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Butyrate Induces Production of Heat Shock Protein 27, 70 and 90 and Protects Against Carbon Tetrachloride Hepatotoxicity in Rats

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Abstract

The liver plays important roles in the body including blood cell formation, metabolism of carbohydrates, proteins and lipids, detoxification, bile production and excretion, and hepatic regeneration. It is however prone to various hepatotoxins that cause damage and diseases including consumption of alcohol whose toxicity is at an alarmingly increasing prevalence globally. Since both butyrate and heat shock proteins (Hsps) can protect various body systems against several perturbations, we investigated whether butyrate protects the liver against carbon tetrachloride (CCl_4) hepatotoxicity via production of Hsps. Rats were treated with sodium butyrate (SB) for 8 days then CCl_4 on the 8^{th} day and sacrificed 1 to 2 days later. Sacrificed animals were autopsied, liver samples taken, fixed in formalin and routinely processed. Tissue sections were stained with haematoxylin and eosin or anti-Hsp90, Hsp70 and Hsp27 monoclonal antibodies to assess morphological changes and expression of Hsps. Pretreatment with SB reduced the severity of CCl_4 induced hepatotoxicity which was associated with expression of Hsp90, 70 and 27. It is concluded that the protective potency of butyrate against CCl_4 hepatotoxicity is mediated, at least in part, through overexpression of Hsps.

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1. Introduction

The liver is the largest digestive gland in the human body that plays important roles such as blood cell formation, metabolism of carbohydrates, proteins and lipids, detoxification, bile production and excretion as well as hepatic regeneration [1]. It is made up of various systems that allow the liver to operate, including biliary and vascular systems, hepatocytes and hepatic lobules, hepatic sinusoidal cells, and the stroma [2, 3]. Of all the organs, the liver has the most difficult circulation. The portal vein, which gathers all the blood that leaves the spleen, stomach, small and large intestine, gallbladder, and pancreas, supplies 75 to 80% of the blood entering the liver, according to the anatomical peculiarity of the double afferent blood supply of the liver [4]. The remaining 25% of the blood is well-oxygenated and travels through the hepatic artery. The total hepatic blood flow is between 800 and 1200 mL/min, or roughly 100 mL/min per 100 g of wet liver weight. The liver receives over 25% of the cardiac output although making up only 2.5% of the total body weight [4]. Being an important organ for body health maintenance, this organ needs to be kept healthy. Noteworthy however, the liver is prone to various stimuli such as toxins from chemicals, dietary supplements, pharmaceutical drugs and medicinal plants [5], as well as bacteria, viruses, alcohol and trauma which could also lead to acute and chronic liver damage and impair its functions [6]. Often, initial liver damage caused by hepatotoxins in centrilobular regions leads to production of reactive oxygen species (ROS), lipid peroxidation, and release of pro-inflammatory cytokines due to high levels of cytochrome P450 oxidases that mediate their conversion to toxic intermediates [7]. This damage subsequently affects the liver performance.

Heat shock response (HSR) is a reaction to heat and other stresses shown by most body cells and organisms. It is characterized by rapid synthesis of a group of evolutionary conserved proteins, called heat shock proteins (Hsps) [8]. The Hsps are also produced by minerals such as sodium arsenite and zinc ions [9, 10]. Production of Hsps requires heat shock factors (HSFs) which bind to heat shock elements and induce transcription of Hsps [11]. These proteins range in size from 8 to 150 kDa and are classified as Hsp 150, Hsp 110, Hsp 90, Hsp 70, Hsp 60, Hsp 40, Hsp 27, Hsp 20 and Hsp 8.5 families according to their molecular weight [12, 13]. The HSR has been proved to be protective to various body cells through shielding the already synthesized proteins by acting as molecular chaperons [14]. As a result, the HSR protects against ischemia, inflammation and infection.

Carbon tetrachloride (CCl_4) model of hepatotoxicity has been widely studied in rats. At present the accepted mechanism for CCl_4 hepatotoxicity is by formation of free radicals and a resulting peroxide chain reaction [15]. CCl_4 forms trichloromethyl and trichloromethyl peroxyl radicals after activation by hepatic cytochrome p450. These radicals cause hepatic lesions by inducing lipid peroxidation reactions [15, 16]. CCl_4 induced hepatotoxicity is grossly characterized by an enlarged pale liver with smooth surface and tinny red spots on the surface [17]. Histologically the toxicity is characterized by mass tissue degeneration and necrosis, congestion and dilation of central vein, fatty infiltration and infiltration of inflammatory cells [18, 19]. CCl_4 rat model for hepatotoxicity remains to be the preferred model to study liver toxicity [17].

Butyrate, a short chain fatty acid (SCFA) produced by microbial fermentation of carbohydrates and fibers in the

colon, has been proved to be protective against various injuries in different body organs including the liver [20]. According to Zhou *and his colleagues* [21] butyrate reduces high fat diet (HFD)-induced microbiota dysbiosis and endotoxemia and prevents HFD-induced steohepatitis by modulating the immune response in the gut and the liver. In addition, Zhou *and his colleagues* [22] found that, butyrate prevents valproate-induced hepatotoxicity both in vivo and in vitro. Moore and Dalley [3] and Malago *and his colleagues* [10] observed production of Hsps following butyrate intake. These observations have been linked to the protective potency of butyrate. Relative effects of various SCFAs on histone acetylation, cellular growth arrest, differentiation, and death in colon cancer cells have been identified by Pirozzi *and his colleagues* [23] whose findings unequivocally demonstrated the significance of SCFAs impact on histone modification. Other researchers have found that butyrate has a novel anti-inflammatory function with extensive ramifications for the control of immune responses and may thus be used against inflammation in various body organs including the liver [24]. The mechanism through which butyrate protects body cells is however not well known. Therefore, because HSR and the subsequent production of Hsps protect against various perturbations in the body, it is compelling to explore whether butyrate is beneficial against CCl_4 hepatotoxicity via at least in part, induction of Hsps production.

2. Materials and methods

2.1. Experimental animals

A total of 48 adult (20-25 weeks) male Wistar rats (Sokoine University of Agriculture, Morogoro, Tanzania) were used in this experiment. The animals were kept in cages in a restricted access room with controlled room temperature and 12 h light/dark cycle and allowed to acclimatize for 1 week before commencement of the experiment. They were randomly allocated in 5 groups with a minimum of 4 animals each and kept up to 16 animals per cage in multiples of 4 (Table 1). The rats were provided with water and food (broilers mash) adlibitum.

2.2. Experimental setting

Five experimental groups were established, and animals treated as shown in Table 1. Animals in group 1 received injection water (IW) for 8 consecutive days; group 2 received olive oil (OO) (Well's, Madrid, Spain) as vehicle for CCl_4 on day 8; group 3 received 50% CCl_4 in OO on day 8; group 4 received 100 mg/kg sodium butyrate (SB) for 8 consecutive days and 50% CCl_4 in OO after 8 hours of SB on day 8; and group 5 received 100 mg/kg SB for 8 consecutive days. All treatments were through intraperitoneal injection of 2 ml of the respective agent, solely or combined. The experiment lasted 48 hours after administration of CCl_4.

2.3. Tissue sampling and processing

At the end of the experiment, animals were humanely sacrificed by over dosage of chloroform. The sacrificed animals and those dying during the experiment were opened by midline laparotomy. The liver was accessed, observed for any gross changes, a portion was taken, fixed in 10% neutral buffered formalin, processed routinely, embedded in paraffin wax, sectioned and stained with haematoxylin and eosin or Hsp antibodies as

described under immunohistochemistry section here below. Stained sections were observed under light microscope (Olympus BX 41, Japan) and photographs taken by DP21 camera (Olympus, Japan).

Table1: Animal grouping and treatments.

GROUP	TREATMENT		SACRIFICE				TOTAL
	Days		Day	Hours after CCl_4			
	1 to 7	8	8	15	24	48	
1	IW	-				4	**4**
2	-	OO				4	**4**
3	-	CCl_4		4	4	4	**12**
4	SB	SB + CCl_4		4	4	4	**12**
5	SB	SB	4	4	4	4	**16**
TOTAL ANIMALS			**4**	**12**	**12**	**20**	**48**

IW - Injection water, SB - Sodium butyrate, CCl_4 - carbon tetrachloride, OO - olive oil

2.4. Histological evaluation of CCl_4 *hepatotoxicity*

For histological evaluation of the liver damage, toxic changes were assessed blindly and quantitated. The scoring was performed by measuring the circular area of necrosis surrounding the central vein (centrilobular necrosis) or portal triad (portal necrosis) and extending throughout the lobule using a standalone system (SAL) of DP21 microscope digital camera (Olympus, Japan).

2.5. Immunohistochemistry

2.5.1. Subbing of microscopic slides

To prevent detachment of tissue sections during immunohistochemistry procedures, microscopic slides were subbed in chrome-gelatin (10 g of gelatin and 1 g $CrK(SO_4)_2.12H_2O$ in 1 l of warm non-boiling distilled water). After stirring until dissolved, a thymol crystal was added, then the solution was allowed to cool before further dissolving of 0.188 g $CrK(SO_4)_2.12H_2O$ in the solution. The slides were then placed on racks and soaked in soap solution for 1 h before rinsing in distilled water that was changed several times to remove all the soap. Thereafter, the slides were dipped into the subbing solution then drained in a paper towel to allow drying. When thoroughly dry, the slides were stored in slide boxes until use.

2.5.2. Deparaffinization

The obtained formalin-fixed paraffin-embedded 4 µm thick tissue sections were applied to the subbed slides. They were then deparaffinized in xylene (3 times, 5 min each) then hydrated in descending ethanol of absolute and 95% (2 changes each solution, 10 min each change) followed by distilled water (1 min with agitation). Any excess liquid on slides was aspirated.

2.5.3. Antigen unmasking (heat induced epitope retrieval)

To unmask antigens, the sections were covered with 10 mM sodium citrate buffer (pH 6.0) and heated at 95°C for 5 min. Heating was repeated after topping off the sections with fresh buffer before cooling (room temperature, 20 min) in the same buffer. The sections were then washed in distilled water (3 changes, 2 min each) and any excess liquid was aspirated from the slides.

2.5.4. Quenching endogenous peroxidase activity

Endogenous peroxidases were inactivated by incubating the sections in 3% hydrogen peroxide in distilled water for 10 min followed by washing (3 changes, 5 min each) in phosphate-buffered saline (PBS) (0.01M Na_2HPO_4, 0.01M NaH_2PO_4, 0.9% (w/v) NaCl; pH 7.3).

2.5.5. Immunoperoxidase staining using Horseradish peroxidase-Streptavidin system

Immunostaining was done by Horseradish peroxidase-Streptavidin system using SPlink HRP detection bulk kit (GBI Labs, Mukilteo, WA, USA, Cat No D01-60) according to manufacturer's recommendations. The sections were incubated for 10 min in ready for use pre-blocking solution and then blotted to remove any excess pre-blocking solution. Subsequently, the sections were incubated in a humid chamber with monoclonal Hsp27, Hsp70 or Hsp90 (Novus biological, Littleton, CO, USA) each separately (diluted in 2% normal blocking goat serum in PBS) for 60 min at room temperature. The sections were then rinsed in PBS (3 changes, 2 min each), incubated (10 min) in broad spectrum biotinylated secondary antibody and rinsed in PBS (3 changes, 2 min each). This was followed by incubation in HRP-Streptavidin (10 min, room temperature), washing in PBS (3 changes, 5 min each) and incubation in DAB+ enzyme (premixed 3, 3-diaminobenzidine plus) chromogen) (adding 1 drop of DAB chromogen concentrate in 1 ml of DAB substrate buffer and mixing well; GBI Labs, Mukilteo, WA, USA, Cat No C09-12) (5 min or until desired colour was attained). The sections were then counterstained in hematoxylin (20 sec) and immediately rinsed thoroughly in tap water (about 2 min) and blued in PBS (60 sec) before rinsing in distilled water. Subsequently, the sections were dehydrated in ascending ethanol (95% then absolute, 2 changes each solution, 10 sec each change) and then dipped in xylene (3 changes, 10 sec each). The sections were then mounted with DPX before examining under light microscope (Olympus BX 41, Japan) and photographs taken by DP21 camera (Olympus)

3. Statistical analysis

Statistical significance between the mean values of controls and those for CCl_4, SB and CCl_4+SB treated rats was assessed by one way ANOVA with comparison of means. The differences were considered significant at $p < 0.05$ level using Student's t-Test. All numerical values were presented as mean ± SEM.

4. Results

4.1. Clinical observation of rats following CCl_4 injection

After injection of CCl_4, the rats became dull and off feed with decreased range of movement. The signs persisted and increased in severity with time but decreased in rats treated with SB prior to CCl_4. In the latter group, the ill-

health signs were lost, and the animals were bright and feeding well after 48 hours of CCl_4 administration.

4.2. Mortalities following CCl_4 administration

As shown in Figure 1, none of the animals died following administration of IW, OO or SB. Instead, in the course of CCl_4 toxicity 50% of the animals died within 15 hours of CCl_4 injection. The mortality increased to 62.5% at 39 hours and 75% at 43 hours to the end of the experiment. Prior administration of SB significantly ($p < 0.05$) reduced the mortality rate to 12.5% at 15 hours, 25% at 19 hours and 37.5% at 23 hours to the end of the experiment.

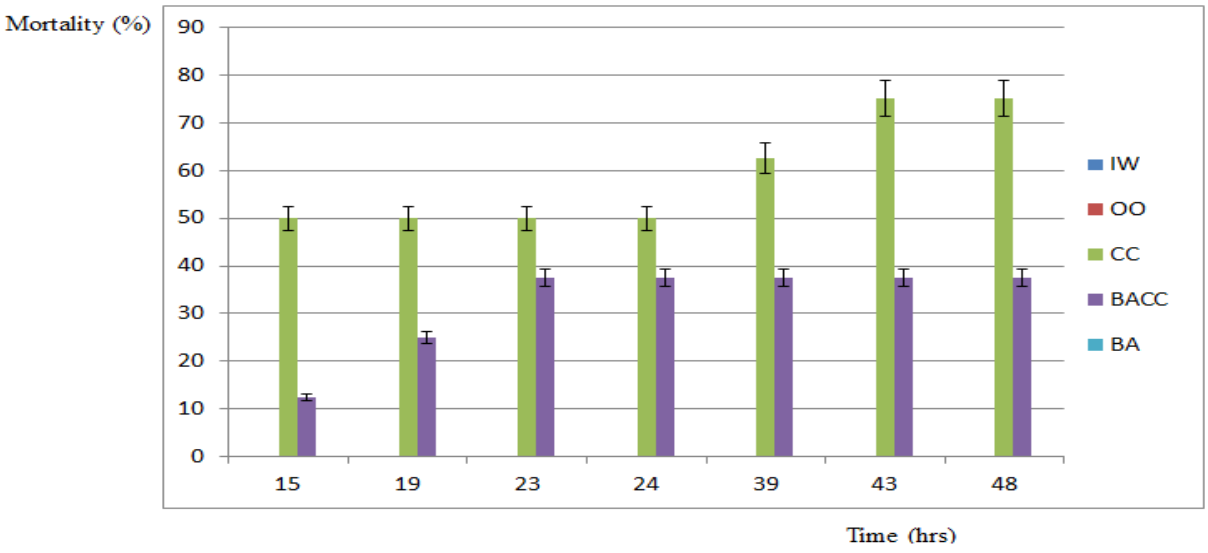

Figure1: Percentage mortality of rats under different treatments.

4.3. Histological changes following administration of CCl_4 with or without preconditioning to SB

Compared to normal liver section from control animals (Figure 10) exposure to CCl_4 for 15 hours resulted to vacuolarization in the cytoplasm and diffuse nuclear condensation or pyknosis in which case the nuclei were roundish, decreased in size, homogeneous, conspicuously stained and lacked nucleoli and chromatin granules (Figure 2). Prior exposure to SB induced moderate expression of Hsp27 (Figure 19, 20), intense expression of Hsp70 (Figure 15) and mild expression of Hsp90 (data not shown). Subsequently, animals preconditioned to SB exhibited less severe pyknosis and vacuolarization. As seen in Figure 3, the nuclei stained less conspicuous, and the cytoplasm had milder vacuolarization when compared to sections from rats not receiving SB (Figure 2). The expression of Hsps was still vivid as intense (Figure 13), moderate (Figure 17 / 18) or mild (Figure 21) and seem to be protective (Figure 5). Liver sections from protected rats preconditioned to SB then exposed to CCl_4 for 24 hours had recognizable hepatic cords, mild centrilobular necrosis and hemorrhages, nuclear condensation, and marked ballooning in midzonal and portal areas (Figure 5). On high magnification, healthy hepatocytes (nuclei with recognizable nucleoli and chromatin granules) were recognized in the periportal and midzonal areas (Figure 6). On the contrary, rats receiving CCl_4 devoid of prior treatment with SB had unrecognizable hepatic

cords, pyknosis and vacuolarization (vesicular fatty change). Pyknosis and vacuolarization were severe and extensive especially on the midzonal and periportal areas of the hepatic lobule (Figure 4). Additionally, there were marked haemorrhages and cell death around the central vein (centrilobular necrosis). According to Figure 7, exposure of rats to CCl_4 for 48 hours led to massive hepatic necrosis involving the whole liver lobule sparing few hepatocytes in the midzonal area that had marked nuclear condensation and vacuolarization. Prior treatment with SB rats exposed to CCl_4 for 48 hours had centrilobular and central bridging necrosis (Figure 8). On high magnification, healthy hepatocytes (intact nuclei with recognizable nucleoli and chromatin granules) were vivid in the midzonal and periportal areas (Figure 9). It was obvious that these animals had benefited from SB compared to their counterparts. Interestingly, even at this time, the expression of Hsps was vividly mild (Figure 14) to low and trace (Figure 22)

Exposure to CCl_4 or OO alone did not induce Hsp expression (data not shown)

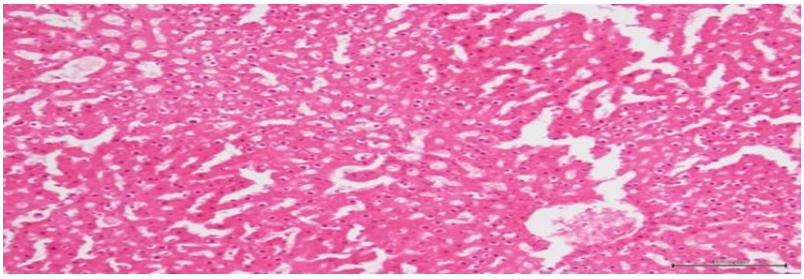

Figure 2: CCl_4 15 hrs Diffuse pyknosis and vacuolarization, HE, 100×.

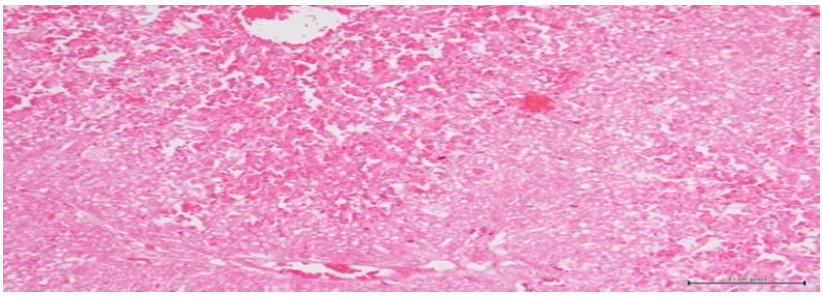

Figure 3: SB+ CCl_4 15 hrs, mild pyknosis and vacuolarization, HE, 100×.

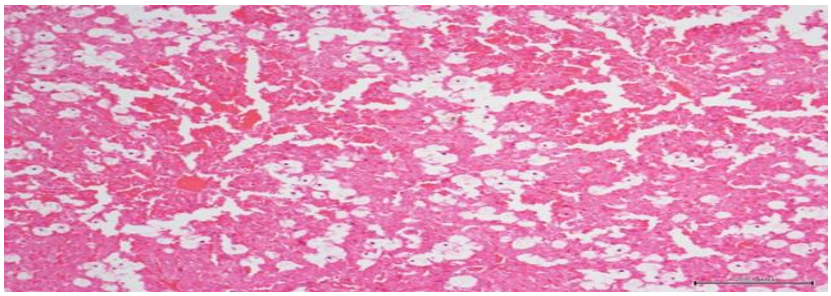

Figure 4: CCl_4 24 hrs, centrilobular necrosis, marked vacuolarization, unrecognizable hepatic cords, HE, 100×.

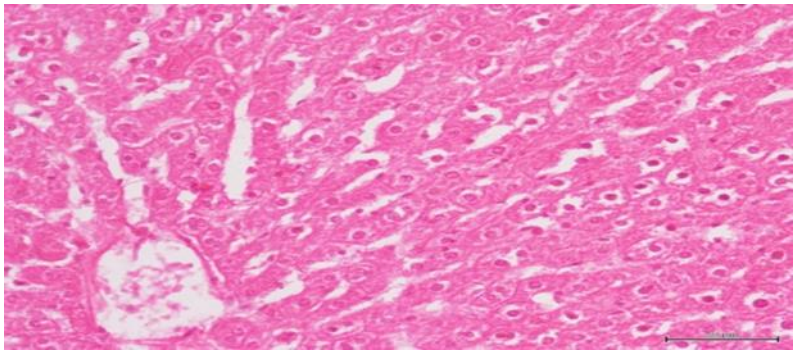

Figure 5: SB +CCl4 24 hrs, hepatic necrosis, pyknosis, vacuolarization and hemorrhages, HE, 100×.

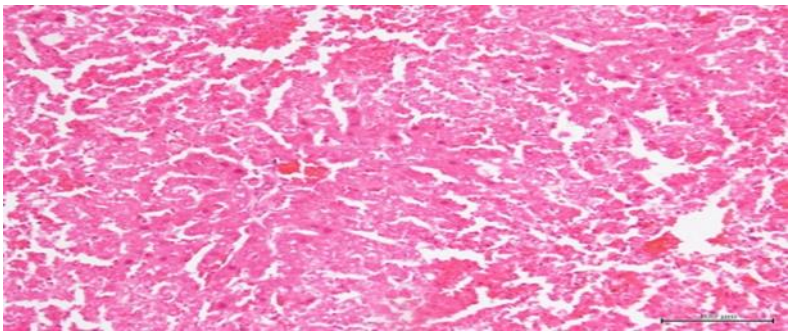

Figure 6: SB+CCl_4 24 hrs, ballooning of hepatocytes, health hepatocytes in the midzonal and periportal areas, HE, 400×.

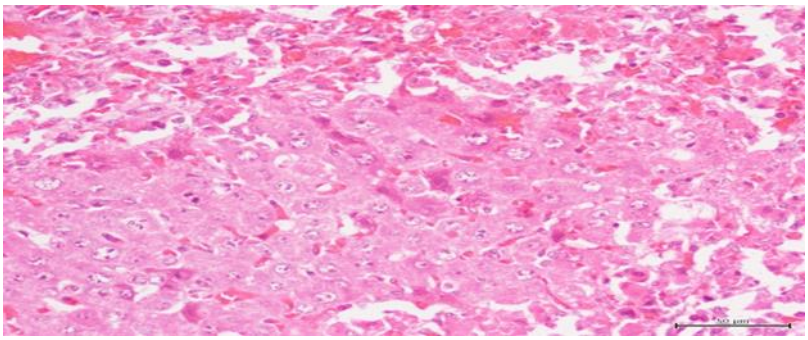

Figure 7: CCl4 48 hrs, diffuse hepatic necrosis, pyknosis, vacuolarization, HE, 100×.

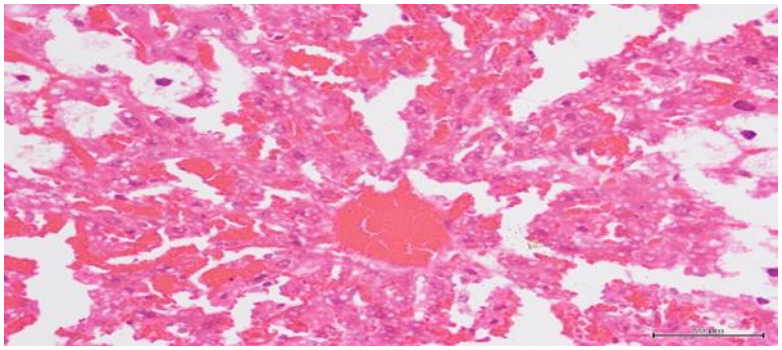

Figure 8: SB+CCl4 48 hrs, centrilobular and central bridging necrosis, HE, 100×.

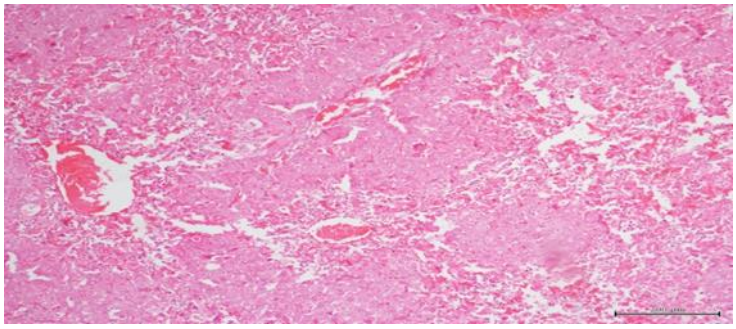

Figure 9: SB +CCl448 hrs, central and centro-central necrosis, health hepatocytes visible in Mmidzonal area, HE, 200×.

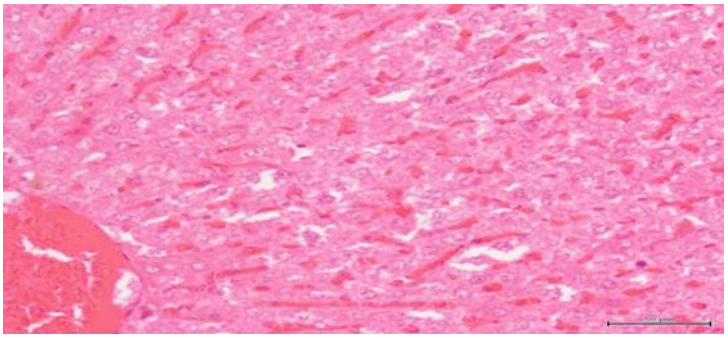

Figure 10: Control, normal liver achtecture, HE, 100×.

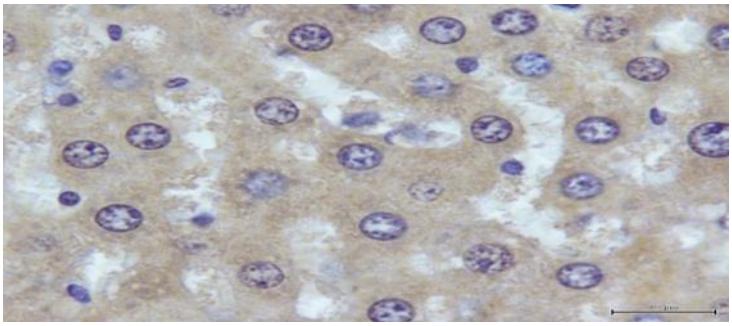

Figure 11: SB, Hsp90 15hrs, moderate expression, 200×.

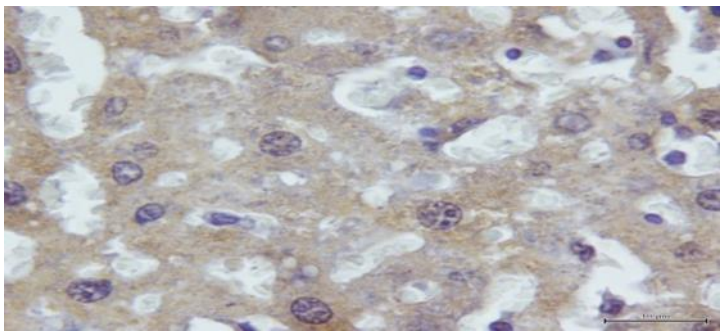

Figure 12: SB +CCl_4, Hsp90 15hrs, moderate expression, 200×.

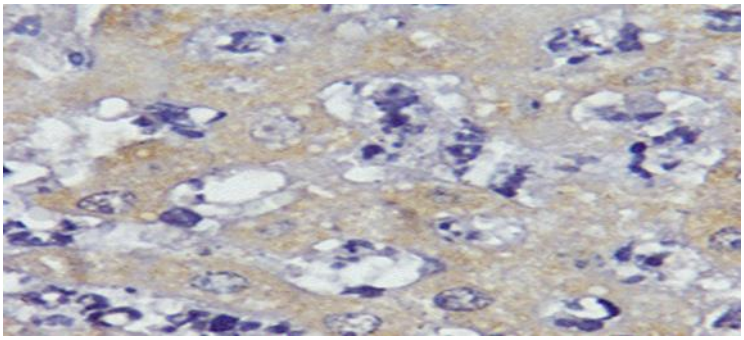

Figure 13: SB +CCl_4, Hsp90 24 hrs, intense expression, 200×.

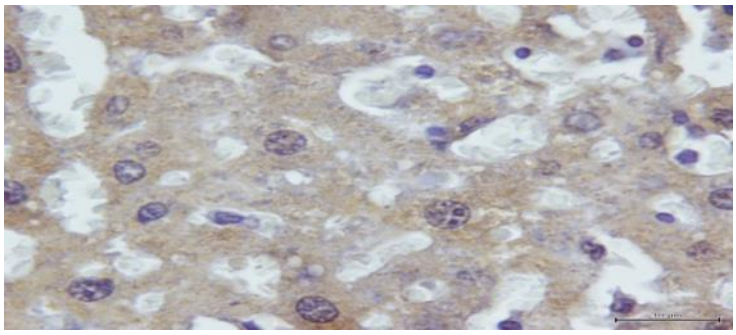

Figure 14: SB +CCl_4, Hsp90 48 hrs, mild expression, 200×.

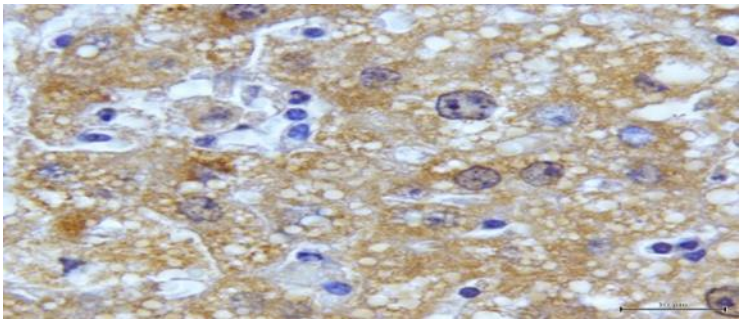

Figure 15: SB, Hsp70 8 hrs, intense expression, 200×.

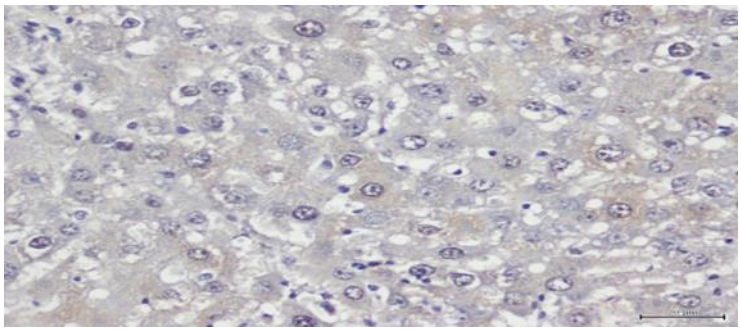

Figure 16: SB+CCl_4, Hsp70 15 hrs, moderate expression, 200×.

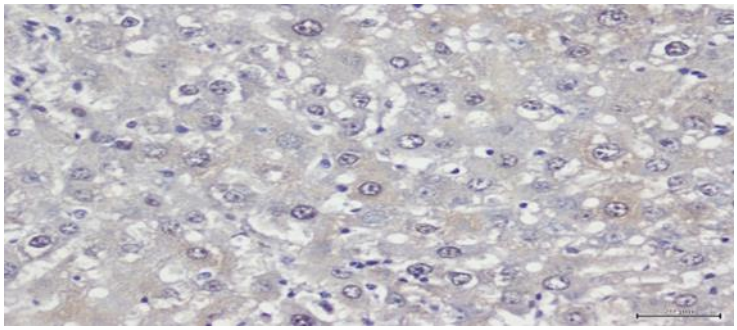

Figure 17: SB+CCl_4, Hsp70 24 hrs, moderate expression, 200×.

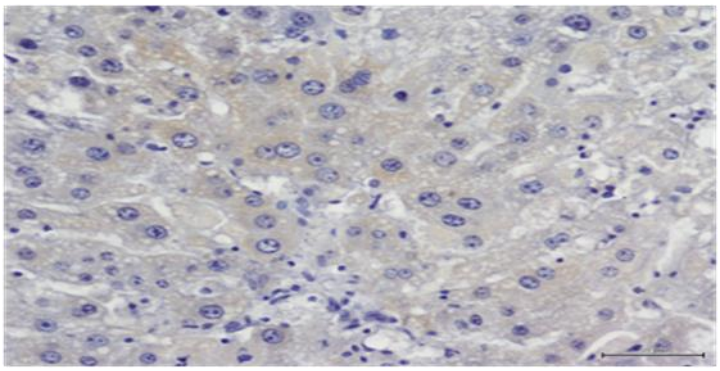

Figure 18: SB+CCl_4, Hsp70 24 hrs, moderate expression, 400×.

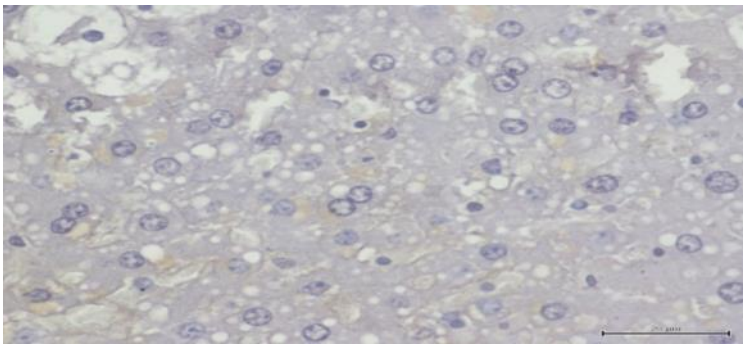

Figure 19: SB, Hsp27 8 hrs, moderate expression, 200×.

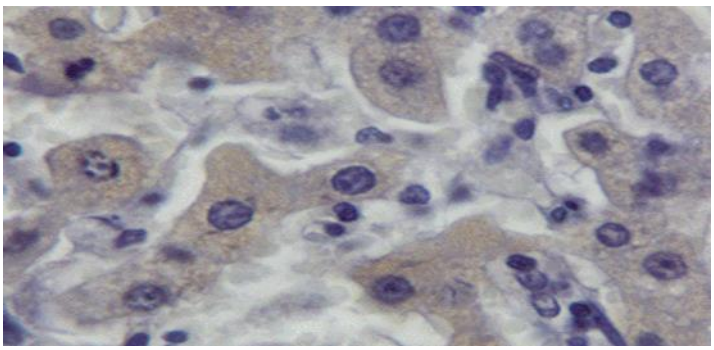

Figure 20: SB, Hsp27 8 hrs, moderate expression, 400×.

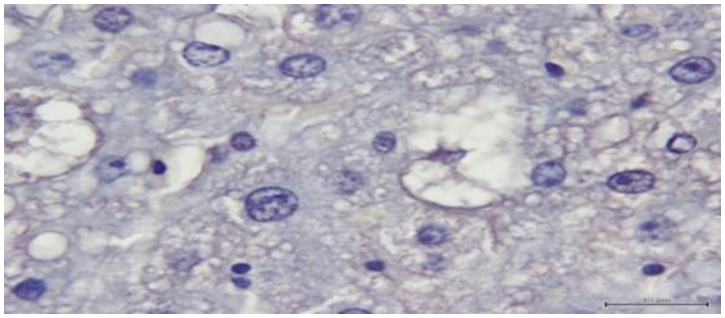

Figure 21: SB+CCl_4, Hsp27 24 hrs, low expression, 200×.

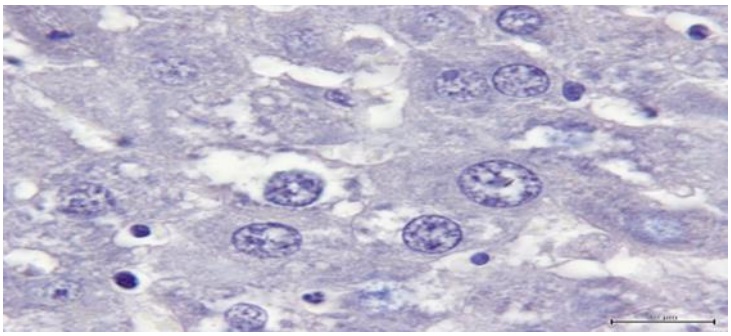

Figure 22: SB+CCl_4, Hsp27 48hrs, no expression, 200×.

5. Discussion

We investigated the hepatoprotective effects of SB and a possible molecular mechanism using a rat model of CCl_4 induced acute hepatotoxicity. It was clearly observed that SB protects against CCl_4 hepatotoxicity. The protective effect is accompanied with expression of Hsp 27, 70 and 90.

In studying mechanisms for liver toxicity and protection, the CCl_4 model of hepatotoxicity has been extensively used. Administration of CCl_4 to rats results in peroxidation of lipids by free radicals from CCl_4 namely trichloromethyl and trichloromethyl peroxyl. These are formed following bioactivation by hepatic cytochrome p450 [15]. Consequently these free radicals are covalently bound to macromolecules such as proteins, lipids and nucleic acids leading to increased concentration of highly reactive lipoperoxide and free peroxide radicals in the cell. The final outcome is cell injury or necrosis [25]. Either acute or chronic lesions in the liver can be observed depending on intensity and exposure duration. Acute toxicity is characterized by hemorrhages, fatty change, degeneration of hepatocytes and necrosis [6]. These changes may be limited to hepatocytes around central veins or spread to midzone or entire [26, 27]. Chronic cases are mainly featured by hepatic fibrosis. Consistently, the current study has reproduced the acute form of CCl_4 toxicity

Several previous studies have shown that butyrate can protect against various liver injuries [28, 29]. Similarly, Hsps have been proved by some studies to be cytoprotective against various liver injuries and diseases through attenuation of cellular death and oxidative stress [30, 31, 32]. Interestingly, different studies have separately indicated that Hsp 70 [33] and butyrate [28, 34] protect the liver against ischemia reperfusion injury. It appears

that butyrate induces production of Hsps that subsequently mediate the protective function. The fact that butyrate induces production of Hsps and subsequently confers cytoprotection has been proposed by Malago and colleagues [10]. In that study, butyrate produced Hsp 70 that modulated inflammatory cytokines to protect intestinal epithelial cells against *Salmonella* infection. The current study extends further the protective potency of butyrate through over-expression of Hsps. Based on our current and previous results [10], we hypothesize that butyrate induces production of Hsps that subsequently protect the liver against CCl_4 hepatotoxicity. Several researchers have demonstrated the Hsps protective phenomenon to the liver against CCl_4. A study by Lu and colleagues [35] reported that administration of dioscin to mice induces production of Hsp5 that subsequently spare the animals from CCl_4-induced acute liver damage. Another study demonstrated that induction of Hsp25 and Hsp70 production by an herbal product Wei Kang Su protects rat liver against CCl_4 [36]. In the latter study, the expressed Hsps were found to enhance mitochondrial antioxidant status. Yet in another study it was observed that preconditioning rats to hyperthermia and recovering for 48 hrs leads to over-expression of Hsp72 that protects against CCl_4-induced liver injury [37]. In this study, pre-treatment of rats with SB protected against CCl_4 hepatotoxicity as demonstrated by reduced mortality rate (figure 1) and amelioration of histopathological liver lesions (figures 3, 5, 6, 8 and 9). SB pre-treatment was also associated with expression of Hsps 27, 70 and 90 (figures 11-22) which are evolutionary conserved proteins that protect body cells against various body perturbations. In this very study, Hsps were induced early during SB treatment and their expression remained elevated for at least 48 hrs after last SB treatment. The Hsp expression coincided with reduced hepatic damage by CCl_4. Indeed, these findings strongly suggest that the observed reduction in hepatic damage by CCl_4 could potentially be attributed to butyrate's protective capacity via production of Hsps.

Modulation of inflammatory cytokines by butyrate has also been observed to mediate cytoprotection. According to Ahmed and Mohammed [38], butyrate inhibits expression of TNF-α and ameliorates hepatotoxicity caused by lead poisoning in rats. A recent study by Sarkar and colleagues [39] reported that butyrate reduces the expression of TNF-α and limits the inflammatory macrophage niche in non-alcoholic steatohepatitis (NASH). Interestingly, Hsp 70 has been shown to inhibit production of TNF-α [40] and its action on inducing secretion of proinflammatory cytokines like interleukin (IL) -6, IL-8 and monocyte chemoattrant protein 1 (MCP-1) leading to protection against inflammation. Most probable the produced Hsps in this study also inhibited the inflammatory role of TNF-α to confer cytoprotection against CCl_4 hepatotoxicity.

6. Conlusion

The current investigation demonstrates that Hsps has a considerable favourable effect on hepatoprotection induced by butyrate pre-treatment. This could mean that individuals consuming diets that provide adequate butyrate may be less vulnerable to hepatotoxins. As a result, liver damage due to various disorders can be avoided by increasing consumption of butyrate-containing foods such as whole (unpolished) cereals. Given that butyrate is a naturally occurring non-toxic product in the body, it may offer a promising strategy for hepatoprotection. Further, because butyrate is produced by fermenting gut microbiota, protecting these microbes might be beneficial in increasing butyrate synthesis. Eating foods high in resistant starch, such as potatoes, yam, soybeans, and legumes, can be advantageous as they provide raw materials for gut fermentation and butyrate production.

7. Recommendation

From the current study, butyrate has protective effects against hepatotoxicity which is mediated at least in part via production of Hsps. More studies are needed to explore more the protective potential of butyrate regarding hepatic health. Indeed, such a knowledge will be beneficial in avoiding liver damage via changes in eating habit.

8. Limitations of the study

Expression of Hsps is just one of several other mechanisms of action for butyrate to protect cells against injury. Being an important product of microbiotal fermentation, further studies are needed to explore how butyrate confers protection to other organs apart from the gut where it is produced and primarily protective to intestinal epithelial cells.

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