

Araştırma Makalesi

THE ASSOCIATION OF THE CARCINOGENIC EFFECT OF N-NITROSO-N-METHYLUREA WITH THE BLOOD PLASMA VITAMIN E AND SELENIUM LEVELS IN RATS\*

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Sıçanlarda N-Nitroso-N-Methylurea'nın Karsinojen Etkisinin Kan Plazmasındaki E Vitamini ve Selenyum Düzeyleriyle İlişkisi

**Özet:** Bu çalışmada kimyasal bir karsinojen olan N-Nitroso-N-Methylurea (NMU) ile oluşturulan kanserlerde E vitamini (Vit.E) ve selenyumun (Se) etkisi araştırıldı.Çalışmada 50 adet Wistar-albino dişi sıçan kullanıldı. Hayvanlar dört deney ve bir kontrol grubu olmak üzere beş gruba ayrıldı (n=10). Bir ve ikinci gruplara NMU enjeksiyonundan 45 gün önce başlanarak içme sularına günlük 3ppm/lt sodyum selenit eklendi. İki ve üçüncü gruplara aynı süre içerisinde haftada üç kez Vit.E intraperitoneal (i.p) uygulandı. Belirtilen sürenin sonunda bütün gruplardaki hayvanların kuyruk venalarından kan alındı ve deney gruplarına (1-4) 45 gün boyunca haftada üç kez 20mg/kg NMU i.p uygulandı. Bu uygulamanın bitiminden çalışmanın sonuna kadar 170 gün daha (toplam 260 gün) Vit.E ve Se uygulamasına devam edildi.

Çalışmanın sonunda hayvanlardan tekrar kan alındıktan sonra ötanazi edilerek nekropsileri yapıldı.Histopatolojik inceleme sonunda birinci grupta (Se+NMU) bir hayvanda (%10), ikinci grupta (Se+Vit.E+NMU) beş hayvanda (%50), üçüncü (Vit.E+NMU) ve dördüncü grupta (NMU) ikişer hayvanda (%20) tümör oluşumu saptandı.

Sonuç olarak Se verilen birinci gruptaki tümör oranının dördüncü gruba göre birazda olsa düşük çıkması Se'un tümör oluşumunu engellemede kısmen de olsa etkili olduğunu, Vit.E verilen üçüncü grup ile yalnızca NMU verilen dördüncü grupta tümör oranlarının eşit çıkması nedeniyle de Vit.E'nin tümör oluşumunu engellemede etkisiz olduğunu göstermektedir. Ancak Se+Vit.E verilen ikinci gruptaki tümör oranının diğer gruplardan belirgin olarak yüksek çıkması, ayrıca bu gruptaki serum Se düzeyinin istatistiksel olarak anlamlı olmamakla birlikte birinci gruba göre düşük, serum Vit.E düzeyinin ise üçüncü gruba göre

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belirgin olarak yüksek ( $p<0.05$ ) çıkması nedeniyle, serumda yüksek düzeyde bulunan Vit.E'nin tümör oluşumunu provake edebileceğini düşündürmektedir.

**Anahtar Kelimeler:** E vitamini, Selenyum, N-Nitroso-N-Methylurea, Kanser, Sıçan

**Summary :** Effects of vitamin E and Se on cancer induced by N-Nitroso-N-Methylurea, which is a chemical carcinogen were investigated in this study. The study was carried out with 50 female Wistar-Albino rats. The animals were divided into five groups: Four experimental and 1 control (n: 10). The animals in groups I and II were daily given 3ppm/lit sodium selenit via drinking water starting 45 days prior to NMU injection, while groups III and IV were injected vitamin E intraperitoneally (i.p) three times a week. At the end of the 45-day-period blood samples were taken from each animal in individual group via tail veins and the experimental groups (1-4) were administrated 20 mg/kg of NMU intraperitoneally three times a week during the second 45-day-period. After this stage vitamin E and Se application was prolonged for 170 days (experimental stage totally lasted for 260 days). All animals were sacrificed at the end of the study after the blood samples were obtained. Histologically, tumoural mass development was obtained in one (10%), five (50%), two (20%) and two animals (20%) in groups I (Se+NMU), II (Se+Vit.E+NMU), III (Vit.E+NMU) and IV (NMU), respectively. In conclusion, the ratio of tumoural development in group I, which was slightly lower than in group II pointed out at least the partial effectiveness of Se on prevention of neoplastic development. On the other hand, vitamin E was demonstrated to be ineffective in the prophylaxis of tumoural development due to equal tumour incidences detected in groups III (Vit.E) and IV (Only NMU). The ratio of tumoural development in group II (Se+Vit.E) was detected to be significantly higher than the other groups. Serum Se and vit.E levels in the relevant group were demonstrated to be lower than in group I (despite the insignificance of the estimated value) and significantly higher ( $p<0.05$ ) than in group III, respectively, which all together indicated the likelihood of the inductive role of high serum levels of vit.E in tumour development.

**Key Words:** Vitamin E, Selenium, N-Nitroso-N-Methylurea, Cancer, Rat

## Introduction

Many factors play role in the ethiology of cancer. One of the most important factors in the ethiology is the chemical carcinogens (12). The most important ones among the chemical carcinogens are polycyclic hydrocarbons, aromatic amines and nitrosamines (12, 16). Most of these carcinogens are electrophilic substances which form free radicals by the interaction in the tissue (4). The occurring free radicals result in mutations and tumour formation by causing DNA damage directly or indirectly (5).

Nitrosamines and amides which are chemical carcinogens are especially found in foods like smoked, soused meat, salami, sausage, fish meat, cheese, soy oil and in cigarette smoke. Nitrosamines are formed by the combination of nitrogen oxide (NO) originating from  $\text{NO}_3$  nitrate and  $\text{NO}_2$  nitrite added with the secondary and tertiary amines formed by the destruction of proteins and amino acids in gastrointestinal tract (10, 13).

Vitamin E (Vit.E) and selenium (Se) have immunostimulatory and anticarcinogenic effects due to their antioxidant properties (6, 8, 9). In vast majority of the studies, investigating the anticarcinogenic effects of Vit.E and Se, both substances were reported to be effective against carcinogenesis (2, 7, 9, 11, 24), while in other they were reported to be ineffective alone or in combination (1, 22) and further more Vit.E was reported to have provoked cancer development (6, 23).

The aim of this study was to develop cancer in rats with the nitrosamine compound N-Nitroso-N-Methylurea (NMU) and to investigate the effects of Se and Vit.E alone and in combination.

### Materials and Methods

Fifty adult female 60-day-old Wistar-albino rats were used in the study. Each containing 10 animals, four test groups and one control group was formed. The animals received rat food throughout the study (this food contains 15 mg/kg vitamin E). Drinking water was given ad libitum. The procedures applied to the test groups are shown in Table 1.

**Table 1.** The procedures that were applied to the test groups

**Tablo 1.** Gruplara yapılan uygulamalar

Groups	Time Periods		
	Day 0-45	Day 45-90	Day 90-260
I Group	Se	Se+NMU	Se
II Group	Se+Vit.E	Se+Vit.E+NMU	Se+Vit.E
III Group	Vit.E	Vit.E+NMU	Vit.E
IV Group	-	NMU	-
V Group (control)	-	-	-

Tree ppm/L sodium selenite (K21667607- Merck) was added to the drinking water and 500 mg/kg vitamin E ( $\alpha$ -tocopherol acetate, dissolved in chremoform) was administered intraperitoneally (i.p.) three times a week. N-Nitroso-N-Methylurea (Sigma, N-4766) was dissolved in saline solution and 20 mg/kg was administered three times a week i.p (18). Blood samples were taken from tail veins before the onset of NMU injections and intracardiac blood samples were taken at the end of the study during sacrifice and the sera of these samples were separated. For Vit E level measurements 200  $\mu$ L serum were taken into tubes containing ethanol ascorbic acid and for Se level measurements 200  $\mu$ L serum were taken into normal serum tubes and these were stored at -18 °C .

On day 126, five animals with poor general conditions from the 2<sup>nd</sup> and 3<sup>rd</sup> groups, and on the 260<sup>th</sup> day the remaining animals were sacrificed by ether anesthesia and systemic necropsies were conducted. Tissue samples were taken from the mammary gland, liver, pancreas, stomach, spleen and kidney of all animals were placed in 10% formal saline solution, passed through the alcohol and xylol series, embedded into paraffin blocks, sections of 3-5  $\mu$ m were taken with Rotary microtome, stained with

Hematoxylin-Eosine (H&E) and examined under light microscopy. In addition some tissue sections were stained with the Masson's trichrome, Mallary's triple, van Gieson and Gomori's stain for iron (15).

The serum Se concentration was determined by atomic absorption spectrophotometer (Perkin Elmer 3030 type ). The serum Vit E concentration was conducted with high performance liquid chromatographic method (3). The obtained data were evaluated using the Student *t*-test. The comparisons were made with control group. The results were expressed as mean  $\pm$  SD.

## Results

### *Biochemical Findings*

Serum Vit.E and Se levels, mean values and standard error before and after the NMU administration are shown in table 2.

Before NMU administration Serum Vit.E levels of second and third groups were significantly higher than the control group ( $p < 0.001$ ). In Se administered groups, serum Se levels of first and second groups were higher than the control ( $p < 0.001$ ). After NMU administration serum Vit.E levels of second and third group were again higher than the control group ( $p < 0.001$ ), but the serum Vit.E level of second group was significantly higher than third group ( $p < 0.05$ ). Although the Se administered first group serum levels were higher than the control group ( $p < 0.05$ ), there were no difference between second group and the fourth group.

### *Histopathological Findings*

The ratio of malignant and benign tumours that developed in groups I, II, III and IV in which tumour induction was done with NMU are shown in Table 3.

In the animals of the first group oedema in liver, parenchymal degeneration, hemosiderosis in spleen, atrophy in white pulp, tubular degeneration in kidneys were generally observed. In the animal number 4 of this group adenocarcinoma originating from mammary alveolar epithelium was observed (Figure 1). Squamous metaplasia was observed in mucosal membrane of forestomach of the same animal. In addition, hyperplasia was detected in the mammary gland epithelia of some animals.

**Table 2.** The mean serum Vit.E and Se levels before and after NMU administration (The comparisons were made with control group).**Tablo 2.** NMU enjeksiyonu öncesi serum Vit. E ve Se düzeyleri (kıyaslamalar kontrol grubuyla yapılmıştır).

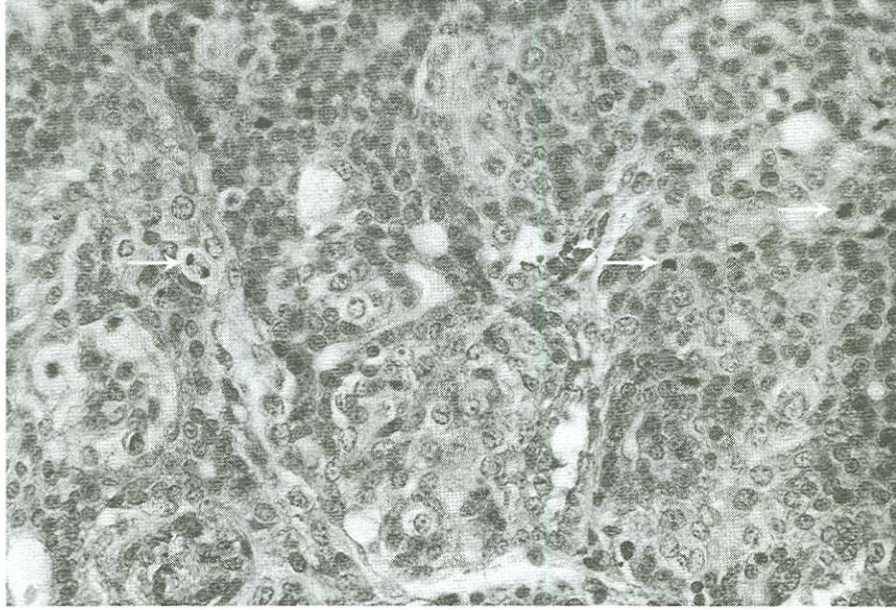
Groups	Before NMU administration		After NMU administration	
	Vit.E (mg/dl)	Se (µg/ml)	Vit.E (mg/dl)	Se (µg/ml)
	X±SD (n=10)	X±SD (n=10)	X±SD	X±SD
I Group (Se+NMU)	0.45±0.27	0.36±0.62***	0.71±0.42 (n=10)	0.21±0.02* (n=10)
II Group(Se+Vit.E+NMU)	1.85±0.62***	0.34±0.04***	3.50±1.49*** <sup>a</sup> (n=8)	0.17±0.06 (n=8)
III Group (Vit.E+NMU)	1.52±0.32***	0.21±0.06	2.16±0.79*** (n=9)	0.17±0.08 (n=9)
IV Group (NMU)	0.50±0.30	0.18±0.03	0.62±0.64 (n=10)	0.16±0.03 (n=10)
V Group (Control)	0.52±0.28	0.19±0.03	0.61±0.13 (n=10)	0.18±0.03 (n=10)

<sup>a</sup> : After NMU administration for Vit.E between the 2<sup>nd</sup> and 3<sup>rd</sup> groups \* = p < 0.05

\* = p < 0.05, \*\*\* = p < 0.001

**Table 3.** The tumour percentages in the groups in which induction was done with NMU.**Tablo 3.** NMU indüklenmesiyle gruplarda görülen tümör oranları

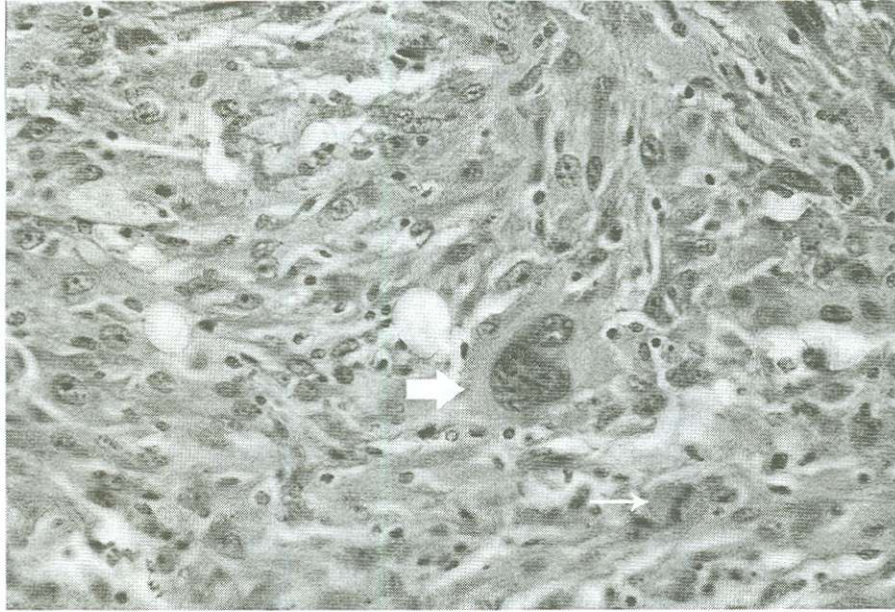
Group	N	Malignant tumour	Benign tumour
I Group (Se+NMU)	10	1/10 (10%)	0
II Group(Se+ Vit.E+NMU)	10	4/10 (40%)	1/10 (10%)
III Group (Vit.E+NMU)	10	2/10 (20%)	0
IV Group (NMU)	10	2/10 (20%)	0
V(µg/ml) Group (Control)	10	0	0



**Figure 1.** Mammary gland adenocarcinoma with numerous mitotic figures (thin arrows) and atypia (H&E, 400x)

**Şekil 1.** Meme bezinde adenokarsinom, çok sayıda mitotik figürler (ince oklar) ve atipi (H&E, 40x)

In the animals of the second group generally, thickening in mesenterium and cell clusters were observed which were formed by the fusiform cells under the serosa, covering a wide area and which were loose in some areas and dense in others. In addition to these, parenchymal degeneration and oedema in liver, hemosiderosis in spleen, and parenchymal degeneration in kidney tubular epithelia were observed. In the animal number one in this group, a highly malign adenocarcinoma originating from the mammary tissue was observed. In animal number two, fusiform cell sarcoma originating from tunica muscularis was detected in the intestine. It was observed that this tumour also invaded to pancreas and liver and caused destruction in these organs by infiltrating the mesenterium. In animal number two, rhabdomyosarcoma originated from tunica muscularis was detected in the intestine which was characterized by high mitotic activity and a high number of giant cells (Figure 2). It was observed that this tumour also infiltrated mesenterium and intestinal wall. In addition, dysplasia in the intestinal gland epithelia and hyperplasia in the mesenterial lymph nodes of animal number three and lipoma and myxoma in the mesenterium of animal number six were observed.



**Figure 2.** Rhabdomyosarcoma originating from tunica muscularis of the intestine. Prominent mitosis (thin arrow), atypia and tumour giant cell (thick arrow), (H&E, 40x)

**Şekil 2.** Bağırsakların tunika muskularisinden köken alan rbdomyosarkom, belirgin mitoz (ince ok), atipi ve tümör dev hücresi (kalın ok), (H&E, 40x).

Fibrous connective tissue proliferation in mesenterium, thickening of hepatic capsule and coagulation necrosis the periphery of this organ were also observed in the third group as in the second group. In some animals hyperplasia in the subcutaneous and intestinal solitary lymph follicles were observed. In the third animal of this group, fusiform cell sarcoma originating from tunica muscularis of the intestine and infiltrating the liver and pancreas via mesenterium was detected. Also, in animal number nine rhabdomyosarcoma originated from abdominal muscles, showing fusiform structures in some areas was detected. Again, this tumour also was observed to have infiltrated the intestinal wall and pancreas through mesenterium.

In the animals of the fourth group, coagulation necrosis foci in liver and activation in the intestinal glandular epithelial cells were observed in general. In this group, leiomyosarcoma originating from stomach wall was seen in animal number two and both adenocarcinoma structure originating from the renal tubular epithelia and leiomyosarcoma in the stomach wall were observed in animal number three.

No pathological alteration was found in the animals of the fifth group, which was the control group.

### Discussion

The occurrence of primary tumours in more than one organ in same animal is consist with the findings in literature (18) reporting that NMU is a multiorgan carcinogenic agent. Although it was reported in some studies (17, 18) that the NMU given animals develop lymphoid originated tumours, in our study no tumour of lymphoid origin developed. Tumours originating from mammary gland, kidney, muscle and connective tissue were detected. The finding that the tumour incidence was 20% in the fourth group, which received NMU and no treatment, was an unexpected result in the study. However, Pazos et al. (19) observed 15% tumour incidence in mouse which was received 5mg/100g body wight NMU and 79% tumour incidence in the animals that received medroxyprogesterone (MPA) together with NMU. In our study, no agents like MPA which could increase the effect of NMU were used. Therefore tumour incidence ratio of 20%, determined in our study is compatible to with the findings reported in the study of Pazos et al (19). In addition, the fact that a high tumour incidence did occur despite NMU administration supports the literatures (12, 16) reporting chemical carcinogens will not necessarily cause cancer.

Tumour incidence was being 10% in the first group, which received Se, while it was 20% in the third group that received Vit.E and in the fourth group which was not given any treatment in the study, shows that Se prevents tumour development at least partially. This result is consistent with the results of the literatures (7, 11, 14) reporting that Se is effective against tumour development.

The detection of tumour incidence in the third group, which was given Vit.E, was equal to the incidence in the fourth group, which received no treatment, is consistent with the literatures' (1, 9, 11, 21) findings reporting that Vit.E alone does not exert a protective effect against the tumours induced by chemical carcinogens.

The tumour incidence in the second group, which received Se+Vit.E, was found 50% and was higher when compared to the fourth group, which did not receive any treatment, and this shows that Se+Vit.E combination increases the tumour development. This result raises the questions of whether the tumour increase results from Se+Vit.E combination, Se alone, or Vit.E alone. Perchellet et al. (20) report that the Se+Vit.E+Glutathione (GSH) combination was inadequate in preventing the tumour development in tumours induced by dimethylbenz(a)anthracene (DMBA) in mice and they also report that this combination even increased the tumour development, when DMBA was administered in repeated subcarcinogenic doses. Horwarth and Ip (9) found that Vit.E was ineffective in tumours induced by DMBA, however it increased the anticarcinogenic effect of Se and reduced the tumour incidence when administered with 4 ppm Se. Horwarth and Ip (9) reports that the tumour formation is low in groups receiving adequate amount of Se+Vit.E in diet or having adequate amount of Se and high amount of Vit.E in diet, but the tumour incidence markedly increases when there is inadequate Se + adequate Vit.E or inadequate Se + excessive amount of Vit.E in diet in DMBA induced carcinogenesis. Gould et al. (6), found that in NMU induced carcinogenesis, not all analogues of Vit.E converted the tumours to the latent state and



on the contrary tumour increase occurred in the group that received tocotrienol. However, **Temple and El-khatib (23)** report that Vit.E increases tumour development in colon tumours induced by 1-2 dimethylhydrazine in mice. Our finding that serum Vit.E level in the second group, which received Se + Vit.E, was significantly higher than the level in the third group that received Vit.E ( $p < 0.05$ ), and that the Se level was slightly lower when compared with the first group while not statistically significant, implies that the tumour increase in this group might have resulted from inadequate Se and excessive amount of vit. E or directly excessive amount of vit. E (6, 23) as reported also in the literature (11).

In conclusion, Se is effective (though partially) in preventing tumour development, and Vit.E is ineffective when administered alone and it is assumed that high levels of Vit E, or low levels of Se together with high levels of Vit.E in serum provacate tumour development.

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