EFFECT OF LEVAMISOLE ON PLASMA CREATININE AND CHOLESTEROL LEVELS AND PLASMA ALKALINE PHOSPHATASE AND LIVER MICROSOMAL DRUG METABOLISING ENZYMES ACTIVITIES IN MALE RATS.

By

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Abstract

Adult male albino rats were injected intra-peritoneally once daily for 14 consecutive days with 20mgkg-1 dose of levamisole dissolved in distilled water. The control received equivalent volumes of distilled water only. Levamisole modestly increased the activities of all the micromisole drug metabolizing enymes determined which include NADPH -Cytochrome C redutase, Cytochrome b5 and Aniline hydroxylase. Out of the three. only Cytochrome b5 was significantly different ($P<0.05$). A comparative evaluation of the hepatic drug metabolizing enzymes from the levamisole treated microsomal pellets and 9000g supernatant showed that NADPH Cytochrome C reductase was higher in the microsomal pellets than in the 9000g supernatant and at the same time was significantly different ($P<0.05$); while the levels of Cytochrome b5 and Aniline hydroxlase were higher in the 9000g supernatant. However, only the level of Cytochrome b5 was significantly different ($P<0.05$) from the microsomal pellets. The activity of alkaline phosphatase and the levels of cholesterol and creatinine in the levamisole treated animals were higher at the same time significantly different ($P<0.05$) from the corresponding control value.

Introduction

Levamisole is the laevo isomer of tetramisole (+-0 -2,3,5,6- tetrahydro - 6- phenylimidazole (2,1-b) thiazole (Thienpont e al. 1966). It is used as a nematocidal agent in veterinary practice and as an antihelmintic drug in has been shown to restore host defence in immunocompromised patients and for this reason it has been employed in a variety of immuno-deficiency diseases, including reheumatoid arthritis, where it is major metabolite (Bossche, 1980). The effect of levamisole on the stabilization of tumour remission has been observed in several models, including various leukemias, carcinomas and sarcomas (Johnson et al. 1975; Hopper et al. 1975, Hadden et al. 1975).

Levamisole is mainly metabolised by the liver in rats essentially through oxidative reactions such as sulfoxidation and hydroxylation (Galtier et al. 1983). Although the effect of levamisole on both reported on hepatic microsomal drug metabolising enzyme system in male mice and on alkaline phosphatase in vitro, there is no published report on the effect of levamisole on both 9000g supernatant and microsomal pellets in male rats and its effect on the vivo plasma level of alkaline phosphatase, cholesterol and creatinine. This study was designed to provide the information.

Materials and Methods

Adult albino rats weighing between 150 -250g were obtained from the Animal House of the College of Medicine, University of Lagos and were housed in metal cages on hardwood chips bedding with access to feeds and water ad libitum.

Potassium dihydrogen orthophosphate, nicotinamide, aniline and magnesium chloride were products of British Drug House (BDH) . Poole England and were of analytical grade. Huma serum albumin, NADP monosodium salt, cytochrome C, glucose - 6-phosphate were obtained from Sigma Chemicals. Alkaline phosphatase and cholesterol assay kits were SGM Italia products. Levamisole HCL substance was a generous gift from ICL Nigeria Limited.

Methods

Five adult male albino rats were injected intraperitoneally daily for 14 days with 20mg Levamisole HCL in 0.5ml distilled water per kg body weight. Five control rats received equivalent volumes of distilled water only.

Preparation of 9000g supernatant and microsomal fraction from the Liver

The rats were starved for 24 hours after the last Levamisole administration. The animals were sacrificed under ether anaesathesia and the Liver quickly with ice-cold 1.15% KCL solution. The liver was minced with a razor blade and homogenized with four volume of 0.1M phosphate buffer pH 7.4 in a Potter- Elvehjem homogenizer immersed in ice. The homogenate was centrifuged in a refrigerated centrifuge Sorvall model at 9000g and 40c for 15min. The supernatant was collected and the pellet discarded. An aliquot of the supernatant designated 9000g supernatant was kept at 40c for enzyme assay and the rest was centrifuged at 1,00,000g and 40c for 60min (Bertram et al. 1982). The 100,000g supernatant was discarded and the pellet was resuspended in the phosphate buffer pH 7.4 equivalent to the volume of the supernatant and designated microsdmes.

Alkaline phosphatase assay

The SGM Italia kit procedure

monitoring the absorbance of the 4 - nitrophencol formed from the enzymatic hydorlysis of P-nitrophenly phosphate (PNPP) at 405 nm in SP8-100UV/VIS PYE UNICAM Spectrophotometer.

PNPP alkaline 4 - nitrophenol + phosphate
Phosphatase

The 10.22mM PNPP was dissolved in 1.0m diethanolamine buffer pH 10.2 and 2.5ml of this solution was put in a cuvette to serve as the enzyme source. The reference cuvette contained 2.5ml of the substrate dissolved in the buffer.

Alkaline phosphatase activity was calculated thus:

Alkaline phosphatase activity = Change in OD per min x 3300 Units/L.

Cytochrome b5 assay

The level of cytochrome b5 was monitored by a slight modification of the procedure reported by Omura and Sato (1964). The content of this haemoprotein was measured from the difference in spectrum between 424 nm and 409 nm in both the sodium dithionite reduced and oxidized enzyme. 1 ml of sodium dithionite solution (1 mg/ml) and 1 ml of 0.1M phosphate buffer pH 7.4 were added to the sample cuvette contained 2 ml of the buffer and 1 ml of the enzyme fraction. The molar extinction coefficient was assumed to be 185 cm-1 mM-1 according to Omura and Sato (1964). A unit of cytochrome b5 activity was defined as that amount of enzyme required to catalyze the reduction of 1 umole of cytochrome b5 under the condition of the assay. The results of the enzyme assays are presented as units per mg protein.

NADPH - Cytochrome C reductase:

NADPH -Cytochrome C reductase was determined by slightly modifying the method of masters et al. (1967). To a small test tube were added the following solution: 5 ml of 0.1M phosphate buffer (pH 7.4) 0.5 ml of Cytochrome C solution (7.36mg ml-1 of water) and 0.1 ml of microsomal enzyme suspension. The resultant solution was mixed well and incubated in a water bath at 370c for 5 min. Following the preincubation period, 2.8 ml of the solution was transferred into each of the two cuvettes, a reference and a sample. These were placed in the compartment of a recording spectrophometer: 0.2 ml of NADPH-generating system consisting of 0.33 nm NADP, 2.5 nm glucose-6-phosphate; 6.37 nm MgCl2 and 12.5 nm nicotinamide was added to the sample cuvette while the reference cuvette received only 0.2 ml of the buffer.

The change in optical density at 550 nm was recorded every 30 seconds for 4 nub. Linearlity was obtained after 90 sec. The amount of Cytochrome C reduced was calculated using the molar extinction coefficient of 19.1 nm-1 cm-1 (Master et al. 1967). A unit of NADPH -Cytochrome C reductase activity was defined as that amount of enzyme required to catalyze the reduction of 1 umole of NADPH-Cytochrome C in 1 min. The results of the enzyme assays are presented as units per mg protein + S.E.M.

Aniline hydroxylase Assay

Aniline hydroxylase activity was measured by following the formation of p-hydroxyanaline from aniline according to a modification of the colorimetric method described by Imai et al. (1966). To a test tube was added 0.3 ml of 0.1M phosphate buffer pH 7.4, 0.3 ml NADPH generating system consisting of 2.5 nm glucose-6-phosphate, C.3 nm NADP and 6.37 nm MgCl2' 0.2 ml aniline dissolved in 0.05M HCl and 0.2 ml enzyme fraction in a final reaction volume of 1 ml. The reference cuvette contained 0.5 ml of buffer, 0.3 ml of NADPH-generating system and 0.2 ml of the substrate. The reaction was carried out in a water bath at 37oC for 20 minutes with moderate shaking and stopped by the addition of 1 ml 20% TCA. After centrifugation at 5000g for 10 minutes, a 1 ml aliguot of the supernatant was taken out and added to 0.5 ml of 10% Na2CO3 in a test tube. A 1 ml aliquot of 2% phenol in 0. 2N NaOH was added. The resulting blue colour was measured at 630 nm after standing for 30 mins. A molar extinction coefficient of p-amino phenol was taken as 10 nm-1 cm -1 as reported by Archakov et al. (1974). A unit of aniline hydroxylase activity was defined as the amount of enzyme required to catalyze the hydroxylation of 1 umole of aniline in 1 min. The results of the enzyme assays are presented as units per mg protein + S.E.M.

Plasma Creatinine level determination

This was determined colorimetrically by the Jaffe reaction method as described by Bonsnes and Taussky (1945). 1 ml of 0.04M picric acid solution and 1 ml. of 0.75 N Naoh solution were added to 3 ml of plasma.

The resulting solution was thoroughly mixed. A red colour was allowed to develop within 15 min. The absorbance was read off at 525 nm in a spectrophotometer using water as the blank. The creatinine content of plasma was calculated from a standard curve prepared with a creatinine concentration range of 0 - 200 um.

Plasma Cholesterol determination:

The SGiM Italia kit procedure was adopted. The assay is based on the enzymatic determination of total cholesterol according to the following reaction:

i. Cholesterolester + 02 Cholesterol esterase cholesterol = fatty acids
ii Cholesterol Cholesterol oxidase cholesten-3-one + H2O2
iii. 2H2O2 + Phenol + 4-amino-antipyrine Peroxidase H2O + red quinone.

The red quinone is monitored spectrophotometrically at 520 nm wavelength. The reagent used in this study was made up of phosphate buffer 50nm, pH 6.85, phenol 10 nm, sodium cholate 5 nm, cholesterol esterase 200 UL-1, cholesterol oxidase 200 UL, peroxidase 1300 UI-1 and 4-aminoantipyrine 0.4 nm. The sample cuvette contained 3 ml of the reagent and 0.03 ml of the standard solution. The cuvettes were incubated for 30 min at room temperature and absorbance read at 520mm. The reference value of the standard cholesterol was between 140-270mg/dl and the average value was taken. Total cholesterol was estimated from the expression.

Optical Density of Sample x conc. of standard
Optical Density of Standard

Protein determination

Sample protein was determined according to the biuret method [Armstrong and Carr 1960].

To 1 ml of sample was added 4ml of biuret reagent and mixed by swirling. The mixture was allowed to stand for 30 min at room temperature and the extinction determined at 540 nm spectrophotometrically. The protein concentration in the sample was exrapolated from a pre-determined calibration curve.

RESULTS AND DISCUSSION.

Table 1 shows that levamisole increased the activity of hepatic drug metabolizing enzymes. Although levamisole is predominantly metabolized in the liver, it has not been reported to be harmful to the liver at therapeutic levels [Dalvi,1987]. The increase in the levels of the drug metabolizing enzymes in this study shows that levamisole is a modest activator of the microsomal enzyme system in rats. This has earlier been reported by Dalvi and Terse [1990] in mice. These authors further suggested that levamisole dose not belong to the group of xenobiotics which produce liver enlargement and concomitant increase in the values of the hepatic drug metabolizing enzymes [Dalvi,1987].

Levamisole is unlike most type II compounds which have a spectral peak at 422 nm and a trough at 394 nm and were known to cause inhibition of the hepatic microsomal mixed function oxidases (Peeples and Dalvi, 1982). It is now established from this study that not all type II compounds behave in similar manner since Levamisole is a type II compound. For example, Metyrapone which is a type II compound causes both induction and inhibition, depending on the does and frequency of dosing (Dalvi and Terse, 1990). The mild induction of mixed function oxidases by Levamisole suggests that it may give rise to drug intraction by enhancing the metabolism of other concomitantly administered drugs, thus reducing their therapeutic efficacy or increasing their toxicity if their metabolic products are more toxic than their parent compounds (Dalvi and Terse, 1990).

The activities of Cytochrome b5 and aniline hydroxylase were higher in the 9000g supernatant than in the microsomal pellets (Table 2). This is no surprise since Fouts (1971) has said that the "9000g" supernatant of the liver contains most of the liver microsomal enzymes. Kato (1967) used 9000g supernatant to measure aniline hydroxlyase activity. However, he was the microsomal fraction for cytochrome b5 determination.

Alkaline phosphatase activity was highly stimulated by Levamisole (Table 3). Van Belle (1972) and Bhargava (1977), have shown that Levamisole are potent in hibitors of alkaline phosphatase while Hoffman et al. (1983) have established that the horse kidney alkaline phosphatase is resistant to Levamisole inhibition. Engleman and Richardson (1986) have found that Levamisole stimulated the activity of alkaline phosphatase in cultured hepatocytes from rat. Rhodaparast-Sharifi and Dale Snow (1989) have reported that the variation in the extent of enzyme inhibition in the pH 10 to 11 range when different substrates are employed suggests that Levamisole inhibition of alkaline phosphatase may be substrate dependent.

The plasma than in the Levamisole treated animals (Table 3). The bulk of plasma cholesterol from the liver was probably impaired by Levamisole. According to Nydegger and Butler (1972) the low level of cholesterol may be due to inhibition of lipoprotein synthesis or increased lipoprotein catabolism. It is also possible that Levamisole inhibits cholesterol synthesis in the liver by the inhibition of 3-hydroxy-3-methylglutaryl coenzyme A reductase which is the rate-controlling enzyme catalyzing the first step in cholesterol biosynthesis.

As show in Table 3, the creatinine content of the control was higher than the Levamisole treated animals. The decrease in weight of the treated animals could be responsible for the decrease in creatinine concentration; since creatinine is normally formed in am amount proportionate to muscle mass and its urinary excretion is related to the amount of skeletal muscle (Ziemann, 1991). Creatine is an important constituent of muscle. 90% of body creatine is contained in muscles and a constant percentage of this part is converted into creatine each day by a non-enzymatic mechanism (Mitch et al. 1980).

From these findings, it is obvious that Levamisole apart from being a modest activator of hepatic drug metabolizing enzymes also has some other biochemical effects on the treated rats.

Table 1

Effect of Levamisole on drug metabolizing enzymes in liver microsomal pellets of albino rats.

	Control	Levamisole treated
	Enzyme type	Units/mg protein (Mean + S.E.M.)	Units/mg protein (Mean + S.E.M)
	NADPH - Cytochrome C reductase	929.32 + 58.05 (n=n)	1012.22 +36.72 (n=4)
	Cytochrome b5	20.54 + 0.88 (n=4)	*32.00 + 0.55 (n=4)
	Aniline hydroxylase	5.56 + 1.12 (n=3)	8.30 + 0.06 (n=3)

Statistical analysis was made using the student's t-test. Results are express are as mean + S.E.M. (Standard Error of Mean).

*Signficantly different ($P<0.05$) from the corresponding control value.

n= number of determinations made.

Comparative evaluation of the effect of Levamisole on drug metabolizing enzymes in Liver microsomal pellets and the 900g supernatant

	Microsomal Pellets	9000g Supernatant
	Enzyme type	Units/mg Protein (Mean + S.E.M.)	Unit/mg Protein (Mean + S.E.M.)
	NADPH -Cytochrome C	* 1012.22 +36.72 (n=4)	699.37 +35.59 (n=4)

Cytochrome b5	* 32.00 + 0.55	37.77 + 0.82
	(n=4)	(n=4)
Aniline hydroxylas		
8.30+0.06		10.78 + 0.99
	(n=3)	(n=3)

Statistical analysis was made uding the Student's t-test.

Result are expressed as the mean + S.E.M. (Standard Error of Mean).

* Significantly different ($P < 0.05$) from the corresponding 9000g supernatant.

n= number of determinations made.

Effect of Levamisole on the activity of alkaline phosphatase and on the levels of cholesterol and creatinline in Plasma of albino rats

	Control	Levamisole treated
Alkaline Phosphatase UL-1	82.50 + 6.87 (n=4)	* 88.10 +24.77 (n=4)
Cholesterol mg dL-1	217.15+17.15 (n=4)	* 136.67 + 10.45 (n=4)
Creatine umole ml-1	152.00 +9.00 (n=4)	* 108.00 + 8.00 (n=4)

Statistical analysis was made using the student's t-test.

Results are expressed as the mean + S.E.M. (Standard Error of Mean)

* Signficantly different ($P<0.05$) from the corresponding control value.

n= number of determination made.

References

1. Armstrong, W.D. and Carr, C.W. (1960): Physiological Chemistry. Laboratory directions. Burgess Publishing Company Minneapolis Second edition.

2. Archakov, A.I., Karuzine, I.I., Teveritinov, V.N. and Kokareva, I.S. (1974): Hydroxylation of aniline and aminoantipyrine (1-phenyl-2,-3-Dimethylaminopyrasolon-5) derivatives in Liver Endoplasmic Reticulum. Biochem. Pharmacol. 23, 1053-1063.

3. Bertram, B. Schuhmacher, J. Frei, E., Frank, N. and Wiessler (1982): Effects of Disulfiram on mixed function oxidase system and trace element concentration in the liver of Rats. Biochem. Pharmacol. 31, 2613 - 2618.

4. Bhargava, K.K., Lee, M.H. Huang, Y.M., Cunning ham, L.S., Agrawal, K.C. and Sartorelli, A.C (1977): Tetramisole analogues as inhibitors of alkaline phosphatase an enzyme involved in the resistance of neoplastic cells to 6-thiopurines. Jr. Med. Chem. 20: 563 -566

5. Bonsnes, R.W and Taussky, H.A (1954): On the colorimetric determination of creatine by the Jaffe reaction. Jr. Biol. Chem. 158-591.

6. Bossche, H.V.N (1980): Peculiar targets in anthehelmintic Chemotherapy. Biochem. Pharmacol. 29: 1981 -1900.

7. Dalvi, R.R. (1987): Dose - related inhibition of the drug-metabolizing enzyme of rat live by the pyrolizidine alkaloid, monocrtaline. Jr. Pharm. Pharmacol. 39: 386 -388.

8. Dalvi, R.R. and Terse, P.S. (1990): Induction of hepatic microsomal drug metabolizing enzyme system by Levamisole in male mice. Jr. Pharm. Pharmacol. 42: 58 -59.

9. Engelmann, G.L. and Richardson, A.G. (1986): Effects of Levamisole on primary cultures of adult rats heptocytes. Biochem. Pharmacol. 35: 1547-1554.

10 Fouts, J.R (1971): Liver smooth endoplasmic reticulum microsomal drug-metabolizing enzyme system. In methods in pharmacology. Book 1, and 2nd edition (Edited by Schwartz, A.) Appleton-century-crofts. New York pp.287 - 325.

11. Galtier, P. Coche, Y. and Alvavinerie, m. (1983): Tissue distribution and elimination of H-Levamisole in the rat after oral and intramuscular administration. Xenobiotica 13: 407 -413.

12. Hadden, J.W., Coffey, R.G., Hadden, E.M., Lopez-Correlates, E. and Sunshine, G.H. (1975): Effects of Levamisole and imidazole on Lymphocyte proliferation and cyclic nucleotide levels. Cell Immun. 20: 98 - 105.

13. Hoffimann, W.E., Dorner, J.L and Morris, H. (3983): Intestinal alkaline phosphatase - like properties of horse kidney alkaline phosphatase. Enzyme 30: 269-272.

14. Hopper, D.G., Pimm, M.V. and Baldwin, R.W. (1975): Levamisole treatment of local and metastatic growth of trasplanted rat tumors. Br. Jr. Cancer 32: 345 - 351.

15. Imai, Y., Akio, I and Sato, R. (1966): Evidence for biochemically different types of vesicles in the hepatic microsomal fractions. Jr. biochem. 60: 417 -425.

16. Johnson, R.K., Houchens, D.P., Caston, M.R. and Goldin, A. (1975): Effects of Levamisole (NSC -177023) and tetramisole (NSC - 102063) in experimental tumour systems. Cancer Chemother. Rep.59: 697 -705.

17. Kato, R. (1976): The effects of starvation and refeeding on the oxidation of drugs by liver microsomes. Biochem. Pharmacol.16: 871 -887.

18. Khodaparast-Sharifi, S.H. and Dale - Snow, L (1989): Levamisole inhibition of alkaline phosphatase and 5'-nucleotides of alkaline phosphatase and 5' nucleotidase of Bovine milk Fat Globule membrane. Int. Jr. biohem. 21: 401-405.

19. Masters, B.S.S., williams, C.G. and kamin,H. [1967]: The preparation and properties of microsomal TPNH Cytochrome C reductase from pig liver. In Estabrook, R.W. Pullman, M.E. (Eds.) Methods in Enzymology. Vol. X, Taylors and Francis, London, Vol. 10, pp. 573.

20. Mitch, W. E., Collier, V.U. and Waker, M. (1980): Creating metabolism in chronic renal failure. Clin. Science 58: 327 - 335

21. Omura, T. and Sato, R. (1964): The carbon monoxide-binding pigment of liver microsomers. I. Evidence for its hemoprotein nature. Jr. Biol. Chem. 239: 2370 - 2379.

22. Peeples, A. and Dalvi, R.R. (1982): Toxic alkaloids and their interaction with microsomal Cytochrome P-450 in vitro. J. Appl. Toxicol. 2, 300 - 302.

23. Rollo, I. M (1980): Chemotherapy of parasitic diseases. In the Pharmacological basis of Therapeutics, 6th edn. (Edited by Gilmar, A. G. , Goodman, L. S. and Gilman, A.). pp. 1031 - 1037. Macmillian, New York.

24. Thienpont, D. , Vanparijs, O. F., Maeymaekers, A.H.N. Vandenberk, J., Demoen, P.J.A., Allewijnm, R.J.N., Marsbom, R.P.H., Niemegeers, C.J.E. Schellekens, K.H.L. and Janssen, P.A.J. (1966): Tetramisole (R8299) a new potent broad spectrum antihelmintic. Nature 209: 1084 - 1086.

25. Van Belle, H. (1972): Kinetics and inhibition of alkaline phosphatases from canine tissues. Biochim Biophtys Acts. 289: 153 - 168.

26 Zeman, F. J. (1991): Clinical nutrition and dietetics 2nd edn p 288. Macmillian, New York.