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Full Length Research Paper

Effect of methionine on hepatic indices in male Wistar rat dosed to acetaminophen formulation

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Studies' reports in both humans and experimental animals have supported the use of methionine as an effective antidote to counteract the manifestation of hepatotoxicity, which is a common occurrence of acetaminophen at overdose levels of exposure. This study was embarked on to test the hepatoprotective effect of methionine in acetaminophen tablets, produced by a leading paracetamol brand in Nigeria especially in ameliorating the hepatocellular damage for which acetaminophen is noted for. Ten percent methionine was detected in this formulation using HPLC technique. Twenty male Wistar rats were used for this purpose, they were divided equally into four groups, and the first group served as the control and received 2 ml of physiologic saline per rat. The other three groups served as the test groups and received 100, 350 and 1000 mg\kg BW of acetaminophen dissolved in 2 ml of physiologic saline per rat. The drug was introduced into the rats by intra-peritoneal route of administration. The study lasted for 48 h after which the animals were sacrificed and blood obtained by cardiac puncture. Results showed that all these hepatotoxic indices and liver function tests (aspartate and alanine amino transferases, alkaline phoshatase, total and conjugated bilirubin, total proteins, albumin and globulins) of rats in both 350 and 1000 mg\kg levels were not statistically different compared to the controls ($p > 0.05$). The rats in the 100 mg\kg set showed the same pattern except that total proteins and globulins were statistically increased in these rats compared to controls ($p < 0.05$). These results therefore, show that methionine containing acetaminophen in tablet form ameliorated the toxic effects of acetaminophen even at toxic level of 1000 mg\kg level BW.

Key words: Hepatocellular damage, male Wistar rats, acetaminophen formulation.

INTRODUCTION

Acetaminophen also known as paracetamol is a standard antipyretic and analgesic agent. These two names are derived from one parent compound, para-acetyl-aminophenol. Prior to the discovery of acetaminophen in Germany towards the end of the nineteenth century, especially in ancient and medieval times, barks of willow and cinchona trees were considered as good sources of antipyretic agents (Gormely et al., 1996; Brown 1968). The salicins extracted from the white willow trees are good examples of such agents, which were precursors of aspirin (Lafont, 2007; Lévesque and Lafont, 2000).

By 1886 and 1887, respectively, acetanilide and phenacetin were developed (Insel, 1996), but several problems were associated with the use of these agents. Phenacetin was found to cause interstitial nephritis (Boyd and Bereczky, 1966; Insel, 1996). In 1948, Bernard Brodie and Julius Axelrod observed that there was a relationship between acetanilide and methemoglobinemia and that the analgesic effect of acetanilide was due to its active metabolite-paracetamol. This led to the replacement of acetanilide with paracetamol.

The discovery, in 1966 by Davidson and Eastham, of the hepatotoxic effect of acetaminophen in a human subject has paved the way for countless number of studies to highlight one aspect of this chemical induced toxicity or the other. Many antidotes have been

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considered to counteract these toxic effects, the most prominent of them being N- acetyl-L- cysteine (Boutis and Shannon, 2001; Maze and Lee, 1998) . Methionine, the soluble precursor of cysteine has been used in combinational form with paracetamol to prevent signs of toxicity especially at overdose levels (Neuvonen et al., 1985; Krenzelok, 1997; Crome et al., 1976; Hamlyn et al., 1981; Prescott et al., 1976; Vale et al., 1981).

A high abuse rate of therapeutic drugs especially analgesics in Nigeria calls for a study of this nature. The seriousness of analgesic abuse in our environment was demonstrated through the study of Agaba et al. (2004). These workers observed that there is indeed high abuse rate of analgesics in Jos (Nigeria). And according to Ucheya and Igweh (2006), a number of factors are responsible for this, which include illiteracy, medical ignorance, poverty and lack of restriction on many drug types especially over counter drugs. In addition, Alubo (1994) revealed that in 1990, over 109 acetaminophen-related deaths were recorded in children as a result of misuse of acetaminophen.

The issue of fake and adulterated drug, a common phenomenon in Nigeria (Raufu, 2002) might also necessitate a study of this nature. Use of fake drugs causes not only premature death, but sometimes permanent disability (Raufu, 2002; ten Ham, 2003; Seiter 2009). One major cause of this problem is the use of substandard chemicals in the preparation of these drugs, sometimes not only the active ingredient itself, but the additives as well. Studies reports have identified that several times fake and adulterated additives have interacted with the genuine active ingredient to reduce efficacy or even cause tissue damage (Raufu, 2002; ten Ham 2003; Seiter 2009).

With this background knowledge, the aim of this study is to test the efficacy of methionine in EVANS acetaminophen tablets (detected in the tablets by high performance liquid chromatography) in ameliorating the hepatocellular damage for which acetaminophen is noted for.

MATERIALS AND METHODS

Animals and experimental protocol

Twenty male Wistar rats were obtained for this study. All the animals were kept in cages in a well ventilated room at atmospheric temperature. They were given unrestricted access to rat pellets and clean water. The acetaminophen tablets were administered to the rats by dissolving it in physiologic saline few minutes before administration. The preparation was administered through intraperitoneal route. The control group was given 2 ml physiologic saline only, while the treatment groups were divided into three groups and received 100, 350 and 1000 mg\kg BW of the formulation, respectively, to represent the tolerable, subtoxic and toxic levels of exposure (Abraham, 2004). The study was terminated 48 h post administration. The animals were sacrificed and the blood obtained through cardiac puncture, liver sections were preserved in 10% neutral buffered formalin for histologic examination.

Sample preparation and biochemical assays

The blood samples were collected into anti coagulant free bottles, left to clot for two hours. Serum samples were obtained by centrifuging clotted blood at 3000 r.p.m. and the serum aspirated into plain bottles, which were preserved at -20°C until the time of analysis. AST, ALT and histologic examination were used to access hepatic injury. Serum ALT and AST were determined using the method of Bergmeyer et al. (1978) with the kits supplied by DIALAB® (Holland). Other tests were carried out to assess other functions of the liver – total proteins, albumin and alkaline phosphatase using the method of Method of Kingsley (1982) and Mc Comb and Bowers (1972), kits were also supplied by DIALAB®. Levels of bilirubin (total and conjugated) were determined using the method of Jendrassik-Groff (1982) . The level of globulins was computed by subtracting the level of albumin from that of total proteins. The actual quantity of methionine in the tablets was estimated using the high performance liquid chromatography technique (HPLC).

Histological examination

Tissues of about 5 mm thickness obtained from the middle portion of the kidney and liver were fixed in 10% neutral buffered formalin. These tissues were processed for histopathological examination using a routine paraffin-wax embedding method. Sections of 5 m thickness were stained with haematoxylin and eosin. The sections were then examined under a light microscope at magnification of × 400.

Data analysis

The results obtained were subjected to statistical analysis using SPSS and were expressed as mean ±SD (standard deviation). Analysis of variance (ANOVA) was used to determine the level of significance among all test groups, while Student 't' test was employed to determine the statistical difference between each exposure group and the control group. Values of $p < 0.05$ were considered as significant. Pearson's correlation coefficient was used to assess the level of significant correlation among hepatoxic indices.

RESULTS

Chemical-induced hepatotoxicity is commonly assessed by the estimation of serum aminotransaminases (ALT & AST), total proteins, albumin and globulin. Table 2 shows that administration of this drug caused a significant increase in the level of total proteins (7.28 ± 0.88) and globulin (4.06 ± 0.77) at the tolerable level of 100 mg\kg BW ($p < 0.05$). At 350 mg\kg level, the total proteins (6.92 ± 0.86) and globulins (3.42±0.47) were not significantly different compared to control (5.80 ± 0.39, 3.70 ± 1.34) ($p > 0.05$). Also at 1000 mg\kg level of exposure, the mean ± SD of total proteins and globulins were not statistically different from the control ($p>0.05$) with values of 7.38 ± 1.46 and 3.70 ± 1.34 respectively. Moreover, total and conjugated bilirubin were equally not significantly different

Table 1. Results of hepatic enzymes (Mean ± SD) in acetaminophen-exposed and control male Wistar rats.

Exposure groups	ALT. (IU\L)	AST (IU\L)	ALP (IU\L)
Control	38.2 ± 13.11	37 ± 12.10	134.8 ± 75.30
100 mg\kg	54 ± 19.20 0.115	40.2 ± 18.0 0.730	167.2 ± 43.75 0.734
350 mg\kg	30 ± 16.23 0.166	39.0 ± 18.20 0.891	182.0 ± 40.90 0.614
1000mg\kg	45.1 ± 05.1 0.618	42.1 ± 06.10 0.476	I42.6 ± 52.60 0.217

Abbreviations: AST and ALT aspartate and alanine amino transferase; ALP, alkaline phosphatase. Results are expressed as mean ±standard deviation; $p < 0.05$ is considered significant.

Table 2. Results of biochemical indices (Mean ± SD) of liver function of acetaminophen-exposed and control male Wistar rats.

Exposure groups	Bil . (Tot.) (µmol\L)	Bil . (Con.) (µmol\L)	Total protein (g\dl)	Albumin (g\dl)	Globulin (g\dl)
Control	10.0 ± 2.63	2.02 ± 0.72	5.80 ± 0.39	3.08 ± 0.40	2.72 ± 0.78
100 mg\kg	8.86 ± 2.17 0.411	0.85 ± 0.98 0.076	7.28 ± 0.88 ***0.031**	3.22 ± 0.47 0.627	4.06 ± 0.77 ***0.025**
350 mg\kg	8.34 ± 1.65 0.227	2.02 ± 0.72 1.000	6.92 ± 0.86 0.080	3.50 ± 0.64 0.249	3.42 ± 0.47 0.123
1000 mg\kg	8.0 ± 2.17 0.227	1.68 ±1.17 0.94	7.38 ± 1.46 0.074	3.68 ± 0.53 0.074	3.70 ± 1.34 0.195

Abbreviations: BIL. (TOT. and CON.); bilirubin (total and conjugated). Results are expressed as mean ±standard deviation; $p< 0.05$ is considered significant.

in exposed groups compared with control ($p > 0.05$). The mean± SD of total bilirubin at 100, 350 and 1000 mg\kg levels were 8.86 ± 2.17, 8.34 ± 1.65, 8.0 ± 2.17, respectively, while mean ± SD of conjugated bilirubin were 0.85 ± 0.98, 2.02 ± 0.72, 1.68 ± 1.17, respectively. Table 1 shows the results of hepatic enzymes. The transaminases (ALT & AST) were not significantly different at 100 mg\kg (54±19.20, 40.2±18.0), 350 mg\kg (30±16.23, 39.0±18.20) and 1000 mg\kg (45.1±05.10, 42.1±06.10) compared with control values of 38.2 ± 13.11 and 37±12.10. Alkaline phosphatase (ALP) was also not significantly different in exposed groups compared to control ($p > 0.05$) with mean ± SD of 167.2 ± 43.75 to 100 mg\kg; 182.0 ± 40.90 to 350 mg\kg and I42.6 ± 52.60 to 1000 mg\kg and the control value of 134.8 ± 75.30. Inter-group comparison using ANOVA did not show any significant difference in all the analytes estimated ($p > 0.05$). Using the HPLC, ten percent methionine was detected in this formulation, which translates to 10, 35 and 100 mg\kg of methionine being administered to 100, 350 and 1000 mg\kg exposure groups, respectively as shown in Table 3.

DISCUSSION

Acetaminophen induced toxicity was first reported in a human subject in 1966 by Davidson and Eastham. In experimental animals, it causes hepatic depletion of glutathione at overdose level. Glutathione is a naturally occurring, tripeptide, consisting of glycine, glutamic acid and cysteine. Methionine even though is not one of the amino acids, which constitute glutathione *in vivo*, it is converted to cysteine, which bears the -SH group

Table 3. Quantity of methionine administered to animals in each exposure group.

Quantity of methionine	Exposure groups
0 mg\kg methionine	Control
10 mg\kg methionine	100 mg\kg of formulation
35 mg\kg methionine	350 mg\kg of formulation
100 mg\kg methionine	1000 mg\kg of formulation

responsible for the detoxification of xenobiotics or their reactive metabolites (McLean and Day, 1975). The presence of methionine in this formulation may probably be responsible for the non-significant differences ($p > 0.05$) observed for ALT and AST at 350 mg\kg as well as 1000 mg\kg levels of exposure.

Methionine detoxifies or inactivates *N*-acetyl- *p*-benzoquinoneimine (NAPQI), the reactive metabolite of paracetamol, through the process of conjugation leading to the formation of nontoxic mercapturate and cysteine conjugates (Jagenburg and Toczko, 1964), which are subsequently excreted through urine. This process ameliorates its toxic effect, which is an observation that is consistent with the result of this study, where even doses that are well above the tolerable level did not result in hepatoxicity, going by the result of the indices (ALT, AST, e.t.c.) that were not significantly different ($p > 0.05$) at all levels of exposure compared to the control group. Another way in which methionine acts is through its demethylation and then transulfuration to generate cysteine (Stramentinoli et al., 1979; Vina et al., 1980). Cysteine (generated from methionine) might have also acted as a source of sulfate for conjugation with unchanged acetaminophen (Glazenberg et al., 1984). This may explain the non significant difference observed as studies have revealed that co-administration of acetaminophen and methionine caused an increase in the amount of acetaminophen excreted as mercapturate and sulfate conjugates with no significant change in the quantity eliminated in urine as glucuronide conjugates. It also did not alter the amount of acetaminophen in its unchanged form. Miners et al. (1984) noted that pretreatment of experimental animals with buthionine sulfoximine (an inhibitor of glutathione synthesis) abolished the protective effect of methionine, which may confirm that methionine probably acted through generation of glutathione in the hepatocytes.

Neuvenon et al. (1985) confirmed the hepatoprotective effect of methionine that simultaneous administration of 20% L-methionine and paracetamol, reduced paracetamol toxicity in the mouse from a mean of 610 to 1096 mg/kg. Maxwell et al. (1975) showed that methionine reduced mortality in dogs from 67% to zero when acetaminophen (750 mg/kg) and methionine (150 mg/kg) were administered orally. These observations are in agreement with results of our study that methionine hepato-protected the animals by causing a lack of acetaminophen-induced liver damage especially when it was co-administered with acetaminophen. Hepatoprotective effect is not species restricted. Legros (1976), by treating mice with an oral lethal dose of acetaminophen 875 mg/kg and subsequent intraperitoneal administration of methionine at 2 and 6 h, later also observed this hepatoprotective effect through a reduction in mortality rate in this species.

Exposure to acetaminophen as low as (750 mg\kg) level has been reported to cause hepatic damage in Wistar rats; resulting in statistically significant increase in the level of hepatic enzymes, aminotransaminases; AST and ALT. The inclusion of an antidote in this formulation, prevented such damage such that at doses as high as 1000 mg\kg, this formulation did not cause significant changes in the levels of these enzymes compared with control group. According to Hemabarathy et al. (2009) estimation of total proteins level in serum is an important way to assess acetaminophen-induced hepatic damage and they demonstrated this by observing a significant decrease in the level of total proteins in experimental animals exposed to high doses of paracetamol.

This study, because of the hepatoprotective effect of methionine, did not reveal a significant decrease in total proteins level especially as a number of proteins in the globulin fraction with half-lives of hours to days. These are also synthesized by the hepatocytes and any alteration of the hepatocytes would have resulted in a corresponding alteration in total protein levels. On the contrary, a significant increase was observed, which was contributed by the globulin fraction; albumin was not different in the paracetamol-exposed rats compared with controls. A study reported earlier in which protein bound trace elements; zinc and copper were significantly increased, may point to methallothionine as one of the protein components of the globulin fraction contributing to the significant increase in the levels of fraction in exposed rats compared with controls (Szentmihályi et al., 2009; Mocchegiani et al., 2008). The result of this study is in complete agreement with the observation made by Neuvonen et al. (1985) who noticed that a paracetamol: methionine ratio of 5:1 caused a decrease in the acute toxicity by at least 50%. In a number of studies in the past, acetaminophen alone was able to induce hepatotoxicity at 750 mg\kg BW; this is in contrast to this study, in that, the physical signs and biochemical indices did not confirm toxicity even at 1000 mg\kg BW, the presence of methionine, which was detected by qualitative and quantitative means must have conferred this protection.

In conclusion, this study proved the protective effect of methionine in rats administered with this formulation and showed lack of interaction between the indices of hepatotoxicity even in the presence of altered levels of total proteins and globulin at tolerable level of exposure.

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