Pharmacia-JTPA
30 (1), 5-12, 1990

INFLUENCE OF NICKEL ON HEPATIC MONOOXYGENASES, LIPID PEROXIDATION, GLUTATHIONE AND GLUTATHIONE S-TRANSFERASE OF MICE *IN VITRO*

Tülay ÇOBAN (*)   Mümtaz İŞCAN (*)

NİKELİN FARE KARACİĞERİ MONOOKSİJENAZLARI, LİPİT PEROKSİDASYONU, GLUTATYONU VE GLUTATYON S-TRANSFERAZI ÜZERİNE *İN VİTRO* ETKİSİ

ÖZET

İnkübasyon ortamına, **in vitro**, olarak nikel klorür ($NiCl_2$) 10^{-7} ile 10^{-5} M arasındaki konsantrasyonlarda ilave edildiğinde erkek fare karaciğeri anilin 4-hidroksilaz enzim aktivitesini etkilemediği ancak daha yüksek konsantrasyonlarda (10^{-3} - $2.5x10^{-2}$ M) inhibe ettiği gözlenmiştir. ($NiCl_2$) ün etilmorfin N-demetilaz enzim aktivitesi üzerine ise farklı etkidiği saptanmıştır. Enzim aktivitesi 10^{-5} M konsantrasyonunda artmış olmasına karşın yüksek konsantrasyonlarda aktivitenin oldukça dik bir şekilde düştüğü saptanmıştır. Mikrozomal aminopirin N-demetilaz enzim aktivitesi 10^{-7} ile 10^{-3} M ($NiCl_2$) konsantrasyonları arasında kontrol değerin biraz üzerinde kalmıştır. Daha yüksek konsantrasyonlarda enzim aktivitesinin inhibe olduğu saptanmıştır. Ancak ($NiCl_2$) **in vitro** olarak inkübasyon ortamına ilave edildiğinde lipit peroksidasyon, glutatyon ve glutatyon S-transferazın hiçbir değişikliğe uğramadığı gözlenmiştir.

SUMMARY

In vitro addition of nickel chloride ($NiCl_2$) to an incubation mixture caused no changes between 10^{-7} M and 10^{-5} M concentrations but produced a concentration-dependent inhibition of liver microsomal aniline 4-hydroxylase activity of male mice at higher concentrations (10^{-3} - $2.5x10^{-2}$ M). $NiCl_2$ produced a different pattern of effect on ethylmorphine N-demethylase activity. At

(*) Department of Toxicology, Faculty of Pharmacy, Ankara University, 06100, Tandoğan, Ankara

10^{-5} M concentration an increase and at higher concentrations a rather abrupt decline was observed in the enzyme activity. Aminopyrine N-demethylase activity was slightly above the control value between 10^{-7} and 10^{-3} M $NiCl_2$ concentrations. At higher cocentrations $NiCl_2$ inhibited the enzyme activity. However, no alterations were observed in the lipid peroxidation, glutathione and glutathione S-transferase by in vitro addition of $NiCl_2$ to the incubation mixtures.

Key words: Nickel, liver, monooxygenases, lipid peroxidation, glutathione, glutathione S-transferase.

INTRODUCTION :

Recently we have studied the **in vivo** effect of nickel (Ni), which has been reported to be widely distributed throughout the environment (1, 2) and carcinogen (3,4), on mice liver monooxygenases (5). It was shown that monooxygenase activities were significantly inhibited by Ni treatment. However, Ni sensitivity differences have been determined between substrates of monooxygenases. Previous studies from our laboratory (6) and others (7-9) revealed that the direct, **in vitro**, effect of Ni on microsomal enzymes also varies depending on the substrate of monooxygenases, organ and animal species. Morever, discrepancies have been well established between **in vitro** and **in vivo** effects of metals on monooxygenases (10,11).

On the other hand it has been reported that the decrease in monooxygenases has been related with increase lipid peroxidation (12). Furthermore the elevated concentration of lipid peroxides have been attributed to the depletion of glutathione (GSH) levels (13,14).

Therefore, this study was designed to examine the **in vitro** effect of Ni on liver monooxygenases of mice in order to see whether **in vitro** there exist metal sensitivity differences between substrates of monooxygenases of mice liver. We have further extended our studies so as to determine whether changes in the monooxygenases are related with lipid peroxidation and GSH level. Ni effect on glutathione S-transferase activity has been well examined.

MATERIALS AND METHODS :

Chemicals

Nicotinamide adenine dinucleotide phosphate sodium salt ($NADP^+$), D-glucose-6-phosphate monosodium salt, glutathione, 1-chloro-2, 4-dinitrobenzene (CDNB), 5,5'-dithiobis-(2-nitrobenzoic acid) were purchased from Sigma Chemical Co., St. Louis, Missouri, USA. p-Aminophenol and formaldehyde were obtained from Fluka A.G., Switzerland. Ethylmorphine HCI was purchased from Knoll, W.Germany. Nickel chloride was purchased from Merck A.G., W.Germany. Aniline was purchased from Riedel A.G., W.Germany.Crystalline bovine serum albumin was obtained from BDH Chemicals Ltd., Poole UK. All the other chemicals were of analytical grade.

Animals and tissue preparation

Male albino mice weighing 25-30 g were used throughout the experiments. The mice were fed with standard laboratory mice pellet chow **ad libitum.** All animals were fasted for 24 h before they were killed.

Animals were killed by cervical dislocation. The livers were removed immediately and washed in ice cold distilled water to remove the excess blood. All subsequent steps were carried out at 0-4°C. 10.000 g supernatant fractions were prepared as described previously (6).

For the determination of GSH, the tissues were homogenized in ice-cold 1.15 % KCl solution using a motor driven Teflon Potter-Elvehjem homogenizer, and

macromolecules were precipitated by mixing equal volumes of tissue homogenates 20 % (w/v) and 10 % (w/v) trichloroacetic acid (TCA) containing 5 mM ethylenediamine tetraacetic acid(EDTA) and centrifuged at 10.000 g for 10 min. This tissue extract supernatant is used in the determination of GSH level.

Analytical procedures

Liver enzyme activities and lipid peroxidation were determined by using 10.000 g supernatant. In the preliminary studies optimum conditions for all the assays were determined. In this study, for the assays, those determined optimum conditions have been used.

Aniline 4-hydroxylase activity was measured by the quantitation of p-aminophenol (p-AP) as described by Imai **et al.** (15). A typical optimized assay mixture contained 10 mM aniline, 100 mM potassium phosphate buffer, pH 6.8 (at 25°C), 0.25 mM $NADP^+$, 6.25 mM $MgCl_2$, 2.5 mM glucose-6-phosphate and 3.0 mg 10.000 g supernatant protein in a final volume of 1.0 ml.

Ethylmorphine N-demethylase and aminopyrine N-demethylase were measured by the quantitation of formaldehyde as described by Nash (16) and modified by Cochin and Axelrod (17). A typical assay mixture contained 10 mM ethylmorphine HCl or 10 mM aminopyrine, 100 mM potassium phosphate buffer, pH 7.8. (at 25°C), 0.25 mM $NADP^+$, 6.25 mM $MgCl_2$, 2.5 mM glucose-6-phosphate and 4.0 mg 10.000 g supernatant protein in a final volume of 1.0 ml.

GSH was assayed as a major non-protein sulfhydryl according to the method of Ellman (18) and Ellman and Lysko (19) in which the rate of reduction of 5,5'-dithiobis (2-nitrobenzoic acid) (DTNB) is proportional to the amount of GSH. The assay mixture comprized 0.1 M potassium phosphate buffer, pH 8.0, containing 5 mM EDTA. To this 200 ul of tissue extract supernatant added followed by 5 μ mole of DTNB. The rate of DTNB reduction was determined spectrophotometrically by recording the increase in absorbance at 412 nm. Also, every daily assay included a standart curve with known amounts of GSH.

Lipid peroxidation was estimated by measuring the product malonyl aldehyde (MDA) using the thiobarbituric acid (TBA) assay (20). 1.0 ml of 10.000 g supernatant (8.0 mg protein) was incubated for 1 h at 37°C in a shaking water bath. Protein was precipitated by the addition of 0.5 ml of 30 % TCA and centrifuged at 10.000 g for 15 min. A portion (1 ml) of the supernatant was removed and 0.25 ml of 1 % TBA in 0.05 N NaOH were added. The test tubes were heated in a boiling water bath for 30 min. After cooling the absorbance at 532 nm was recorded.

Glutathione S-transferase (GST) activity was determined in 10.000 g supernatant as detailed by Habig **et al.**(21), using 1-chloro-2, 4-dinitrobenzene (CDNB) as a substrate.

Protein content of 10.000 g liver supernatant was determined by the method of Lowry **et al.** (22) with the use of bovine serum albumin as a standard.

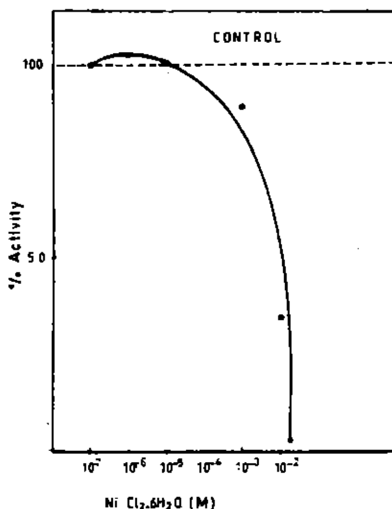

Figure 1. Concentration related effect of $NiCl_2$ on liver microsomal aniline 4-hydroxylase activity. Aniline 4-hydroxylase activity was determined at optimum conditions as described under Materials and Methods. Each point, expressed as a percentage of control value, represents the mean of three determinations. Variation between the determinations was always less than 5 %.

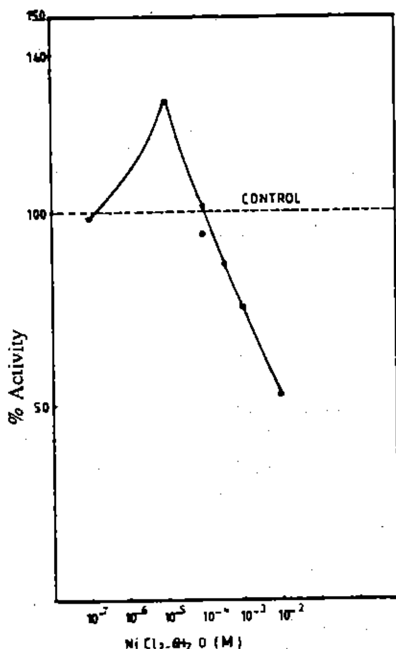

Figure 2. Concentration related effect of $NiCl_2$ on liver microsomal ethylmorphine N-demethylase activity. The enzyme activity was determined at optimum conditions as described under Materials and Methods. Each point, expressed as percentage of control value, represents the mean of three determinations. Variation between the determinations was always less than 5 %.

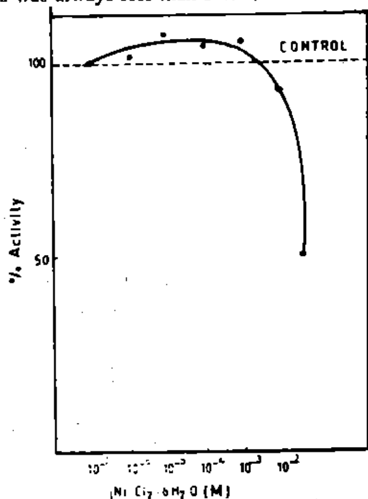

Figure 3. Concentration related effect of $NiCl_2$ on liver microsomal aminopyrine N-demethylase activity. The enzyme activity was determined at optimum conditions as described under Materials and Methods. Each point, expressed as a percentage of control value, represents the mean of three determinations. Variationbetween the determinations was always less than 5 %.

RESULTS:

In the experiments, the average control values of liver microsomal AH, EMND and AMND were found to be 3.33 ± 0.16 nmol/25 min/mg protein (N=6, mean ± SE), 6.48 ± 0.04 nmol HCHO/10 min/mg protein (N=6, mean ± SE) and 7.70 ± 0.13 nmol HCHO/10 min/mg protein (N=6, mean ± SE), respectively.

The effects of various concentrations of $NiCl_2$ on liver monooxygenases are shown in Figs 1,2 and 3. As seen in Fig. 1 between 10^{-7} and 10^{-5} M concentrations no changes were observed in the AH activity but concentration-dependent inhibition was noted in the enzyme activity at higher concentrations.

$NiCl_2$ produced different pattern of effect on EMND activity (Fig.2). At 10^{-5} M concentration an increase and at higher concentrations a rather abrupt decline was observed in the enzyme activity. The **In vitro** effect of the metal on AMND activity was not similar to those observed on AH and EMND activities. Between metal concentrations 10^{-7} and 10^{-3} M the enzyme activity was determined to be slightly over the control value and at higher concentrations it decreased the enzyme activity.

As illustrated in Table 1,$NiCl_2$ did not alter the lipid peroxidation. Similarly, the metal was unable to change the level of GSH and glutathione S-transferase (CDNB) activity when added in increasing concentrations to the reaction mixtures (Table 2).

DISCUSSION:

The present study revealed that Ni at high concentrations inhibited the monooxygenase activities but at lower concentrations the pattern of effect varied depending on the substrate of monooxygenases.

The result obtained for AH activity in this study is in good agreement with our

Table 1. Concentration related effect of $NiCl_2$ on lipid peroxidation in mice liver*.

$NiCl_2.6H_2O$ (M)	Lipid peroxidation (nmol MDA/mg protein)
Control	159.5 ± 6.5
10^{-7}	158.0 ± 8.0
10^{-6}	174.5 ± 8.5
10^{-5}	166.5 ± 16.5
10^{-4}	171.5 ± 11.5
10^{-3}	173.0 ± 7.0
10^{-2}	155.0 ± 5.0

* The 10.000 g supernatant protein concentration for the assay was 2 mg protein/ml. Each value represents the mean of three determinations (mean, ± SE).

Table 2. Concentration related effect of $NiCl_2$ on glutathione level and glutathione S-transferase activity in mice liver*.

$NiCl_2.6H_2O$ (M)	GSH (μmol/g tissue)	GSH-S-Transferase (CDNB) (nmol/min/mg protein)
Control	3.06 ± 0.08	1652 ± 45
10^{-7}	3.18 ± 0.02	1682 ± 15
10^{-6}	3.19 ± 0.06	1786 ± 119
10^{-5}	3.23 ± 0.03	1742 ± 45
10^{-4}	3.15 ± 0.02	1756 ± 89
10^{-3}	3.02 ± 0.06	1682 ± 15

* The reaction mixture (3 ml) of GSH assay contained 0.05 ml of tissue extract supernatant corresponding to 5 mg tissue. The 10.000 g supernatant protein concentration for GSH-S transferase assay was 0.025 mg/0.75 ml. Each value represents the mean of three determinations (mean, ± SE).

previous finding in the guinea-pig liver (6) and with the result of Arinç and İşcan (23) in sheep liver. With respect to EMND activity previously we observed biphasic effect of Ni in the guinea-pig liver (6) as observed in this study in mice liver. In the sheep liver Ni was reported to inhibit EMND (9). **In vitro** Ni was shown to inhibit the microsomal benzo (a) pyrene hydroxylase in mice liver (8), while causing a marked increase in hamster fetus cell culture (7). The sensitivity of AMND appeared to be different from that of AH and EMND. These findings show that the direct, **in vitro,** effect of Ni on monooxygenases of mice liver also differ depending on the substrate of monooxygenases, possibly due to alteration of different isozymes of cytochrome P-450, which are responsible for their metabolism, to different extends since various isozymes of cytochrome P-450 are reported to be present in mice liver (24). Furthermore these studies demonstrate that direct effect of Ni on monooxygenases may as well vary depending on substrate, organ and animal species.

In the current study since Ni has been observed to be ineffective on lipid peroxidation, it seems likely, in the case of Ni and at least **in vitro,** that lipid peroxidation is not responsible for the changes observed in monooxygenases. This finding is in contrast with the result of Kamatagi and Kitagawa (12) but in accordance with that of Baird (25).

Due to its pronounced nucleophilicity, glutathione is expected to react readily with metals. However, Ni **in vitro** was unable to react with glutathione. However, several heavy metals have been shown to evoke a nearly total depletion of GSH in isolated rat hepotocytes and as a consequence lipid peroxidation (26-28). Nevertheless, the lack of alteration in the GSH level probably resulted unalteration of lipid peroxidation. This finding seems to confirm the previous report (13-14) that there exist relationship between GSH levels and lipid peroxidation.

Glutathione S-transferase activity towards CDNB was not influenced by any Ni concentrations studied, possibly due to unalteration of GSH level. Nevertheless, the possibility still exists that other isozymes of GSH-S transferases can be influence[illegible] direct effect of Ni. However, in rat li[illegible] **in vitro** was also found to be ineffective on glutathione S-transferases towards another substrate styrene oxide (29).

In conclusion, the results of the present study show that (i), **in vitro** there exist Ni sensitivity differences between substrates of monooxygenases,(ii) lipid peroxidation is not responsible for the changes in the monooxygenase activities, probably, due to the lack of alteration in the GSH level (iii) Ni, **in vitro,** has no influence on glutathione S-transferase towards CDNB.

ACKNOWLEDGEMENT:

This work was supported by Institute of Health Sciences, Ankara University (Grant No: 87-30-00-01).

REFERENCES

1. Hammond, P.B., Beliles, R.P.(1980), "Metals". In Toxicology (Ed. Doull, J. Klaassen, C.D., Amdur, M.O.) pp. 409-467 Mac Millan, New York.

2. Leonard, A., Greber, G.B., Jacquet, P., (1981) "Carcinogenicity, mutagenicity and teratogenicity of nickel", **Mutation Res.** 87, 1-15.

3. Sunderman, F.W.Jr., "Carcinogenic effects of metals", (1978) **Fend. Proc. Fend. Am. Socs. exp. Biol.** 37, 40-46.

4. Holbrook, D.J.Jr., "Chemical carcinogenesis" (1980) **In Biochemical Toxicology** (Ed. Hodgson, E., Guthrie, F.E.) pp. 310-329. Elsevier North Holland, New York.

5. Iscan, M., Coban, T., (1989) "Nickel effect on hepatic cytochrome P-450 dependent monooxygenases in mice". In Cytochrome P-450: **Biochemistry and Biophysics** (Ed.Schuster, I.) pp. 787-789, Taylor and Francis, London.

6. Iscan, M., (1984) "A comparative study of the effects of cadmium and nickel on liver microsomal drug metabolizing enzymes of guinea-pig in vitro" **Comp. Biochem. Physiol.** 79 C, 429-433.

7. Nebert, D.W., Gelboin, H.V. (1968) "Substrate-inducible microsomal aryl hydroxylase in mammallian cell culture". **J.Biol.Chem.** 243, 6242-6249.

8. Tsang, S., Furst, A., (1976) "In vitro inhibition of aryl hydrocarbon hydroxylase by heavy metals". **Onco-logy** 33, 201-204.

9. Inci, M., (1983), "Characterization of microsomal ethylmorphine N-demethylase from sheep liver". M.Sc. Thesis. METU, Ankara,

10. Lui, E.M.K., Lucier, G.W., (1981), "Hypophysial regulation of cadmium-induced depression of the hepatic monooxygenase system in the rat" **Molec. Pharmac.** 20, 165-171.

11. Karakaya, A., Iscan, M., (1988), "Effects of cadmium on pulmonary and renal microsomal drug metabolizing enzymes of the guinea-pig". **Comp. Biochem. Physiol.** 90C, 241-244.

12. Kamatagi, T., Kitagawa, H., (1973), "Effects of lipid peroxidation on activities of drug metabolizing enzymes in liver microsomes of rats". **Biochem. Pharmac.** 22, 3199-3207.

13. Anundi, I., Hogberg, J., Stead, A.H.,(1979) "Glutathione depletion in isolated hepatocytes: its relation to lipid peroxidation and cell damage". **Acta. pharmac.tox.** 45, 45-55.

14. Younes, M., Seigers, C.P., (1981), "Mechanistic aspect of enhanced lipid peroxidation following glutathione depletion **in vitro". Chem. Biol. Interact.** 34, 257-266.

15. Imai, Y., Ito, A., Sato, R., (1966), "Evidence for biochemically different types of vesicles in the hepatic microsomal fraction". **J.Biochem.** 60, 417-428.

16. Nash, T., (1953) "The colorimetric estimation of formaldehyde by means of the Hantzsch reaction". **Biochem j.** 55, 416-421.

17. Cochin, J., Axelrod, J., (1959) "Biochemical and pharmacological changes in the rat following chronic administration of morphine, nalorphine and normorphine". **J. Pharmac. exp. Ther.** 125, 105-110.

18. Ellman, G.L., Lysko, H., (1978), "A precise method for determination of whole blood and plasma sulphydryl groups". **Analyt. Biochem.** 93, 98-105.

20. Placer. Z.A., Cushman., L., Johnson, B.C., (1966) "Estimation of product of lipid peroxidation (malonyldialdehyde) in biochemical systems "**Analyt. Biochem.** 16, 359-364.

21. Habig, W.H., Pabst, M.J., Jakoby, W.B., (1974) "Glutathione-S transferases. The first enzymatic step in mercapturic acid formation". **J. Biol. Chem.** 249, 7130-7139.

22. Lowry, O.H., Rosebrough, H.J., Farr, A.L., Randall, R.J., (1951), "Protein measurement with the Folin phenol reagent". **J.Biol.Chem.** 193, 265-275.

23. Arınç, E., Işcan, M.Y., (1983) "Comparative studies of sheep liver and lung microsomal aniline 4-hydroxylase". **Comp. Biochem. Physiol.** 74C, 151-158.

24. Gonzalez, F.J., (1989) "The molecular biology of cytochrome P450s". **Pharmac. rev.** 40, 243-288.

25. Baird, M.B., (1980), "Microsomal NADPH-dependent lipid peroxidation does not require the presence of intact cytochrome P-450". **Biochem. Biophys. Res. Commun.** 95, 1510-1516.

26. Stacey, N.H., Cantilena, L.R., Klaassen, C.D., (1980), "Cadmium toxicity and lipid peroxidation in isolated rat hepatocytes". **Toxicol. Appl. Pharmac.** 53, 470-480.

27. Stacey, N.H., Klaassen, C.D., (1981), "Comparison of the effects of metals in cellular injury and lipid peroxidation in isolated rat hepatocytes". **J.Toxicol. Environ.Health** 7, 139-147.

28. Stacey, N.H., Kappus, H. (1982), "Cellular toxicity and lipid peroxidation in response

to mercury". **Toxicol.Appl. Pharmacol.** 63, 29-35.

29. Aitio, A., Ahotupa, M., Parkki, M.G., (1978) "Inhibition of drug metabolizing enzymes by heavy metals **in vitro**". **Biochem. Biophys. Res. Commun.** 83, 850-856.