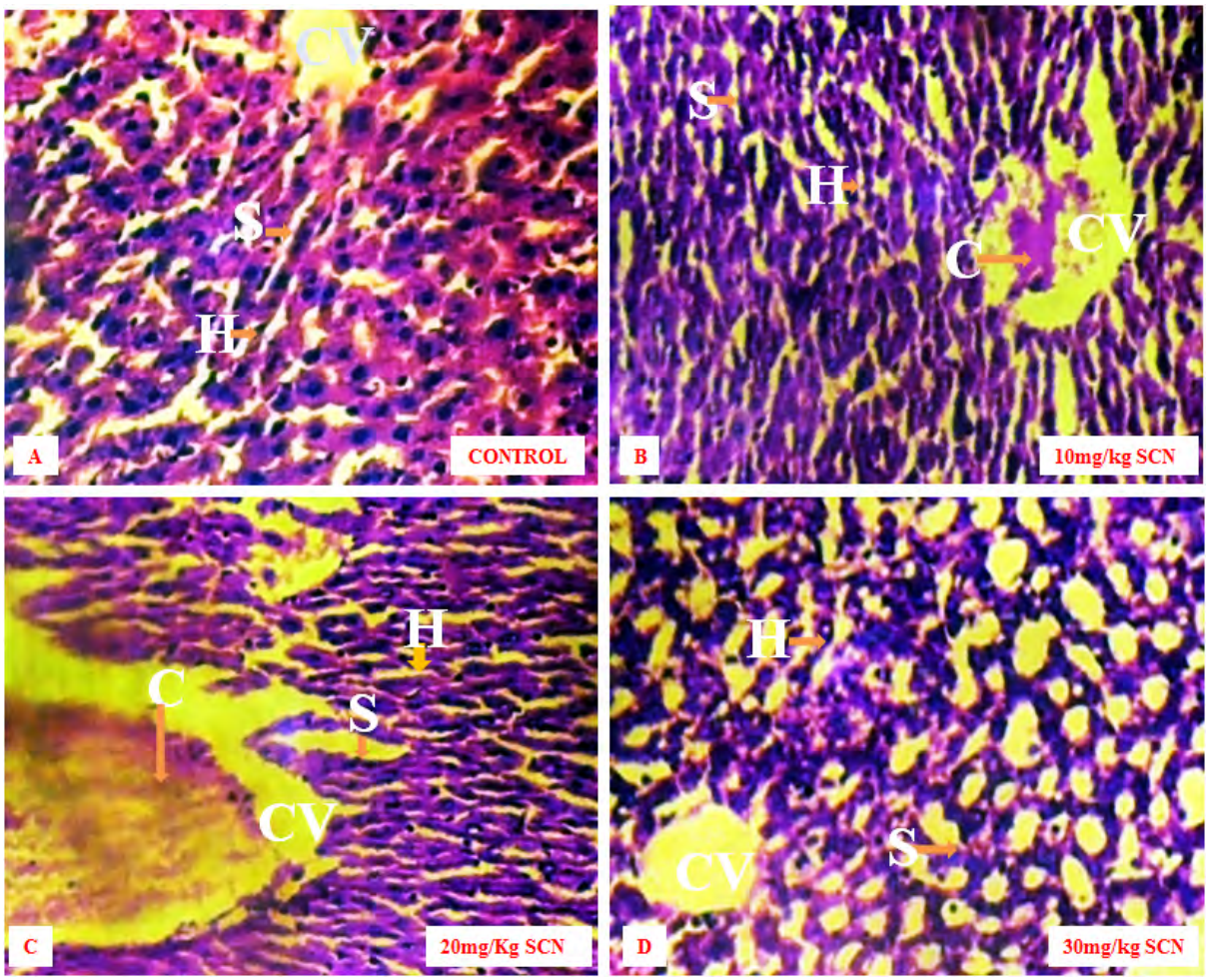

Figure 2: Liver (H&E×400): Histology of the rat liver that was given 1ml distilled water, 10mg/Kg/day, 20mg/Kg/day & 30mg/Kg/day SCN respectively. (A) Given 1ml distilled water/day for 28 days, shows normal central vein (CV), hepatocyte (H) and sinusoid(S). (B) Given 10mg/Kg/day SCN for 28 days, shows mild cellular hyperplasia and vascular congestion due to lysed red blood cells (RBCs). (C) Given 20mg/Kg/day SCN for 28 days, shows severe cellular hyperplasia and disintegration. The central vein also contains lysed RBC. (D) Given 30mg/Kg/day SCN for 28 days, show multi-foci cellular and fatty degeneration with its hepatocytes(H) compressed by ballooning sinusoids.

Biochemical assay

The analysis of biochemical enzymes (ALT&AST) in tissue homogenate showed a relative increase though not absolutely consistent. Quantitatively determined serum total bilirubin also increased progressively from the control to group D which received 30mg/Kg/day for the twenty eight day period. The elevation in the levels of AST and total bilirubin was not significant among the treated groups. However the increase observed in ALT levels was significant in groups C & D but not in group B when compared with the control. This is illustrated in Table 2 and Figures 4 - 6 below:

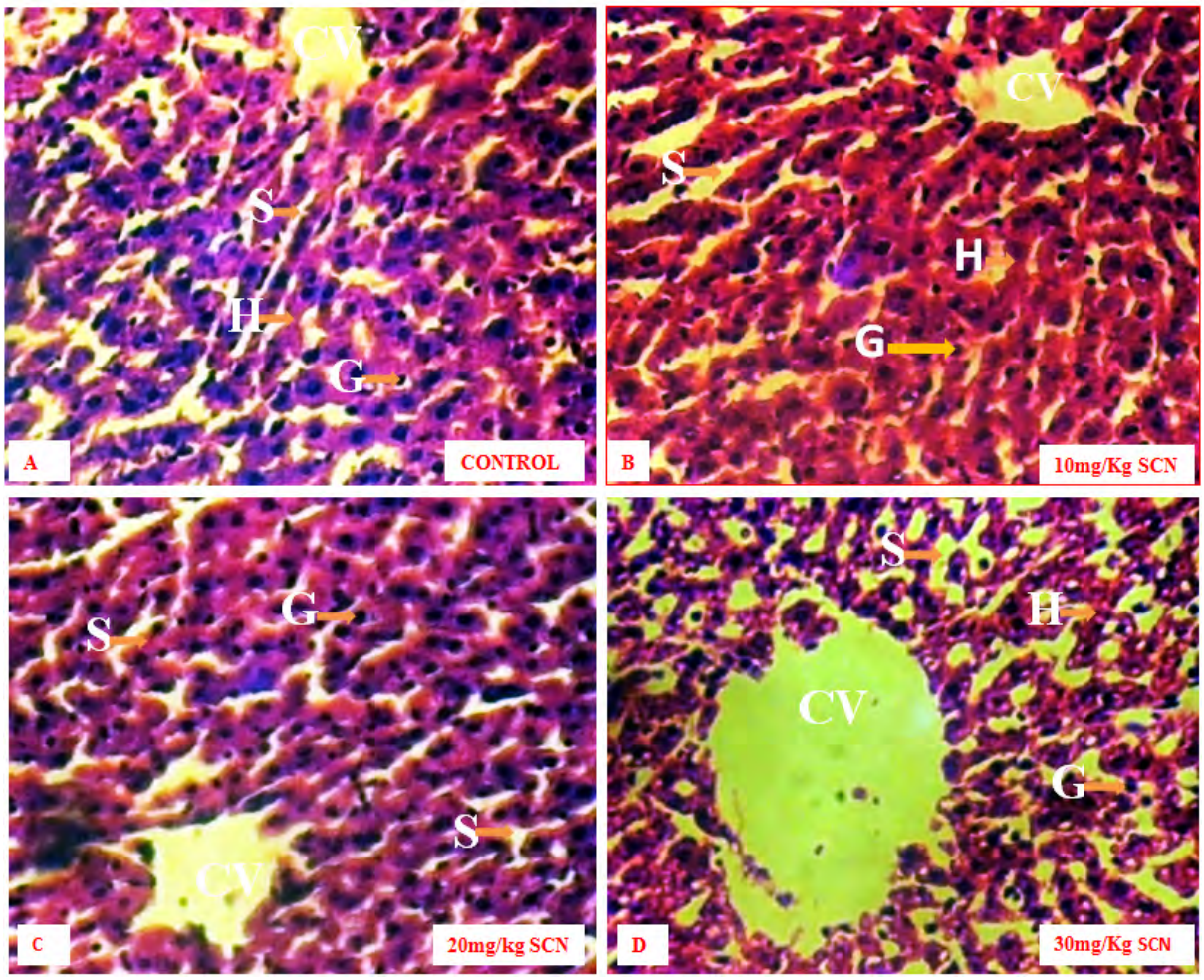

Figure 3: Liver (PAS×400): Histology of the rat liver that was given 1ml distilled water, 10mg/Kg/day, 20mg/Kg/day & 30mg/Kg/day SCN respectively. (A) Given 1ml distilled water/day for 28 days, shows normal hepatocytes(H), central vein(CV) and sinusoids(S); PAS positive substance(PPS) stained magenta observed. (B) Given 10mg/Kg/day SCN for 28 days, shows normal hepatocytes(H), central vein(CV) with well-defined wall and sinusoid(S) but mild cytoplasmic distortion; PPS abundant in the cytoplasm. (C) Given 20mg/Kg/day SCN for 28 days, shows moderate cellular hyperplasia and fatty degeneration; PPS also abundant though mildly distorted. (D) Given 30mg/Kg/day SCN for 28 days, shows severe apoptosis, ballooning sinusoids and fatty degeneration and sparse PPS.

Table 2: Results of tissue alanine aminotransferase (ALT) and aspartate aminotransferase (AST) and serum total bilirubin activities.

GROUPS	ALT(IU/L)	AST(IU/L)	TOTAL-BILIRUBIN(mg/dl)
A (control)	895.0±37.815	1215.0±23.345	2.22±0.34554
B (10mg/Kg/day)	1046.8±38.668	1238.8±22.258	3.84±0.54644
C (20mg/Kg/day)	1124.6±43.228*	1207.8±8.163	4.12±0.82183
D (10mg/Kg/day)	1097.6±32.528*	1238.4±22.919	4.47±0.69325

Values are mean± SEM of five animals.
*indicates significant difference with regards to the control ($p<0.5$)

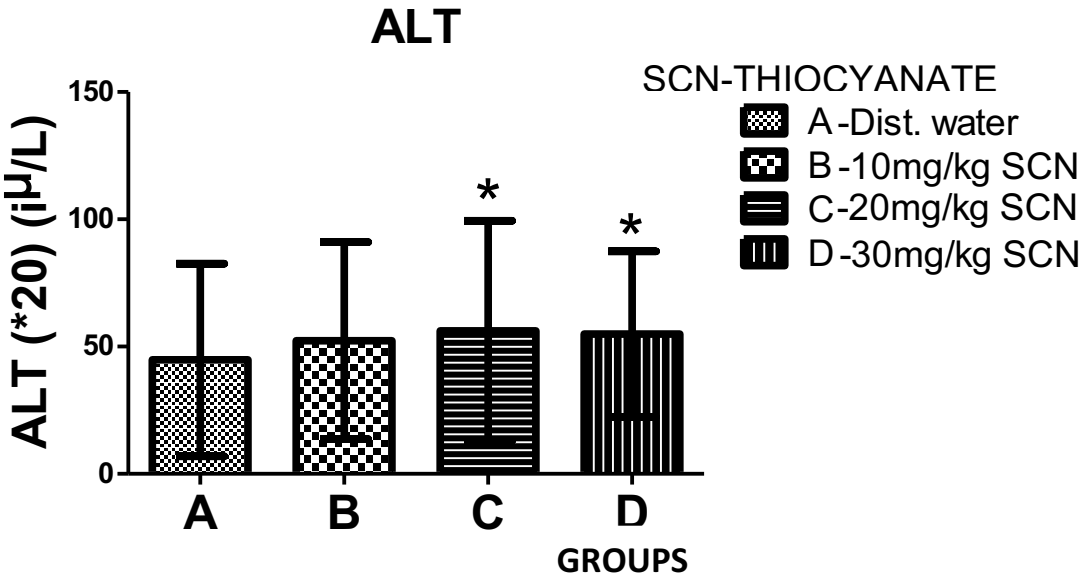

Figure 4: Bar chart showing the result of alanine aminotransferase (ALT) activities in the liver. *Significant difference ($P<0.05$) exist between ALT levels of animals in groups C&D compared to A&B.

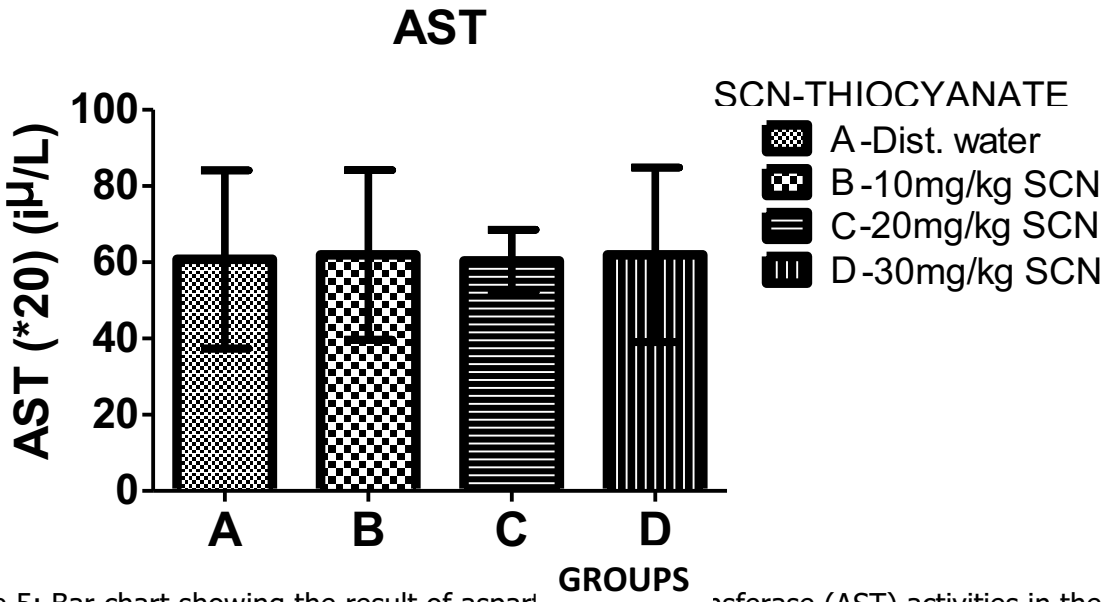

Figure 5: Bar chart showing the result of aspartate aminotransferase (AST) activities in the liver.

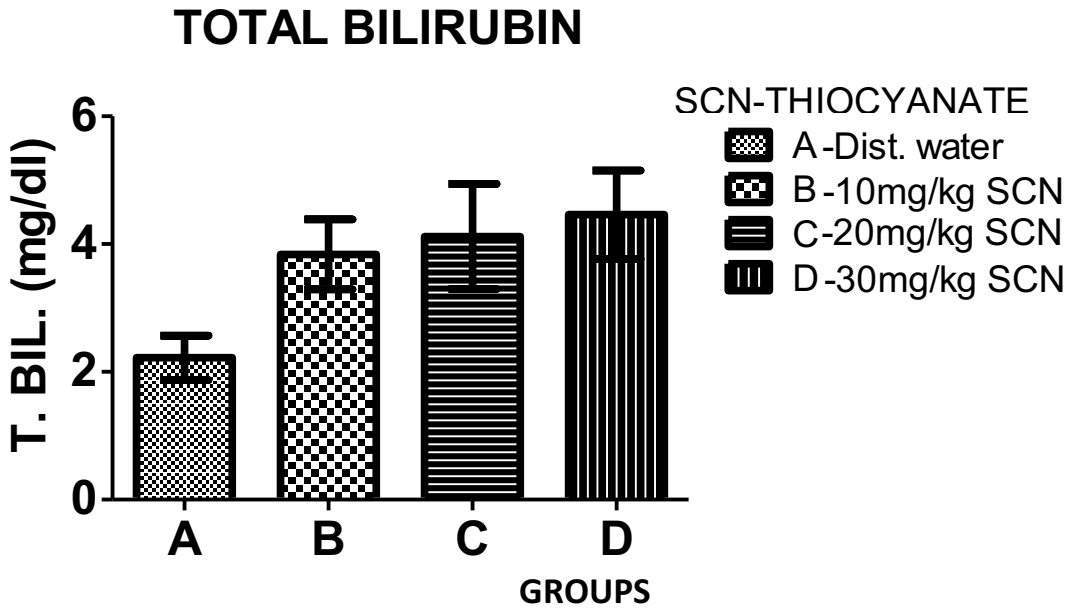

Figure 6: Bar chart showing the result of total bilirubin activities in the serum.

DISCUSSION

From observations made, the experimental animals maintained a constant rate of food and water intake from the beginning to the end of the experiment. This resulted in to a subtle weight increase that is not significant when a comparison of the initial and final weights was made. The increased weight can be attributed to normal growth and development of the rats as well as lack of interaction between appetite and SCN. The

liver is often vulnerable to the effects of xenobiotic-induced injury due to its vital role in the metabolism of foreign compounds and portal location within the circulatory system (Babalola *et al.,* 2001).

The histological tissue sections stained with H & E had a normal appearance of the sinusoids and hepatocytes radiating from the central vein in the control group. However an observation of those in groups B, C, and D had appearances varying from mild cellular hyperplasia, severe cellular hyperplasia and prominence of sinusoid to multi-foci cellular and fatty degeneration as well as ballooning sinusoid. As for the sections stained with periodic acid-Schiff (PAS), hepatocytes of control group shows normal histoarchitecture and magenta coloured PAS positive substance (PPS)-glycogen. Glycogen granules revealed by the periodic acid-Schiff was observed in abundance across the treated groups B and C except in group D where it was only sparse. A progressive disruption of hepatocyte histoarchitecture was also noticed with mild cytoplasmic distortion in group B; moderate cellular hyperplasia and fatty degeneration in group C; and severe cell death, fatty degeneration, and ballooning sinusoids in group D, indicating hepatocellular damaging effects of SCN in dose dependent order in male wistar rats. This study suggests that mild to severe hepatocyte hyperplasia, multi-foci cellular and fatty degeneration are prominent responses to SCN administration with increasing dose. The predominant or persistent hyperplastic response should therefore be considered to be an adverse effect.

Nevertheless, hepatic injury is usually related with alterations in the concentration of serum total bilirubin and tissue levels of some enzymes i.e. ALT, AST (Whitby *et al.,* 1984). Bilirubin is formed by the catabolism (breakdown) of haemoglobin in the liver, spleen and bone marrow (Vasudevan and Sreekumari, 2007). Bilirubin measurement is a useful index of determining the excretory function of the liver and assessment of haemolytic anaemia. An increase in tissue or serum bilirubin concentration results in jaundice and it occurs in toxic or infectious disease of the liver e.g. hepatitis or bile obstruction (Edem and Usoh, 2009).These results showed an increase in tissue ALT level of group B that was not significant relative to the value in the control group. However significant elevation of ALT levels in groups C and D was observed compared to the control. The increased levels of liver enzymes are an indicator of disturbance of hepatocytes membranes (Rezaei-Moghadam*et al.,* 2012). Thus the observed significant ($p<0.05$) increase is a pointer of hepatocellular damage arising from the use of SCN at doses higher than the therapeutic. This finding conform with the result obtained by Bolaji and Olabode (2011) where a significant increase in liver enzymes (ALT & AST) was observed following the administration of 3mg/Kg of cyanide to rats for 15 days. Similarly a significant increase in the concentration of ALT and AST that relates to the necrosis of hepatocytes was reported from the administration of 10mg/Kg SCN daily for twenty-eight (28) days (Oyewole and Malomo, 2009).Analysis also show that serum total bilirubin concentration increased in all the treated groups, though not significantly. The increase in total bilirubin can be explained by the thiocyanate induced inhibition of transporters mediating bilirubin uptake into hepatocytes (OATP1B1) or release of conjugated bilirubin from hepatocytes into bile (MRP2). This may cause hyperbilirubinaemia which eventually leads to jaundice. The analysis of AST showed insignificant increase in some treated groups (groups B and D) and decrease in group B when compared to the control. The corresponding histological and biochemical enzymes results of this study can be used to infer that SCN use in sickle cell disease management regimen can cause hepatocellular damage in wistar rats.

In conclusion, administering SCN in sickle cell disease management regimen can affect the cellular make-up of the liver at the therapeutic dose. A similar potentially toxic effect resulted from doses higher than the therapeutic. So an uninterrupted use of SCN at the therapeutic dose must be avoided and not considered at all for doses beyond the therapeutic.

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