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Petroleum Refining Chemicals Enhance Aflatoxin B_1 induced Toxicities in Wistar Rats

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The incidence of hepatocellular cancer is one of the highest among the population in the tropics. This has been associated with the ingestion of foods contaminated by aflatoxin B_1 (AFB_1) a potent liver carcinogen elaborated as metabolite of *Aspergillus* fungi and some other fungi. Petrochemical refineries workers are in addition exposed to chemicals used in the refining of crude petroleum oil. Four petroleum-refining chemicals: N-methylpyrrolidone, Phenylenediamine, methylethylketone and Dialkylketonoxine, all obtained from a refinery situated in Nigeria were investigated in this study. Male Wistar albino rats, exposed subcutaneously to different amount of these chemicals and also orally given different concentrations of AFB_1 in corn oil for 12 weeks, were observed to have higher levels of γ-glutamyl transferase enzyme activity in their livers and sera than the rats treated with either the chemicals or AFB_1 only. They also have higher alkaline phosphatase (AP) activity in their sera. The activities of these enzymes were also higher than those observed in control rats treated with the carrier vehicle of corn oil only. Micronuclei and histopathology analysis results correlate with the results obtained in the enzyme assays. We therefore concluded that the petrochemicals are significant factor in hepatocellular cancer development in the refinery workers.

Key words: Aflatoxin B_1 (AFB_1), N-methylpyrrolidone (NMP), phenylenediamine (PHEN), methylethylketone (MEK), dialkylketonoxine (MEKOR-70)

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INTRODUCTION

Aflatoxin B_1 (AFB_1) is the most mutagenic, clastogenic, carcinogenic and hepatotoxic member of the aflatoxin group of mycotoxins (Wogan, 1975; Wogan *et al*., 1971). Almost all agricultural products and animal feeds in the tropics are contaminated by this mycotoxin because the fungi producing them only require high humidity, high temperature and any carbon source to grow. For instance, aflatoxins have been shown to contaminate especially rice, maize and groundnuts (Gang *et al*., 2004) and virtually most other foods sold in the markets in Nigeria (Miller 1978). After ingestion, AFB_1 is usually bio-transformed in the liver to AFB_1-8, 9-epoxide, the electrophilic proximate that binds to cellular macromolecules, particularly DNA, forming adducts, which mediate the deleterious effects of AFB_1 (Miller, 1978). Point mutation at codon 249 of p53 (tumour suppressor gene) by AFB_1 has been reported to play a critical role in the development of hepatocellular carcinoma (Hsu *et al*., 1991).

Primary liver cancer is one of the most common cancers in the world (Parkin *et al*., 1999) and one of the leading malignancies in sub-Saharan Africa and South-east Asia (Chen *et al*., 1997; Yu and Chen, 1994). Ingestion of foods contaminated with AFB_1 has been associated with the relatively high incidence of primary hepatocellular carcinoma in the tropical countries of the World (Qian *et al*., 1994).

AFB_1-induced toxicities in man and laboratory animals have been shown to be enhanced by co-exposure to other chemicals and biological factors (Wild and Turner, 2002) such as hepatitis B virus (Wang *et al*., 1996), malaria (Angsubhakorns and Bhamarapravati, 1986), arsenite contaminated ground water (Hernberg, 1972), alcohol consumption (Radike *et al*., 1981), other mycotoxins and industrial chemicals (IARC Working Group, 1980).

The present study assesses the effect of co-exposure to four petroleum-refining chemicals on aflatoxin B_1 induced toxicities in Wistar rats by monitoring the liver and serum enzymes, gamma-glutamyl transferase and alkaline phosphatase. Elevated levels of these enzymes have been linked with liver insults and diseases (Lum and Gambino, 1972). The refining chemicals tested are N-methylpyrrolidone (NMP), a rejuvenation catalyst solvent; Phenylenediamine (PHEN), a gasoline processing antioxidant; methyl-ethyl ketone (MEK), a lubricant oil solvent dewaxing and dialkylketonoxine (MEKOR-70), a boiler water additive. These chemicals are used for refining of crude petroleum oil to produce different fractions such as waxes, asphalt, aviation fuel, kerosene, domestic gases and so on. To our knowledge the study of the hepatotoxicity of these petrochemicals alone and in combination with AFB_1 is the first of its kind.

MATERIALS AND METHODS

Chemicals and reagents: AFB_1, gamma glutamyl transferase reagent kit, alkaline phosphatase reagent kit and other chemicals were purchased from Sigma Chemical Co. St Loius Mo. All other reagents and solvents were of analytical grade. The refining chemicals NMP, PHEN, MEK and MEKOR-70 were obtained from a petroleum refining company in Warri, Delta State, Nigeria.

Animals and treatment: The study was conducted between January and November 2004. Fifty male albino Wistar rats weighing between (120-150) g and of ages between (10-15) weeks were obtained from the Primate Colony, Department of Biochemistry, University of Ibadan, Ibadan, Nigeria. They were housed in standard metabolic cages in the Animal House, Department of Biochemistry, University of Ibadan, Ibadan and were fed with commercial pellets from Bendel Feeds and Flour Mills (BFFM) Ewu, Edo State and given water *ad libitum*. The animals were placed into ten groups of five rats each. Rats in group 1 were fed corn oil only, this is the negative control group. Those in group 2 (the positive control) were given aflatoxin B_1 in corn oil (100 $\mu g\ kg^{-1}$ bd wt.). Animals in groups 3-6 were exposed subcutaneously to the refining chemicals, NMP, PHEN, MEK and MEKOR-70, respectively for 12 weeks. Those in groups 7-10 were exposed simultaneously to the refining chemicals and aflatoxin B_1. After 12 weeks the animals were bled via cardiac puncture and their livers and bone marrow were also excised at different time interval.

Analytical procedures: Serum was prepared from blood that has been allowed to clot at room temperature for about two hours. The clotted blood samples were centrifuged at 3,000 g for 10 min the supernatant was decanted and used immediately or stored at -20°C until required. Livers were washed in cold phosphate buffered saline, blotted dry on filter paper and weighed. They then were homogenized in 4 volume of saline at 4°C. The homogenate was centrifuged at 900 g for 20 min. The supernatant was then saved at -20°C. Gamma-glutamyl transferase (γGT) activity was assayed in the serum and liver homogenates by using the reconstituted γGT reagent; 0.5 mL of the serum or liver homogenate was mixed with 0.05 mL of the reconstituted reagent. The absorbance of the mixture was read at 405 nm four times with a minute interval between the readings. The mean

changes in absorbance per minute were also obtained. Alkaline phosphatase (AP) activities were also assayed in the serum and liver homogenate using the reconstituted AP reagent. 2.5 mL of the reagent at 25°C was mixed with 0.05 mL of the sample. The mixture was then incubated at 25°C; the absorbance of the mixture was also read twice at a minute interval at 405 nm. The change in absorbance per min was evaluated.

Bone marrow cells from the femurs were flushed out and slides prepared as previously reported (Matter and Schmid, 1971). This was followed by fixing in methanol and air-drying; the dried slides were stained in 5% Giemsa solution and induced in phosphate buffer for 30 sec. Thereafter it was rinsed in distilled water, air-dried and mounted on DPX for proper drying. The slides were scored under a microscope for micronucleated polychromatic erythrocytes. Limited histopathological analysis of the liver was done at the Anatomy and Physiology Departments, University of Ibadan.

Statistical analysis: The data were analysed by one-way analysis of variance (ANOVA). p-values less than 0.05 were considered statistically significant.

RESULTS

Effect of AFB_1 and the petroleum refining chemicals on serum and liver gamma-glutamyl transferase (γGT) activities: Treatment with AFB_1, a known hepatotoxin and the four refining chemicals independently increase the γGT activity in the serum and liver of the experimental animals significantly ($p<0.05$) as compared with what was observed in the negative control rats treated with corn oil alone. When the rats were treated with individual petroleum refining chemicals and AFB_1, there were greater than 1.5 fold increase in the mean serum γGT across all the treated groups (groups 7-10) and about 2 times raised level of liver γGT observed compared with the animals treated with AFB_1 only (group 2 Table 1).

Effect of AFB_1 and the petroleum refining chemicals on serum alkaline phosphatase (AP) activity: Findings with measured serum alkaline phosphatase activity were similar to the pattern observed for the γGT activity. Treatment with either AFB_1 or each of the petrochemicals resulted in serum alkaline phosphatase activity that is significantly ($p<0.05$) greater than what was observed for the negative control group. In addition, combined treatment with AFB_1 plus each of the petroleum refining chemicals gave serum alkaline phosphatase activity that is significantly ($p<0.05$) greater than that observed in the positive control (AFB_1 only treated) group (Table 2).

Table 1: Serum and liver γ-glutamyl transferase (GGT) activity in U/L[a]

Groups	Treatments	Means SGGT (U L^{-1})	Mean liver GGT (U L^{-1})
1	Negative control (corn oil)	7.83±2.5	6.59±2.3
2	Positive control (AFB_1 only)	38.32±3.3*	29.47±2.5*
3	NMP	18.03±1.9*	16.52±1.7*
4	PHEN	16.82±3.6*	22.60±3.2*
5	MEK	15.23±1.9*	14.00±2.0*
6	MEKOR-70	19.34±2.2*	19.55±2.0*
7	NMP + AFB_1	63.50±2.2**	54.36±2.1**
8	PHEN + AFB_1	61.42±1.0**	60.41±2.1**
9	MEK + AFB_1	64.00±2.0**	56.20±1.6**
10	MEKOR-70 + AFB_1	66.22±3.1**	58.16±3.0**

[a]Values are means (n = 10)±standard deviation, *Significantly greater than the negative control,**Significantly greater than the positive control, Abbreviations: SGGT, serum γ-glutamyl transferase; AFB_1, Aflatoxin B_1, NMP, N-methylpyrrolidone, PHEN, Phenylenediamine, MEK, Methylethylketene and MEKOR-70, Dialkylketonoxine

Table 2: Serum alkaline phosphatase activity in U/L[a,b]

Groups	Treatments	Mean alkaline phosphatase activity (U L^{-1})
1	Negative control (corn oil)	37.25±1.3
2	Positive control (AFB_1 only)	109.75±2.2*
3	NMP	51.50±2.2*
4	PHEN	67.30±1.4*
5	MEK	45.50±1.5*
6	MEKOR-70	53.45±2.5*
7	NMP + AFB_1	130.95±2.5**
8	PHEN + AFB_1	142.35±2.1**
9	MEK + AFB_1	128.58±3.3**
10	MEKOR-70 + AFB_1	136.00±3.0**

[a]Values are means (n = 10)±standard deviation, *Significantly greater than the negative control, **Significantly greater than the positive control, [b]Abbreviations: As in legends to Table 1

Table 3: Micronucleated cells/1000 polychromatic Erythrocytes scored[b]

Groups	Treatments	No. of micronuclei	Polychromatic erythrocyte scored
1	Negative control (corn oil)	2	1000
2	Positive control (AFB_1 only)	28*	1000
3	NMP	6*	1000
4	PHEN	8*	1000
5	MEK	6*	1000
6	MEKOR-70	7*	1000
7	NMP + AFB_1	38**	1000
8	PHEN + AFB_1	30**	1000
9	MEK + AFB_1	32**	1000
10	MEKOR-70 + AFB_1	36**	1000

*Significantly greater than the negative control, **Significantly greater than the positive control, [b]Abbreviations: As in legends to Table 1

Effect of AFB_1 and the petroleum refining chemicals on micronucleated polychromatic erythrocytes: The number of micronucleated polychromatic erythrocytes per 1000 erythrocytes scored in the bone marrow of the rats treated with AFB_1 is more than 10 times that of the negative control group, given corn oil only. The petrochemicals also produced increased number of micronuclei in the range of 200-300% above the negative control. Treatment with AFB_1 plus each of the petroleum refining chemicals suggested that the petrochemical enhanced the ability of AFB_1 to produced micronucleated cells (Table 3).

DISCUSSION

Nigeria is one of the largest producers of crude oil in the world. Current data shows that it produces about 1.8 million barrel/day of crude petroleum product (NNPC, 2005); export about 70% of these and the remaining 30% is refined into various fractions for domestic use and export (NNPC, 2005). The refinery in which crude oil petroleum is refined into its constituent products employs various chemical feedstock and catalysts for its production efficiency, blending and enrichment of its fine products. Workers are thus occupationally exposed to these chemicals on a daily basis for prolonged period of time with its attendant hazardous risks on their health.. In addition to occupational exposure, the refinery workers are also non-committally co-exposed to AFB_1 in contaminated foods and food products they ingest.

Findings from this study are in line with earlier observations that AFB_1 markedly induce γGT and AP activities in laboratory animals (Gbadegesin *et al*., 2000; Friedman *et al*., 1996). Although there is dearth of information on the toxicity of the refining chemicals (viz., NMP, PHEN, MEK and MEKOR-70) used, present findings suggest that all the chemicals are capable of inducing γGT activity in the serum and liver and serum AP activity. The elevation in the level of the two enzymes is an indication of a liver lesion (Ideo *et al*., 1972). Thus suggesting that the chemicals are hepatotoxic in the experimental animals. The ability of the four chemicals to modulate AFB_1 activity seems to be similar.

Present findings also show that AFB_1 induce micronuclei formation in the polychromatic erythrocyte (PCEs) of the bone marrow of the rats. This is in line with early reported cases (Friedman and Staub, 1977; Hanumantharao *et al*., 1998). The individual refining chemicals alone have mild micronuclei formation induction activity as compared with the treatment with AFB_1. However, in the presence of the refining chemicals the ability of AFB_1 to induce micronuclei formation significantly ($p<0.05$) increased suggesting that the refining chemicals may potentate AFB_1 induced hepatotoxic activity. We suggest that the petrochemicals, NMP, PHEN, MEK and MEKOR-70 synergically interact with AFB_1 to produce greater genotoxic effect in the experimental rats. The petroleum oil refining workers are therefore put at increased risk of development of hepatocellular carcinoma. Further work is in progress in our laboratory to elucidate the mechanism of the interaction between the petrochemicals and AFB_1.

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