



The Effect of Some Inducers On The N-Nitrosomorpholine Metabolism in Diabetic Rats

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Abstract. N-Nitrosomorpholine (NMOR) a cyclic nitrosamine, is a carcinogenic and mutagenic compound found in various foodstuffs and tobacco and used together with polyacrylonitril in the production of rubber and rubber products. In this work, nitrite and acetaldehyde formation kinetics have been studied with the aim of investigating the NMOR metabolism in the rat liver microsomal system. The NMOR metabolism was investigated kinetically in rats in which experimental diabetes was induced by way of streptozotocin and following administration of acetone, isopropanol and ethanol to these rats. In addition, the effects of nickel and cadmium ions on the NMOR metabolism were investigated in these experimental groups. At the end of the investigations, it was observed that dealkylation increased in diabetic rats (V_{max} : 10,02 nmol/mg protein/min) when compared with that of the controls (V_{max} : 5,29 nmol/mg protein/min) It was determined that dealkylation increased in diabetic groups administered solvent when compared with that of diabetic controls. Similar results were also obtained in nitrate (the denitrosation product) formation. It was denitrosation and cadmium of identical concentration had a greater effect than nickel.

Keyword: N-Nitrosomorpholine, Streptozotocin, Diabetes Mellitus

Abbreviations used: NMOR, N-Nitrosomorpholine; STZ, streptozotocin; DM, diabetes mellitus; NDMAd, nitrosodimethylamine demethylase ; PB, phenobarbital ; 3-MC, 3-methylcholanthrene

Diyabetik Ratlarda N-Nitrozomorfolinin Metabolizmasına Bazı İndükleyicilerin Etkisi

Özet. Halkasal bir nitrozamin olan N-Nitrozomorfolin (NMOR) çeşitli yiyeceklerde ve tütünde yer alan endüstride poliakrilonitril ile lastik ve kauçuk üretiminde kullanılan karsinojenik ve mutajenik bir bileşiktir. Çalışmamızda NMOR'un rat karaciğer mikrozomal sisteminde metabolizmasını incelemek amacıyla nitrit ve asetaldehit oluşum kinetikleri izlendi. Streptozotocin verilerek deneysel diyabet oluşturulan ratlarda ve bu ratlara aseton, izopropanol ve etanol uygulanmasından sonra NMOR metabolizması kinetik olarak incelendi. Ayrıca bu deney gruplarında nikel ve kadmiyum iyonlarının NMOR metabolizması üzerindeki etkileri araştırıldı. Çalışmalar sonunda, kontrollerle kıyaslandığında (V_{max} : 5,29 nmol/mg protein/dk) diyabetik ratlarda (V_{max} : 10,02 nmol/mg protein/dk) dealkilasyonun arttığı gözlemlendi. Çözücü uygulanan diyabetli gruplarda dealkilasyonun diyabetli kontrollere göre arttığı saptandı. Nitrat (denitrozasyon ürünü olan) oluşumunda da benzer sonuçlar elde edildi. Kadmiyum ve Nikel iyonlarının denitrozasyonu inhibe ettiği, aynı derişimdeki kadmiyumun nikelden daha etkili olduğu saptandı.

Anahtar Kelimeler: N-Nitrozomorfolin, Streptozotocin, Şeker hastalığı

Kullanılan kısaltmalar: NMOR, N-Nitrozomorfolin; STZ, Streptozotocin; DM, Şeker hastalığı; NDMAd, nitrozodimetilamin demetilaz ; PB, fenobarbital ; 3-MC, 3-metilkolantren

1. INTRODUCTION

It is well established known that nitrosomorpholine, NMOR, has carcinogenic and mutagenic properties [1]. It has been demonstrated that NMOR may enter in our body through certain food, tobacco and its products and it is used in the production of rubber and rubber products [2,3]. NMOR can be synthesized *in vivo* from nitrite and secondary amines. NMOR and its metabolites have been found in human urine

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[4]. It has been shown that NMOR binds to aromatic amino acids and inhibits the activities of some enzymes [5-7].

The metabolic activation of nitrosamines is mainly catalyzed by cytochrome P450 enzymes. The distribution of the various P450 forms in different tissues are important in determining the tissue specificity of different nitrosamines. P4502E1 has been shown to be a key enzyme in the activation of nitrosamines with low molecular weight, especially with methyl and ethyl groups. However, in the bioactivation of more complex nitrosamines such as the tobacco carcinogen 4-(methylnitrosamino)-1-(3-pyridyl)-1 butanone, other enzymes may be more important. [8-11]. NMOR is metabolized by cytochrome P450, producing nitrite (NO^-) by denitrosation reaction and gets converted into stable metabolites such as acetaldehyde, formaldehyde, glyoxal and N-nitroso-2-hydroxymorpholine [12]. High fat diet, diabetes, fasting, acetone, isopropanol and ethanol have been reported to induce enzyme activity of P-4502E1 [13-14]. We previously showed an increased cytochrome P-450 reductase activity in diabetic rats and also diabetic rats received acetone or isopropanol or ethanol in comparison to controls [15].

Nickel compounds are known to be carcinogenic to humans and animals. Cobalt compounds produce tumors in animals and are probably carcinogenic to humans. The mechanisms of the carcinogenicity of these metal compounds, however, have remained elusive [16-20].

In the present study, we have administered streptozotocin to induce diabetes in rats, followed by acetone or isopropanol or ethanol and studied the alterations in dealkylation and denitrosation of NMOR in liver microsomes, and we also studied the effect of Ni^{2+} and Cd^{2+} ions on NMOR metabolism. The results are presented in this communication.

2. MATERIALS and METHODS

2.1 Materials

Streptozotocin (STZ), glucose-6-phosphate and glucose-6-phosphate dehydrogenase, NADPH, N-nitrosomorpholine, bovine serum albumin, Coomassie Brilliant Blue G250, acetylacetone, sulfanilic acid, N-1-naphthylethylenediamine, acetone, ethanol, isopropanol, acetaldehyde, cadmium chloride, nickel chloride and sodium nitrite were purchased from Sigma Chemical Company. All other chemicals were of analytical grade.

2.2 Animals and injections

Wistar albino rats, (n=30) weighing 150-260g were divided into five groups of six in each. Of these, the control group received injections (ip) of physiological saline and the remaining 4 groups received 65 mg/kg streptozotocin in 0.05 M citrate buffer pH 4.5 (ip). Glucose concentrations in the blood from

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the tail veins were measured to monitor the development of diabetes. After ten days when development of diabetes was confirmed, the control group and group 1 of diabetic rats were decapitated. Group 2 of was administered 2.5 ml/kg of 25 % acetone 18 hrs before decapitation and the group 3 received 2.5 ml/kg isopropanol 24 hrs before decapitation by gavage. In the case of group 4, 15 % ethanol was added to the drinking water for 3 days and the animals were decapitated [13,21,22].

2.3 Microsomal pellet

The rats were fasted for 8 hrs, anaesthetized by ether and blood was drawn from heart to determine blood-glucose by glucose oxidase method. The liver was removed, 3 ml of 154 mM KCl containing 10 mM EDTA was added per gram fresh tissue, homogenized in a tissue homogenizer in four strokes and centrifuged at 15,000g for 30 min at 4 °C to remove the membrane fragments and mitochondria. The supernatant was centrifuged at 105,000g for 60 min at 4°C (Beckman L5-75 B Ultracentrifuge). The pellet was re-suspended in fresh buffer, centrifuged again at 105,000g for 60 min. at 4°C and microsomal pellet was separated. Protein was estimated by the method described by Scopes [23], using bovine serum albumin as standard.

2.4 pH Effect on NMOR denitrosation

Buffers containing 10 mM MgCl₂, 150 mM KCl and 50 mM Tris-HCl of different pH (6.0, 6.5, 7.0, 7.5, 8.0 and 8.5) were used. NMOR 17.5 mM, buffers and microsomal fractions (0.5 mg/ml) in a total volume of 1 ml solution was incubated at 37°C for 2 min. Reaction was initiated by adding NADPH-regenerating system containing 0.4 mM NADP, 10 mM glucose-6-phosphate and 0.2 U glucose-6-phosphate dehydrogenase, incubated at 37 °C for 15 min, reaction was stopped by adding 150 µl of 25 % ZnSO₄ and 150µl saturated Ba(OH)₂, centrifuged at 1000g for 10 minutes and nitrite (NO₂⁻) in the supernatant was determined by sulfanilic acid and alpha-naphthylamine, undergoes a diazotization reaction with nitrites, forming a red azo dye [24]. Similar experiment in the control and the four other experimental groups were done to study the effect of pH on NMOR denitrosation.

2.5 The effects of Cd²⁺ and Ni²⁺ on the NMOR denitrosation

CdSO₄ and NiCl₂ solutions of varying (0-20 mM) concentrations containing 10 mM MgCl₂, 150 mM KCl, 50 mM Tris-HCl (pH ;7.5) buffer, 17.5 mM NMOR and 0.5 mg protein/ml microsomal fractions were incubated in a total volume of 1 ml at 37°C for 2 min. The reaction was initiated by adding NADPH-regenerating system and the nitrite was assayed as described above [25] in the control and the four experimental groups.

2.6 Kinetic investigation on the formation of acetaldehyde

NMOR solutions of final concentration 1.7, 7, 17.5, 35 and 70 mM were prepared for the determination of K_m and V_{max} the reaction leading to formation of acetaldehyde, one of the NMOR metabolites. Tris-HCl buffer (50 mM), pH 7.5 containing NMOR solutions and 0.5 mg protein/ml microsomal fraction in a total volume of 1 ml 10 mM MgCl₂, 150 mM KCl was incubated at 37 °C for 2 min. The reaction was started by adding NADPH-regenerating system, incubated for 15 min at 37 °C and the reaction was stopped by adding 150 µl of 25 % ZnSO₄ and 150 µl saturated Ba(OH)₂, centrifuged at 1000g for 10 minutes and acetaldehyde in the supernatant was determined by colorimetric with p-hydroxydiphenyl. [24] in the control and the four experimental groups.

3. RESULTS AND DISCUSSION

It was observed that fasting blood glucose levels of streptozotocin administered rats increased 2.5-4 times compared to those of the control group. The mean blood glucose level of the control group was found to be 90 mg/dl. The growth rate of diabetic rats was lower than the control group rats (Table 1). NMOR is a potent liver carcinogen in rats when administered orally [26]. It has been reported that it binds to the liver DNA at very low levels in treated rats [27].

Table 1 Physiological parameters of rats injected stz. †

| Group | Injection | No. of rats | Body Weight (g) | | Liver Weight (g) | Blood Glucose (mg/dl) |
|-----------|----------------------|-------------|-----------------|--------|------------------|-----------------------|
| | | | Initial | Final | | |
| Control | Physiological saline | 6 | 208±11 | 230±10 | 7.45±0.35 | 90±08 |
| Group I | Streptozotocin(Stz) | 6 | 220±22 | 235±24 | 7.15±0.65 | 262±40 |
| Group II | Stz + acetone | 5* | 264±03 | 275±05 | 7.85±0.45 | 256±05 |
| Group III | Stz + isopropanol | 6 | 183±09 | 197±13 | 7.10±0.15 | 344±08 |
| Group IV | Stz + ethanol | 6 | 150±03 | 162±03 | 5.75±0.85 | 334±41 |

* One of the rats died

Values are given as mean ±SD

The optimum pH for NMOR denitrosation was found to be 7.5 (Fig.1). Figures 2 and 3 shows the effects of the Ni²⁺ and Cd²⁺ ions on the NMOR denitrosation. It is seen that with increase in the concentration of both ions, NMOR denitrosation decreased in an inversely proportional manner. In addition, it has been reported that Cd²⁺ causes necrosis and apoptosis and increases the lipid peroxidation in cell. Cd²⁺ and Ni²⁺ was also reported to change the several enzyme activities [28-31].

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V_{max} and K_m were calculated for NMOR concentrations ranging from 1.7 to 140 mM from Michaelis Menten and Lineweaver-Burk plots. There was no significant difference in the K_m values between the control group and the acetone, isopropanol and ethanol groups. However V_{max} increased 2-2.5 fold in diabetic rats (Table 2).

Lorr *et al.* [13]. have shown that the liver microsomal activity increased in diabetic rats induced by STZ, acetone, isopropanol and fasting. It has been reported that the NADPH-dependent nitrosodimethylamine
Table 2 Kinetic parameters of acetaldehyde following NMOR metabolism.

| Group | Injection | V_{max} (nmol/mg/min) | K_m (mM) |
|-----------|----------------------|----------------------------|---------------|
| Control | Physiological saline | 5.29 | 3.78 |
| Group I | Streptozotocin(Stz) | 10.02 | 4.67 |
| Group II | Stz + acetone | 10.85 | 4.54 |
| Group III | Stz + isopropanol | 11.62 | 3.76 |
| Group IV | Stz + ethanol | 13.38 | 3.72 |

demethylase activity of rats administered acetone and isopropanol increased 3-4.5 fold and a mild increase was observed in the Cytochrome P-450 reductase activity [32]. Appel *et al.* [33]. Investigated NMOR denitrosation in rats given Phenobarbital and 3-methylcholanthrene and reported that the former increased nitrite formation by about 200 % but latter did not affect the nitrite formation. The data presented in this paper shows that the metabolization of NMOR in diabetes is accelerated, and it could be further accelerated by introducing a second inducer.

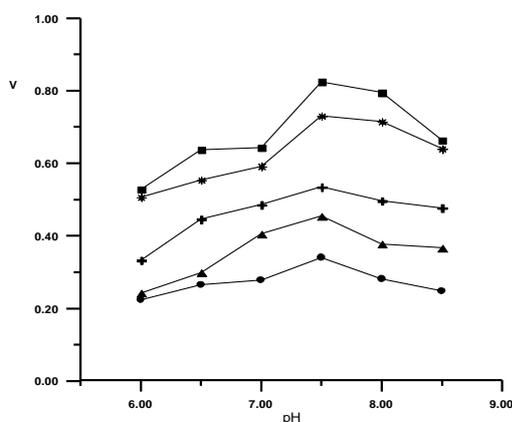


Fig 1 Effect of pH on the activity of denitrosation of NMOR. [(●) Control, (▲) diabetes (DM), (+) DM + acetone, (★)DM + ethanol, (■) DM + isopropanol. V=Nitrite, nmol/mg microsomal protein/min]

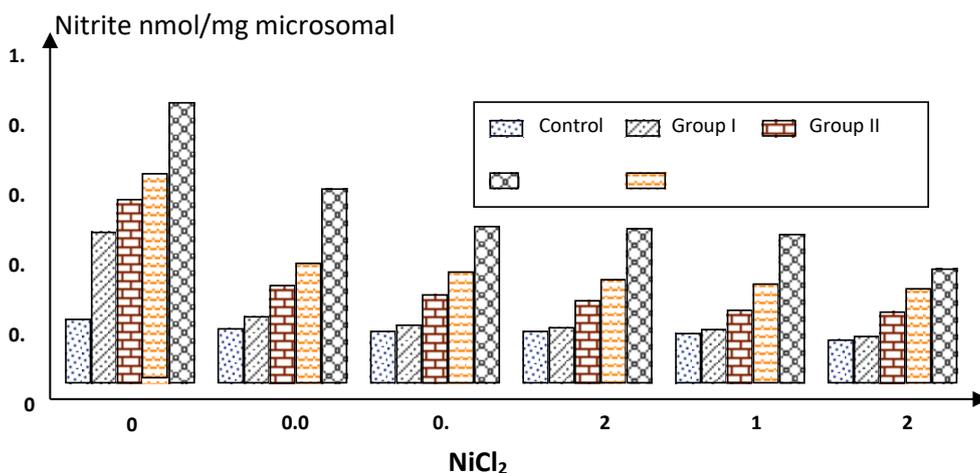


Fig 2 Effect of Ni²⁺ on NMOR denitrosations

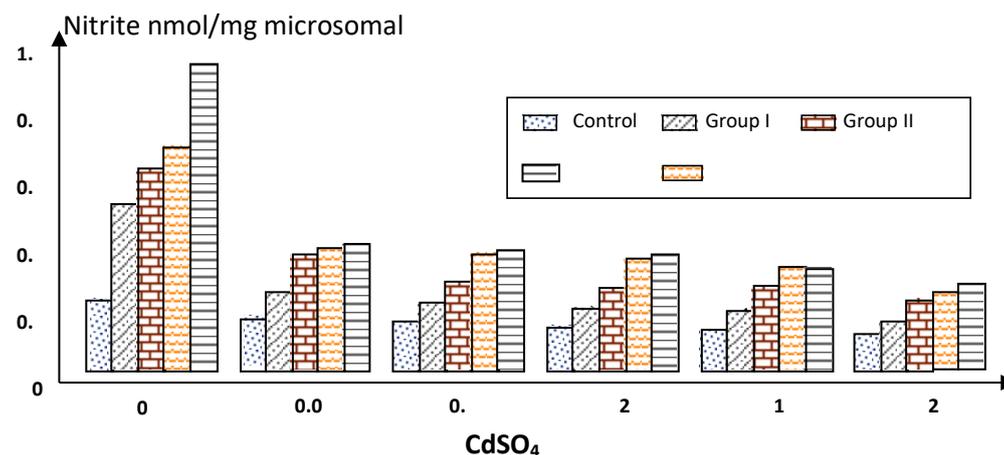


Fig 3. Effect of Cd²⁺ on NMOR denitrosations

REFERENCES

- 1 IARC Monographs on the evaluation of the carcinogenic risk of chemicals to human. Some N-Nitroso Compounds, 17, 263-275 (1978)
- 2 Scanian, R.A. N-Nitrosamines in foods. Crit.Rev. Food. Technol., 5, 357-359 (1975).
- 3 Morrison J.B., Hecht S.S. A sensitive new method for the detection of NMOR formation in vivo. Can.Res. 44, 2873-2877 (1984).
- 4 Kakizoe,T., Wang,T.T. Volatile N-Nitrosamines in the urine of normal donors and of bladder cancer patients, Can.Res., 39, 829-832 (1978).
- 5 Çetinkaya, Ö., Çetinkaya, S., Atalay, A. Effect of intraperitoneal administration of some nitrosamines on mouse liver Na/K ATP ase enzyme activites. Doga Tr. J.Med.Sci, 22, 81-83 (1994).

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- 6 Çetinkaya, S., Çetinkaya, Ö., Atalay, A. Intraperitoneal N-nitrozomorfolin uygulamasının fare karaciğer hekzokinaz, glukoz-6-fosfat dehidrogenaz ve malat dehidrogenaz enzim aktivitesine etkisi, *Doğa Türk Biyo. Der.* 16, 59-67 (1992).
- 7 Çetinkaya, S., Çetinkaya, Ö., Atalay, A. Aromatik amino asitlerin NMOR ile etkileşiminin ince tabaka kromatografisi ve spektrofotometrik yöntemlerle incelenmesi. *Ege Tıp Der.*, 32, 81-84 (1993).
- 8 Tanata E, Terada M & Misawa S. (2000) Cytochrome P450 2E1: its clinical and toxicological role. *J. Clin. Pharm. Ther* 25, 165-175
- 9 Yang CS, Yoo JSH, Ishizaki H & Hong J (1990) *Cytochrome P450IIE1: roles in nitrosamine metabolism and mechanisms of regulation.* 22, 147-59
- 10 Yang C.S., Smith T.J., Hong J. and Zhou S., (1994) Kinetics and enzymes involved in the metabolism of nitrosamines. ACS Symposium Series 553, Chap. 14, 176-169.
- 11 Yang CS & Hong, JY (1995) *Molecular Aspects of Oxidative Drug Metabolizing Enzymes NATO ASI Series Cell Biology* Vol 90, 181-192.
- 12 Jarman M. and Manson D. The metabolism of N-nitrosomorpholine by rat liver microsomes and its oxidation by the Fenton system. *Carcinogenesis* 7, 559-565 (1986).
- 13 Lorr NA, Miller KW, Chung HR, Yang CS. Potentiation of the hepatotoxicity of N-nitrosodimethylamine by fasting, diabetes, acetone and isopropanol. *Toxicol. and App. Phar.* 73, 423-311 (1984).
- 14 Tu Y.Y., Sonnenberg J., Lewis K.F. and Yang C. S. Pyrazole-induced cytochrome P-450 in rat liver microsomes: an isozyme with high affinity for dimethylnitrosamine. *Biochem. Biophys. Res. Com.* 103, 905- 912 (1981).
- 15 Siliğ Y., Çetinkaya, Ö., Cytochrome P-450 Reductase Activities in the Liver Microsomal of Diabetic Rats Induced with Some Chemical Solvents. *Tr. J. Medical Sciences*, 28, 343-346, (1998).
- 16 Nackerdien Z, Kasprzak KS, Rao G, Halliwell B & Dizdaroglu M, (1991) *Cancer Res* 51, 5837-5842
- 17 IARC(1990) *Monographs on the Evaluation of Carcinogenic Risk To Humans.* IARC no.49 Lyon.
- 18 Costa M, (1991) Molecular mechanisms of nickel carcinogenesis. *Annu Rev Pharmacol Toxicol.* 1991;31:321-37.
- 19 Leonard A & Lauwerys R, Mutagenicity, carcinogenicity and teratogenicity of cobalt metal and cobalt compounds. (1990) *Mutat Res.* 1990 Jul;239(1):17-27.
- 20 Jensen A & Tüchsen F, (1990) Cobalt exposure and cancer risk. *Cobalt exposure and cancer risk.*
- 21 Tu Y.Y., Yang C.S. Demethylation and denitrosation of nitrosamines by cytochrome P450 isozymes. *Archives of Biochemistry Biophysics.* 242, 32-40 (1985).
- 22 Dong Z, Hong J, Ma Q, Li D, Bullock J, Gonzalez JF, Park SS, Gelboin HV & Yang CS (1988) *Arch Biochem Biophys* 263, 29-35
- 23 Scopes R K (1982) *Methods for measuring protein concentration.* Chapt 8, pp 239-243, Springer-Verlag, New York
- 24 Lorr N. A., Tu Y.Y. and Yang C.S. The nature of nitrosamine denitrosation by rat liver microsomes. *Carcinogenesis* 3, 1039-1043 (1982).
- 25 Dagani D. and Archer M C. Colorimetric determination acetaldehyde in the presence of formaldehyde. *Anal. Biochem.* 87, 455-459 (1978).

- 26 Klein RG, Spiegelhalter B & Preussmann R, Inhalation carcinogenesis of N-nitrosomorpholine (NMOR) in rats and hamsters. *Exp Pathol.* 1990;40(4):189-95.
- 27 Ashby J & Lefevre PA, (1989) The rat-liver carcinogen N-nitrosomorpholine initiates unscheduled DNA synthesis and induces micronuclei in the rat liver in vivo. *Mutat Res.* 1989 Apr;225(4):143-7.
- 28 Beyersmann D & Hechtenberg S, (1997) Cadmium, gene regulation, and cellular signalling in mammalian cells. *Toxicol Appl Pharmacol.* 1997 Jun;144(2):247-61.
- 29 Miccadei S1, Floridi A. (1993) Sites of inhibition of mitochondrial electron transport by cadmium. *Chem Biol Interact.* 1993 Dec;89(2-3):159-67.
- 30 Rikans LE & Yamano T, (2000) Mechanisms of cadmium-mediated acute hepatotoxicity. *J Biochem Mol Toxicol.* 2000;14(2):110-7.
- 31 Sato M, Yamandoe K & Nagal Y,(1983) Sex-related differences in cadmium-induced lipid peroxidation in the rat. *Life Sci.* 1983 Sep 5;33(10):903-8.
- 32 Yong Y. T., Peng R., Chang Z. and Yang C.S. Induction of a high affinity nitrosamine demethylase in rat liver microsomes by acetone and isopropanol. *Chem.Biol Interactions*, 44, 247-260 (1983)
- 33 Appel K.E., Ruf H.H., Mahr B., Schwarz M., Rickart and Kunz W. Binding of nitrosamines to cytochrome p-450 of liver microsomes. *Chem. Biol. Inter.* 28, 17-33 (1979).