EFFECT OF METRONIDAZOLE ON THE HEPATIC MIXED FUNCTION OXYGENASES (CYTOCHROMES b_5 AND P-450) IN SWISS MICE

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Summary : Effect of metronidazole (MNZ) treatment (oral and ip) on activities of cytochrome b_5 and P-450 was studied in male, virgin and pregnant female mice. Activities of both the cytochromes increased in virgin mice treated with 2 *mg* (ip or PO, per mouse) but not in male and pregnant females. 30 *mg* dose (per mouse) was toxic in pregnant mice but increased the cytochromes activities in males and virgin females. HPLC analysis of liver MNZ levels showed that virgin females had higher MNZ content than male and pregnant females when treated with ip injection of MNZ (250 *mg/kg*).

Key words : metronidazole cytochromes b_5 and P–450

INTRODUCTION

Metronidazole (MNZ) has been reported to be carcinogenic in mice and rats (1,2, 10,11) and mutagenic to the histidine auxotrophs of *Salmonella typhimurium* (5,9). In our laboratory we conducted studies on its tumorigenic and teratogenic action (3). We found it to be tumorigenic and teratogenic in female Swiss mice and mutagenic in strain TA 100 of *S. typhimurium*.

Sufficient data pertaining to MNZ carcinogenicity and levels of Mixed Function Oxygenases is yet not available. Studies were therefore designed to measure the levels of Cyt. P-450 and Cyt. b_5 in male, virgin females and pregnant Swiss mice exposed to two doses of MNZ through different routes (viz., PO and ip). We also studied the levels of MNZ in liver tissue of mice in the three types of groups following its ip injection (250 *mg/kg* to see if the activity of drug metabolizing enzymes can be correlated to the drug levels in these three groups. The present paper reports our observations.

MATERIAL AND METHODS

Animals : Eight to twelve week-old virgin female, pregnant and male Swiss mice housed at 20°C were used.

Two doses of MNZ were administered – 2 *mg* MNZ/mouse/day and 30 *mg* MNZ/mouse/day - through two different routes i.e. oral and ip. The oral feeding was done for the first 18 days of gestation in the pregnant mice and 18 consecutive days in the virgin female and male mice. On the 18th day, the animals were sacrificed after starving them overnight. Single ip administration was done 24 hr prior to the time of sacrifice. These animals were also starved overnight. Control (untreated) and treated mice were killed by cervical dislocation and 20% homogenate was made from liver in 1.15% potassium chloride. After removal of cell debris by centrifugation at 9000 x g for 15 min the microsomal fraction was pelleted by centrifugation of the supernatant at 105,000 X g for 1 h at 4°C and resuspended in 0.1 M phosphate buffer (pH 7.4) to give protein concentration of 2 *mg/ml*. The protein was estimated by the method of Lowry *et al.* (6). Activities of Cyt b_5 and P-450 were determined by the method of Omura and Sato (12), using molar absorption coefficient of 171 cm^{-1} and 91 cm^{-1} mM^{-1} respectively. The activities were expressed in nmoles per *mg* protein.

Analysis of MNZ in liver tissue in mice injected with 250 *mg/kg* weight was carried out by homogenizing the tissue in methanol (7 *ml/g* tissue) at 4°C using potter Elvehjem Homogenizer. Homogenate was centrifuged at 2000 rpm for about 10 min and the pellet was washed thrice in methanol. Supernatants were pooled, flash evaporated at 50°C to concentrate to 1.00 *ml*, kept at —40°C for 30 min, centrifuged at 2000 rpm and filtered through millipore filter (filter type FIT; FITLP 13 mm diameter, 0.5 mm pore-size, Millipore Corp, USA). After filtration the sample was injected into HPLC system (HPLC Associates, USA) fitted with a model 6000 A solvent delivery system using close Bondapack reverse phase column, U6K filter, Chromatographs were recorded on Omnischribe (Houston Instruments, USA) stripchart recorder. The solvent system used was aceronitrile-acetic acid-water (90:0.5:9.5) as a mobile phase. Using MNZ as a standard with a retention time of 7 min the amount of MNZ present in the homogenate was calculated by measuring peak height and peak area. The amount of MNZ was expresed in *µg* of MNZ per g tissue. The results were expressed as mean of 3 experiments with S.E.M. and analysed using 't'-test. MNZ was used as pure compound (May & Baker Bombay, for higher doses) or injectable (Unique Pharmacy Bombay, for lower doses).

RESULTS

Table I shows the effect of ip injection of MNZ (2 *mg*/mouse) on liver microsomal cytochromes b_5 and P-450 in virgin and pregnant females and in males of the same age and strain. In virgin mice a significant increase in Cty. b_5 and Cty. P-450 was observed whereas there was no significant difference in the activities of the two cytochromes of untreated and MNZ treated males and pregnant females. It may also be noted that untreated female mice have relatively low cytochrome activity as compared to untreated males and pregnant females.

2 *mg* MNZ was administered by gavage to pregnant females during gestation. Virgin and male mice were also treated by gavage by the same dose for 18 days for comparison. The 30 *mg* dose of MNZ to pregnant females proved to be toxic, but was well tolerated by male and virgin females. Table II shows that 2 *mg* dose caused an increase in activities only in virgin females and had no effect on male and pregnant females. 30 *mg* dose increased the activities both in males and virgin/females.

TABLE I : Mixed function oxydases in male, virgin and pregnant females injected with metronidazole (2 *mg*/mouse, ip).

Group	Cyt b_5 (nmoles/mg protein)		Cyt P-450 (nmoles/mg protein	
	Untreated	Treated	Untreated	Treated
Virgin	0.18±0.01	0.35±0.02*	0.36±0.02	0.58±0.01*
Pregnant	0.22±0.02	0.24±0.01	0.60±0.03	0.68±0.02
Male	0.26±0.01	0.21±0.01	0.50±0.04	0.63±0.02

*Differ significantly ($P<0.05$) from value for the corresponding untreated mice.

TABLE II : Mixed function oxydases in male, virgin and pregnant females fed with MNZ for 18 days.

Group	Cyt b_5 (nmoles/mg protein)			Cyt P-450 (nmoles/mg protein)		
	Untreated	MNZ		Untreated	MNZ	
Virgin	0.17±0.01	0.23±0.02*	0.25±0.01*	0.36±0.02	0.58±0.02*	0.61±0.03*
Pregnant	0.25±0.01	0.24±0.01	TOXIC	0.61±0.02	0.61±0.02	TOXIC
Male	0.24±0.01	0.21±0.01	0.25±0.02	0.61±0.02	0.58±0.02	0.86±0.03

* Values differ significantly ($P<0.05$) from corresponding groups of male and virgin mice.

TABLE III : The levels of Metronidazole in the liver tissue of virgin female, pregnant and male Swiss mice at various times after its injection (250 *mg*/*kg*, ip).

Group	µg/MNZ/g tissue		
	1 hr	4 hr	18 hr
Virgin ♀	367±55	81.6±10.7	0.49±0.24
Pregnant	94±15	2.4±00.6	0.2±0.2
Male	172±31	22.2±8.2	—

Since Mixed Function Oxydases activity did not increase further on treatment with MNZ either by ip or oral route in males and pregnant females it seemed interesting to see if the level of MNZ itself was of similar magnitude in males, pregnant females and virgins. For that purpose mice were injected with MNZ (250 *mg/kg*, ip) and MNZ levels in liver tissue of males, virgins and pregnant females were measured.

Table III shows levels of MNZ at 1,4 and 18 hours after MNZ injection in all the three groups of mice. MNZ content in liver tissue of virgin mice at all times, was significantly higher than that in males and pregnant females.

DISCUSSION

Above data show clearly that Cyt. b_5 and Cyt. P-450 in liver tissue of pregnant and male did not increase further, after 2 *mg* MNZ treatment. This was probably due to the fact that basal levels of the two cytochromes in untreated pregnant and male mice were high and they could act on relatively low amount of MNZ present in liver tissue. On the other hand, virgins had low enzyme activities which increased in presence of relatively high level of MNZ. This suggests that probably MNZ levels and the drug metabolising enzymes are under hormonal influence and supports the earlier report that drug metabolizing enzymes are modulated by hormones (13). In this study pregnant mice were used on 18th day of gestation when estrogen activity is known to be suppressed by progesterone (4). It is possible that lack of estrogen activity in pregnant and male mice are for less absorption of the drug as well as for non-inducibility of the drug metabolizing enzymes. Munir *et al.* (7) have reported that host factors such as age and sex of the animal modulate the activity of benzo (a) pyrene (BP) hydroxylase in Swiss mice. They showed that activity of hexachlorocyclohexane-induced BP hydroxylase was greater in male than in female mice while in sterilized females it was comparable to that in intact mice.

So far, there has been no report on the effect of MNZ on Cyt P-450 and Cyt b_5. This is the first report on the effect of MNZ on the cytochromes b_5 and P-450 activity in Swiss mice. The function of Cyt b_5 working in concert with P-450 is not well-resolved. It has been proposed that reduced b_5 can denote an electron to only cytochrome P-450 (8). Hence these two cytochromes seem to play an important role in the metabolism of MNZ. It may have implication in MNZ carcinogenicity since the induction of tumors is seen only in virgin females (3) where there is induction of cytochromes b_5 and P-450 due to the drug while males are resistant to MNZ carcinogenicity and also do not show induction of the two enzymes.

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REFERENCES

1. Cavaliere, A., M. Bacci, A. Amorosi, M. Del Gaudio and R. Vitali. Induction of lung tumours and lymphomas in Balb/c mics by MNZ. *Tumori*, **69** : 379-382, 1983.
2. Cavaliere, A., M. Bacci and R. Vitali. Induction of mammary tumors with MNZ in female sprague-Dawley rats. *Tumori*, **70** : 307-312, 1984.
3. Chacko, M. Experimental studies on the tumorigenicity mutagenicity and teratogenicity of Metronidazole (*MNZ*) Ph.D. Thesis, University of Bombay, India, 1984.
4. Leaivitt, W.W., R.G. McDonald and W. Okulitz.C. Hormonal regulation of estrogen and progesterone receptor systems. In : Biochemical actions of hormones, Vol. 10, G. itwack (ed) New York, Academic Press, pp. 323-356, 1983.
5. Legator, M.S., T.H. Connor and M. Sooecket. Detection of mutagenic activity of Metranidazole and niridazole. in body fluids of humans and mice. *Sci.*, **188** : 118-1119, 1975.
6. Lowry, O.H., N.J. Rosenbrough, A.L. Farr and R.J. Randall, Protein measurement with the follin phenol reagent. *J. Biol. Chem.*, **193** : 265-275, 1951.
7. Munir, K. M., U. S. Murdia, and S. V. Bhide. Influence of estradiol on the benzo (a) pyrene hydroxylase activiry induced by hexachlorocyclohexane. *Toxicol. Letters*, **19** : 179-185, 1983.
8. Remmer, H., H. Greim, J. B. Schenkman and R. W. Estabrook. 'Methods for the elevation of hepatic microsomal mixed function oxydase levels and cytochrome P-450. *Methods in Enzymol.*, **10** : 703-708, 1967.
9. Rosenkranz, H. A. and W. T. Speck. Mutagenicity of metranidazole activation by mammalian liver microssmes. *Biochem. Biophys. Res. Comm.*, **66** : 520-525, 1975.
10. Rust. J. H. An assessment of Metronidszole (MNZ) : Tumorigenicity : Studies in the mouse and rat. In MNZ. proceedings of the International MNZ Conference., Finegold. M., J. A. McFadzean and F.J.C. Roe (eds). Montreal *Excerpta Medica*, pp 138-144, 1977.
11. Rutia, M. and P. Shubik. Induction of lung tumors and malignant lymphomas in mice by mettanidazole *J. Natl. Cancer Inst.*, **48** : 721-723, 1972.
12. Omura, T. and R. Sato. The carbon monoxide-binding pigment of liver microsomes. I. Evidence for its homoprotein nature. *J. Biol. Chem.*, **239** : 2370-2378, 1964.
13. Williams, D.C. and M. Smethurst. Enzyme induction by steroid hormones with reference to cancer. In : Enzyme Induction. D. V. Parke (ed) New York, Plenum Press. pp. 145-168, 1975.