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Research Article

The Effect of β-carotene on Acute Phase Response in Diethylnitrosamine Given Rabbits [1]

Oğuz MERHAN [1] Ayla ÖZCAN [2] Emine ATAKİŞİ [1]
Metin ÖĞÜN [1] Abdulsamed KÜKÜRT [1]

[1] *This study was supported by Kafkas University Research Fund (Project No: 2012-VF-24)*
[1] Department of Biochemistry, Faculty of Veterinary Medicine, University of Kafkas, TR-36100 Kars - TURKEY
[2] Department of Biochemistry, Faculty of Medicine, University of Kafkas, TR-36100 Kars - TURKEY

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Abstract

The aim of this study was to investigate the effect of β-carotene in acute phase response (APR) in rabbits which were administered high doses of toxic diethylnitrosamine (DEN). Twenty-one New Zealand race rabbits at 5-7 months of age were divided into 3 groups each having 7 ones. Control group received single dose of 0.9% NaCl solution intraperitoneally (IP); Group 1 received single dose of 100 mg/kg DEN (IP); Group 2 received both 100 mg/kg DEN (IP) and 2 mg/kg/day β-carotene orally for 7 days. After applying administrations, blood samples were obtained on 1st, 4th, and 7th days. The levels of ceruloplasmin was determined by the Colombo and Richterich technique; serum amyloid A (SAA) and haptoglobin using ELISA; iron (Fe) and unsaturated iron binding capacity (UIBC), AST and ALT using a commercial colorimetric kit; total iron binding capacity (TIBC) and transferrin saturation (TS) were determined using a formula based on Fe and UIBC. We determined that the levels of haptoglobin and SAA on 1st, 4th, and 7th days ($P<0.01$) and the level of ceruloplasmin on 1st and 4th days significantly increased ($P<0.01$) relative to the controls. On the other hand, the levels of TS decreased on 4th and 7th days relative to control ($P<0.05$). These results suggest that β-carotene has a protective role on APR resulted from the toxic effect of DEN.

Keywords: *Acute phase protein, β-carotene, Diethylnitrosamine (DEN), Rabbit*

Dietilnitrozamin Verilen Tavşanlarda β-karotenin Akut Faz Yanıta Etkisi

Özet

Bu çalışmada, tavşanlara yüksek dozda verilen dietilnitrozaminin (DEN) toksik etkisine karşı oluşabilecek akut faz yanıt (AFY) üzerine β-karotenin etkisinin araştırılması amaçlandı. Materyal olarak kullanılan 21 adet, 5-7 aylık Yeni Zelanda ırkı tavşan, her birinde 7 adet olacak şekilde 3 gruba ayrıldı. Kontrol: Tek doz %0.9'luk NaCl solüsyonu intraperitoneal (İP), Grup I: Tek doz 100 mg/kg DEN (İP), Grup II: Tek doz 100 mg/kg DEN (İP) + 2 mg/kg/gün β-karoten 7 gün boyunca günlük oral yolla verildi. Tavşanlardan enjeksiyondan sonraki 1., 4. ve 7. günlerde kan örnekleri alındı. Seruloplazmin düzeyi Colombo ve Richterich yöntemiyle, serum amiloid A (SAA) ve haptoglobin ELISA kit ile albümin, demir (Fe), doymamış demir bağlama kapasitesi (DDBK), AST, ALT ticari kit kullanılarak kolorimetrik yöntem ile, total demir bağlama kapasitesi (TDBK) ve transferin doyumu (TD) ise Fe ve DDBK üzerinden formülle hesaplanarak saptandı. Haptoglobin ve SAA düzeylerinin 1., 4. ve 7. günlerde ($P<0.01$), seruloplazmin düzeyinin 1. ve 4. günde kontrol grubuna göre arttığı ($P<0.01$), TD düzeyinin ise 4. ve 7. günlerde kontrol grubuna göre azaldığı ($P<0.05$) gözlenmiştir. Sonuç olarak, DEN'in toksik etkisine bağlı olarak meydana gelen AFY'ye β-karotenin koruyucu etkisinin olabileceği sonucuna varılmıştır.

Anahtar sözcükler: *Akut faz protein, β-karoten, Dietilnitrozamin (DEN), Tavşan*

INTRODUCTION

Nitrosamines which are found in different types of foodstuff including, meat, salted fish, alcoholic beverages, agricultural drugs, insecticides, cigarette and several vegetables are known to have carcinogenic effects [1-4]. Nitrosamines cause tissue damage and inflammation because they increase the levels of free radicals, and ultimately leads to acute phase response (APR) [5]. To evaluate the damage in the liver, the levels of AST and ALT are routinely measured [6].

İletişim (Correspondence)
+90 474 2426807/5145
oguzmerhan@hotmail.com

The precursor of vitamin A, β-carotene, is taken via diet because it cannot be synthesized in the body [7]. Due to conjugated double bonds in their chemical structures, carotenoids have an inhibitory action for superoxide [8-11]. Carotenoids not only remove already existed superoxide and peroxide radicals but also prevent the formation of them [12,13].

Acute phase response is an unspecific reaction in an organism in response to inflammation, tissue damage, neoplastic formations, or immunologic disorders. It actually delineate changes in the concentration of numerous plasma proteins which are produced by the liver in response to above listed events [14,15]. Assessing APR is important for determining diagnosis, prognosis, and treatment strategies [16]. There-fore, it is imperative to better understand the mechanism of APR in various pathological conditions in various animal species.

It is believed that free radicals are one of the main players causing inflammation and tissue damage. Hence, in this study, the toxic effects of high DEN exposure on liver damage at the tissue level, and the role of β-carotene on APR were investigated.

MATERIAL and METHODS

In this study, 21 New Zealand race rabbits at 5-7 months of age were utilized. Before the experimental procedure, the permission for the use of laboratory animals was obtained from Kafkas University Animal Experimentation Ethics Board (Decision no: KAU-HADYEK: 2012/9).

Animals were adapted in an environment with temperature (25±2°C), and light (12 h light/dark cycles), and ventilation. All animals allowed to access *ad libitum* nourishing. According to their weight, 3 groups each having 7 rabbits were formed. The weight and total feed consumption of each group was recorded weekly basis throughout the experiment. Control group received single dose of 0.9% NaCl solution intraperitoneally (IP); Group 1 received single dose of 100 mg/kg DEN (IP); Group 2 received both 100 mg/kg DEN (IP) and 2 mg/kg/day β-carotene (Sigma) daily by oral gavage for 7 days. For biochemical analyses, blood samples obtained from *vena auricularis* at 1st, 4th, and 7th days post-injections, and serum was separated and stored at -20°C until further analysis.

The levels of ceruloplasmin was determined by the Colombo and Richterich [17] technique; SAA and hapto-globin using ELISA (Cusabio Biotech, China); albumin, Fe, and UIBC, AST and ALT using a commercial colorimetric kit (DDS, Turkey and Epoch, Biotech, USA); TIBC and TS were determined using a formula based on Fe and UIBC (TS (%) = Fe/TIBC x 100) [18].

Statistical Analysis

The data were analyzed using SPSS [19] for Windows 16.0.2 software. The difference among groups using ANOVA and Tukey multiple comparison test; and the difference among days was determined via variants analysis of repeated measures. Values were presented as mean +/- standard error.

RESULTS

When comparing groups relative to the control, the levels of haptoglobin and SAA on 1st, 4th, and 7th days in group I and II significantly increased relative to control ($P<0.01$), and the level of haptoglobin peaked on day 1, the levels of SAA peaked on day 4. The level of ceruloplasmin increased relative to control on 1st and 4th days ($P<0.01$) with the highest level on day 4th. The concentration of albumin and Fe in groups increased relative to the control but not significant. The level of TS (%) significantly decreased ($P<0.05$) on day 4 and 7 relative to the control group.

When comparing days, haptoglobin levels in both DEN and DEN + β-carotene were significantly higher on day 1, and significantly lower on days 4 and 7 ($P<0.01$). The level of ceruloplasmin in DEN given group was highest on 4th day ($P<0.01$), and it was lowest on 7th day in DEN + β-carotene group ($P<0.05$). The concentration of albumin significantly lower on 4th and 7th days in DEN and DEN + β-carotene relative to 1st day. The increase in TIBC in those days were not significant relative to day 1. The levels of AST and ALT in response to DEN was highest on 7th day relative to other days ($P<0.05$), and they was significantly higher in DEN and DEN + β-carotene group on 4th and 7th days relative to 1st day ($P<0.05$).

When comparing groups, the levels of AST and ALT in all groups significantly increased in all three days relative to control ($P<0.05$), and reached the highest on 7th day. In addition, the levels of AST and ALT were significantly lower in DEN + β-carotene relative to only DEN given group ($P<0.05$) *(Table 1)*.

DISCUSSION

Nutrients meet animals' nutritional needs and may play roles in preventing diseases; however, they may also cause diseases. N-nitrosamines in the environment and in nutrients are one of the risks [1]. Although the mechanism is not fully understood, nitrosamines which are used as preservative in meat and meat products are proposed to be carcinogenic for numerous tissues [4,20]. DEN metabolism is catalyzed by the enzymes in monooxygenase system which belongs to cytochrome P-450. As a consequence of its metabolic activation, it displays its toxic effects. The intermediate products stemmed from its bioactivation have low binding affinity to the binding sites of various enzymes, and therefore, instead of excretion with urine, they may form covalent bonds with important components

Table 1. *The levels of acute phase protein and the activities of AST and ALT in DEN given rabbits*
Tablo 1. *DEN verilen tavşanlarda bazı akut faz protein düzeyleri ile AST ve ALT aktiviteleri*

Parameters	Groups	Time (day)			P Values
		1	4	7	
Haptoglobin (g/L)	Control	0.108±0.003^{x}	0.105±0.003^{x}	0.104±0.005^{x}	NS
	DEN	1.003±0.021^{y,a}	0.894±0.032^{y,b}	0.873±0.032^{y,b}	P<0.01
	DEN+β-Carotene	0.756±0.021^{z,a}	0.637±0.019^{z,b}	0.594±0.014^{z,b}	P<0.01
	P Values	P<0.01	P<0.01	P<0.01	
SAA (µg/mL)	Control	14.36±0.90^{x}	13.76±0.62^{x}	14.96±0.25^{x}	NS
	DEN	24.88±0.33^{y,a}	25.82±0.15^{y,b}	23.84±1.50^{y,ab}	P<0.01
	DEN+β-Carotene	23.52±0.67^{y,a}	24.34±0.70^{y,a}	21.68±0.76^{y,ab}	P<0.01
	P Values	P<0.01	P<0.01	P<0.01	
Ceruloplasmin (mg/dL)	Control	16.33±1.49^{x}	17.11±2.42^{x}	16.47±2.34	NS
	DEN	27.26±1.44^{ya}	28.87±1.43^{ya}	22.81±1.72^{b}	P<0.01
	DEN+β-Carotene	24.58±2.17^{ya}	25.15±2.25^{ya}	19.20±1.04^{b}	P<0.05
	P Values	P<0.01	P<0.01	NS	
Albumin (g/dL)	Control	3.40±0.11	3.44±0.13	3.41±0.13	NS
	DEN	3.23±0.13	3.06±0.17	3.05±0.09	NS
	DEN+β-Carotene	3.31±0.19	3.12±0.10	3.08±0.12	NS
Fe (µg/dL)	Control	184.70±5.36	181.90±3.25	185.23±5.09	NS
	DEN	176.02±4.92^{a}	171.53±3.45^{b}	170.62±4.60^{b}	P<0.05
	DEN+β-Carotene	180.26±6.16	174.28±4.80	178.58±4.83	NS
TIBC (µg/dL)	Control	264.80±2.49	262.57±2.95	268.89±5.82	NS
	DEN	278.48±4.94	279.46±4.91	282.77±5.93	NS
	DEN+β-Carotene	274.93±3.54	276.77±6.26	277.84±4.07	NS
TS (%)	Control	69.85±2.45^{x}	69.28±1.08^{x}	68.97±1.79^{x}	NS
	DEN	63.33±2.11^{x,a}	61.58±2.13^{y,b}	60.57±2.37^{y,b}	P<0.05
	DEN+β-Carotene	65.72±2.84^{x}	63.13±2.06^{y}	64.29±1.62^{y}	NS
	P Values	NS	P<0.05	P<0.05	
AST (U/L)	Control	100.70±6.05^{x}	99.86±3.20^{x}	99.33±4.44^{x}	NS
	DEN	122.11±6.33^{y,a}	130.90±6.73^{y,a}	148.03±7.85^{y,b}	P<0.05
	DEN+β-Carotene	108.43±3.35^{z,a}	116.79±5.23^{z,a}	121.43±8.30^{z,b}	P<0.05
	P Values	P<0.05	P<0.05	P<0.05	
ALT (U/L)	Control	86.93±5.75^{x}	85.41±6.92^{x}	86.61±5.72^{x}	NS
	DEN	118.17±5.78^{y,a}	122.73±12.76^{y,a}	128.57±6.41^{y,b}	P<0.05
	DEN+β-Carotene	98.50±6.56^{z,a}	99.39±8.95^{z,a}	103.21±6.49^{z,b}	P<0.05
	P Values	P<0.05	P<0.05	P<0.05	

^{a,b} The groups in the same line labeled different letters are statistically significant (P<0.05, P<0.01)
^{x,y,z} The groups in the same column labeled different letters are statistically significant (P<0.05, P<0.01), ***NS:*** *Non Significant*

of the cells, and ultimately cause mutations, necrosis and cancer [5,21]. One of the intermediate products formed by the bioactivation of DEN is superoxide anion free radical that may cause inflammation and tissue damage [22]. Inflammation and tissue damage triggers APR, and as a result, acute phase proteins (APP) are produced by the liver [16].

Atakisi and Ozcan [3], Chiarello et al.[23], Bansal et al.[24] and Pradeep et al.[25] have reported that single dose of 150 mg/kg or 200 mg/kg DEN (IP), respectively caused significant liver damage. In addition to these studies, Sahin et al.[26] reported that exposure of 100 and 200 mg/kg DEN (IP) increased serum concentrations of AST, ALT and GGT in rats. In this study, we believe that the increase in AST and ALT resulted from the hepatotoxicity due to DEN exposure. In order to decrease the damage caused by DEN, Atakisi and Ozcan [3], Atakisi et al.[27] reported that

omega-3 rich fish oils might decrease the toxic effects of DEN. Similarly, in other studies in rats, when Karaca and Baysu Sozbilir [28] administered α-lipoic acid; when Sadik et al.[29] administered blueberries, and when Liu et al.[30] administered barley enriched with selenium, the activities of AST, ALT, ALP, and GGT were decreased relative to only DEN given group, and accordingly they decreased the toxic effects of DEN. Parallel to these studies, in our study, β-carotene decreased the serum levels of AST and ALT on 7th day suggesting the protective effect of β-carotene.

Free oxygen radicals play important roles in stress induced tissue damage and pathogenesis of inflammation [31,32]. The imbalance between protective and damaging mechanisms results in acute inflammation. Cytokines, such as IL-1 and TNF-a, are released from damaged tissues due to inflammation. Because of the effects of these cytokines, APPs are synthesized in the liver [33]. The levels of these proteins are important for diagnosis and prognosis of inflammation, tissue damage, and formation of tumors [34]. There are a very limited number of studies on the effect of DEN on APR up-to-date. Sadik et al.[29] and Sukata et al.[35] have reported that the concentrations of α-fetoprotein and α-macro-globulin positive APPs are increased in response to DEN. Consistently, in our study, a positive APP, haptoglobin, on 1st day, and SAA and ceruloplasmin reached their highest levels on 4th day, and subsequently their concentration significantly dropped on 7th day. The reduction in APP on day 7 might be due to the inhibiting effects of β-carotene on superoxide anions.

Active cytokines during acute phase reaction released from the damaged tissues also affect other organs, such as the brain, liver, and other tissues and cause a reduction in the levels of various minerals, such as Ca, Zn, and Fe [15]. In this study, we found that the level of Fe dropped possibly due to APR. The level of TS is also reduced proportionally to the level of Fe.

In conclusion, our study revealed that toxic effect of DEN led to APR; and as a consequent of this, the synthesis APP and the hepatotoxicity were induced. Our results also support the idea that exogenous β-carotene supplementation has beneficial roles against APR resulted from the toxic effects of DEN, and this should be further scrutinized for both medical and veterinary clinical pathology.

REFERENCES

1. Bayraktar N, Gökçe R, Ergün Ö: The influence of the nitrat and nitrit residues of the foods to the human health. *Ekoloji*, 28, 28-30, 1998.

2. Skog K: Problems associated with the determination of heterocyclic amines in cooked foods and human exposure. *Food Chem Toxicol*, 40, 1197-1203, 2002. DOI: 10.1016/S0278-6915(02)00052-2

3. Atakişi E, Özcan A: Investigation of the protective role of omega-3 fatty acids riched fish oil on rats given diethylnitrosamine. *Turk J Biochem*, 30, 279-284, 2005.

4. Palamutoğlu R, Sarıçoban C: Alternative natural curing agents to nitrate and nitrite in meat products. *Electron J Food Technol*, 7, 46-58, 2012.

5. Yamada K, Yamamiya I, Utsumi H: *In vivo* detection of free radicals induced by diethylnitrosamine in rat liver tissue. *Free Radical Biol Med*, 40, 2040-2046, 2006. DOI: 10.1016/j.freeradbiomed.2006.01.031

6. Atakişi E, Karapehlivan M, Atakişi O, Özcan A, Çitil M: Investigation of protective effect of L-carnitine in the liver tissue of phenylhydrazine given mice. *Kafkas Univ Vet Fak Derg*, 11, 1-4, 2005.

7. Ayaşan T, Karakozak E: Use of β-carotene in animal nutrition and its effects. *Kafkas Univ Vet Fak Derg*, 16, 697-705, 2010. DOI: 10.9775/kvfd.2010.2008

8. Jacob RA, Burri BJ: Oxidative damage and defense. *Am J Clin Nutr*, 63, 985-990, 1996.

9. Edge R, McGarvey DJ, Truscott TG: The carotenoids as anti-oxidants - A review. *J Photochem Photobiol B*, 41, 189-200, 1997. DOI: 10.1016/S1011-1344(97)00092-4

10. Stahl W, Sies H: Antioxidant activity of carotenoids. *Mol Aspects Med*, 24, 345-351, 2003. DOI: 10.1016/S0098-2997(03)00030-X

11. Stahl W, Sies H: Bioactivity and protective effects of natural carotenoids. *Biochim Biophys Acta*, 1740, 101-107, 2005. DOI: 10.1016/j.bbadis.2004.12.006

12. Chaudiere J, Ferrari-Iliou R: Intracellular antioxidants: from chemical to biochemical mechanisms. *Food Chem Toxicol*, 37, 949-962, 1999. DOI: 10.1016/S0278-6915(99)00090-3

13. Young IS, Woodside JV: Antioxidants in health and disease. *J Clin Pathol*, 54, 176-186, 2001. DOI: 10.1136/jcp.54.3.176

14. Eckersall PD, Conner JG: Bovine and canine acute phase proteins. *Vet Res Commun*, 12, 169-178, 1988. DOI: 10.1007/BF00362798

15. Gruys E, Obwolo MJ, Toussaint MJM: Diagnostic significance of the major acute phase proteins in veterinary clinical chemistry: A review. *Vet Bull*, 64, 1009-1018, 1994.

16. Petersen HH, Nielsen JP, Heegaard PMH: Application of acute phase protein measurements in veterinary clinical chemistry. *Vet Res*, 35, 163-187, 2004. DOI: 10.1051/vetres:2004002

17. Colombo JP, Richterich R: Zur bestimmung des caeruloplasmin im plasma [on the determination of ceruloplasmin in plasma]. *Schweiz Med Wochenschr*, 94, 715-720, 1964.

18. Blanck HM, Pfeiffer CM, Caudill SP, Reyes M, Gunter EW, Imperatore G, van Assendelft OW, Strider S, Dearth T: Serum iron and iron-binding capacity: A round-robin interlaboratory comparison study. *Clin Chem*, 49, 1672-1675, 2003. DOI: 10.1373/49.10.1672

19. SPSS, SPSS for windows release 16.0.2. SPSS Inc., Chicago, 2008.

20. Jimènez-Colmenero F, Carballo J, Cofrades S: Healthier meat and meat products: Their role as functional foods. *Meat Sci*, 59, 5-13, 2001. DOI: 10.1016/S0309-1740(01)00053-5

21. Daoust R, Morais R: Degenerative changes, DNA synthesis and mitotic activity in rat liver following single exposure to diethylnitrosamine. *Chem Biol Interact*, 57, 55-64, 1986. DOI: 10.1016/0009-2797(86)90048-7

22. Bartsch H, Hietanen E, Malaveille C: Carcinogenic nitrosamines: Free radical aspects of their action. *Free Radic Biol Med*, 7, 637-644, 1989. DOI: 10.1016/0891-5849(89)90144-5

23. Chiarello PG, Iglesias AC, Zucoloto S, Moreno F, Jordao AA, Vannucchi H: Effects of a necrogenic dose of diethylnitrosamine on vitamin E-deficient and vitamin E-supplemented rats. *Food Chem Toxicol*, 36, 929-935, 1998. DOI: 10.1016/s0278-6915(98)00043-x

24. Bansal AK, Trivedi R, Soni GL, Bhatnagar D: Hepatic and renal oxidative stress in acute toxicity of N-nitrosodiethylamine in rats. *Indian J Exp Biol*, 38, 916-920, 2000.

25. Pradeep K, Mohan CVR, Gobianand K, Karthikeyan S: Silymarin modulates the oxidant-antioxidant imbalance during diethylnitrosamine induced oxidative stress in rats. *Eur J Pharmacol*, 560, 110-116, 2007. DOI: 10.1016/j.ejphar.2006.12.023

26. Sahin S, Türkmen G: The effect of diethylnitrosamine on the levels of sialic acid, lipid-bound sialic acid and enzyme activities of transferase in rat serum. *Turk J Vet Anim Sci*, 29, 607-612, 2005.

27. Atakisi O, Atakisi E, Ozcan A, Karapehlivan M, Kart A: Protective effect of omega-3 fatty acids on diethylnitrosamine toxicity in rats. *Eur Rev Med Pharmacol Sci*, 17, 467-471, 2013.

28. Karaca EG, Bayşu Sözbilir N: Investigation of the protective role of α-lipoic acid on rats given diethylnitrosamine. *Med J Kocatepe*, 7, 11-17, 2007.

29. Sadik NAH, EL-Maraghy SA, Ismail MF: Diethylnitrosamine-induced hepatocarcinogenesis in rats: Possible chemoprevention by blueberries. *Afr J Biochem Res*, 2, 81-87, 2008.

30. Liu JG, Zhao HJ, Liu YJ, Wang XL: Effect of selenium-enriched malt on hepatocarcinogenesis, paraneoplastic syndrome and the hormones regulating blood glucose in rats treated by diethylnitrosamine. *Life Sci*, 78, 2315-2321, 2006. DOI: 10.1016/j.lfs.2005.09.033

31. Griendling KK, FitzGerald GA: Oxidative stress and cardiovascular injury. Part I: Basic mechanisms and *in vivo* monitoring of ROS. *Circulation,* 108, 1912-1916, 2003. DOI: 10.1161/01.CIR.0000093660.86242.BB

32. Kwiecien S, Pawlik MW, Brzozowski T, Konturek PC, Sliwowski Z, Pawlik WW, Konturek SJ: Nitric oxide (NO)-releasing aspirin and (NO) donors in protection of gastric mucosa against stress. *J Physiol Pharmacol*, 59, 103-115, 2008.

33. Murata H, Shimada N, Yoshioka M: Current research on acute phase proteins in veterinary diagnosis: An overview. *Vet J*, 168, 28-40, 2004. DOI: 10.1016/S1090-0233(03)00119-9

34. Whicher T, Bienvenu J, Price CP: Molecular biology, measurement and clinical utility of the acute phase proteins. *Pure Appl Chem*, 63, 1111-1116, 1991. DOI: 10.1351/pac199163081111

35. Sukata T, Uwagawa S, Ozaki K, Sumida K, Kikuchi K, Kushida M, Saito K, Morimura K, Oeda K, Okuno Y, Mikami N, Fukushima S: α_2-Macroglobulin: A novel cytochemical marker characterizing pre-neoplastic and neoplastic rat liver lesions negative for hitherto established cytochemical markers. *Am J Pathol*, 165, 1479-1488, 2004. DOI: 10.1016/S0002-9440(10)63406-2