

The effects of anticancer drugs on levels of nitric oxide and adrenomedullin

Elif Güler¹, Ayşe Balat¹, Mustafa Çekmen², Metin Kılınc¹, Ercan Sivaslı¹
Muhittin Yürekli³, Can Duman²

Departments of ¹Pediatrics, Gaziantep University Faculty of Medicine, Gaziantep, ²Biochemistry, Kocaeli University Faculty of Medicine, Kocaeli, and ³Molecular Biology, İnönü University Faculty of Science and Art, Malatya, Turkey

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Nephrotoxicity is one of the most important complications of anticancer treatment. Ifosfamide, platinum and methotrexate (MTX) affect renal tubular epithelial cells.

Nitric oxide (NO) serves many functions within the kidney. Adrenomedullin (AM) is a potent vasodilator peptide, and may function as a circulating hormone and an autocrine/paracrine mediator involved in the regulation of the cardiovascular system, blood pressure, and renal function. It also has a renoprotective effect and inhibits the generation of reactive oxygen metabolites.

To our knowledge, no studies have investigated the effects of anticancer drugs on levels of AM and NO. We investigated the effects of these drugs on the levels of AM and total nitrite, a stable product of NO, and their relations to renal functions. The study was performed in 18 patients (13 males, 5 females) who received chemotherapeutic regimens including high-dose MTX or ifosfamide and platinum. Total nitrite was quantitated by means of the Griess reaction, while AM level was measured by high performance liquid chromatography (HPLC).

Plasma total nitrite level ($\mu\text{mol/L}$) was decreased after chemotherapy (78.73 ± 47.28 vs. 46.69 ± 13.89 , $p: 0.002$). A statistically significant difference was found between fractional excretion (FE) of total nitric oxide (FE_{NO}) before and after chemotherapy (25.89 ± 23.11 vs. 51.74 ± 40.01 , $p: 0.008$). The differences in plasma AM levels (pmol/ml) before (25.07 ± 4.98) and after (30.20 ± 1.39) chemotherapy were also statistically significant ($p: 0.005$). FE_{AM} after chemotherapy (1.41 ± 1.01) was found to be higher than before chemotherapy (0.64 ± 0.43) ($p: 0.000$).

Our results indicate that some chemotherapeutic agents (high-dose MTX, ifosfamide, and cisplatinium) may cause renal tubular damage. FE_{AM} and FE_{NO} may also be used for the detection of subclinical acute tubular nephrotoxicity. However, further detailed researches will be necessary to establish the certain role of NO and AM in toxicities of chemotherapeutic agents.

Key words: adrenomedullin, chemotherapy, children, nitric oxide.

Nephrotoxicity is one of the most important complications of anticancer treatment. Anticancer drugs can cause reversible or irreversible renal impairment, and it can occur early or late¹. Proximal renal tubular cells are metabolically very active and have rich blood supply, therefore, it is one of the

important targets of adverse effects of many drugs and metabolites². Ifosfamide, platinum and methotrexate (MTX) affect renal tubular epithelial cells³⁻⁵. The nephrotoxic effects of ifosfamide, platinum and MTX are well known, although the molecular pathogenesis of tubular cell damage has not yet been clarified^{5,6}.

Nitric oxide (NO) is synthesized from L-arginine by the enzyme nitric oxide synthase (NOS). It is a labile compound that rapidly decomposes to nitrite (NO_2^-) and nitrate (NO_3^-) in biologic fluids. The concentration of these stable products can be measured in fluids and used as markers of NO production *in vivo*⁷. Intensive research activities have focused on the production and effects of NO in the kidney, since NO affects renal function and hemodynamics. It participates in the mediation of arterial pressure-related changes in urine flow and sodium excretion, and may also influence tubular reabsorption⁸.

Adrenomedullin (AM) is a potent vasodilator peptide (molecular weight 6,028) originally isolated from extracts of human pheochromocytoma tissue⁹. Its presence has been reported in normal adrenal medulla, heart, lung, and kidney as well as in plasma and urine. Circulating AM is cleared by the kidney¹⁰. AM may function as a circulating hormone and an autocrine/paracrine mediator involved in the regulation of the cardiovascular system, blood pressure, and renal function^{9,11}. Owada et al.¹² showed that AM mRNA is localized in the glomerulus, cortical collecting ducts, and outer- and inner-medullary collecting ducts. AM receptors have been found especially in the proximal tubule.

Natriuretic and diuretic actions of AM reflect unique action of the peptide on renal blood flow and tubular function¹³. AM also has a renoprotective effect and inhibits the generation of reactive oxygen metabolites in both mesangial cells (MCs) and macrophages¹⁴.

To our knowledge, no studies have investigated the effects of anticancer drugs on plasma and urinary levels of AM and NO. The aim of this study was to investigate the effects of anticancer drugs on the levels of these agents and their relations to renal functions.

Material and Methods

The study was performed in 18 patients (13 males, 5 females) who received chemotherapeutic regimens including high-dose MTX or ifosfamide and platinum. The clinical characteristics of patients and applied chemotherapy regimens are shown in Table I.

Blood and 24-hour urine samples were obtained before and at least 24-h after the chemotherapy cycles. The patients with hypertension or decreased renal function (serum creatinine >1 mg/dl and creatinine clearance <80 ml/min/1.73 m²) were excluded. A total of 52 samples (26 before, others after chemotherapy cycles) were evaluated, and each patient constituted a

Table I. Clinical Characteristics of Patients and Applied Chemotherapy Regimens

No.	Age	Sex	Diagnosis	Chemotherapy regimens
1	3 ^{6/12}	M	ALL	BFM 95 (MTX 5 g/m ²)
2	5	M	ALL	BFM 95 (MTX 5 g/m ²)
3	15	M	NHL	LMB (MTX 3 g/m ²)
4	4	M	NHL	LMT (MTX 3g/m ²)
5	2	F	Germ cell tumor	BEP
6	1	M	Germ cell tumor	BEP
7	15	M	Ewing sarcoma	VAIA
8	15	M	Osteosarcoma	CDDP 100 mg/m ² (1), Adriamycin 30 mg/m ² (1-3)
9	13	F	PNET	VAIA
10	14 ^{6/12}	F	NHL	LMT (MTX 3 g/m ²)
11	2	M	Medulloblastoma	CDDP 100 mg/m ² (1), VP-16 100 mg/m ² (1-3)
12	13 ^{10/12}	M	Ewing sarcoma	VAIA
13	8 ^{4/12}	M	Ewing sarcoma	VAIA
14	11 ^{3/12}	M	Ewing sarcoma	VAIA
15	8 ^{6/12}	M	NHL	LMB (MTX 3 g/m ²)
16	12 ^{3/12}	M	Ewing sarcoma	VAIA
17	14 ^{5/12}	F	Medulloblastoma	CDDP 100 mg/m ² (1), VP-16 100 mg/m ² (1-3)
18	13	F	PNET	VAIA

ALL: Acute lymphoblastic leukemia. NHL: Non-Hodgkin lymphoma. PNET: Primitive neuroectodermal tumor. BEP: Bleomycin-etoposide-platinim. VAIA: Vincristine-adriamycin-ifosfamide-actinomycin. MTX: Methotrexate.

self-control group. Blood samples were drawn into tubes with heparin, and urine samples into tubes containing sodium tetraboric acid (0.5 g/L). BUN, creatinine, sodium (Na), potassium (K), phosphorus (P), and urinary creatinine and electrolytes were determined by routine methods. Informed consent was obtained from parents of the children.

Plasma and urinary total nitrite levels: We deproteinized 300 µL of plasma by adding 600 µL of 75 mmol/L ZnSO₄ solution, stirring, and centrifuging at 10,000 g for at least 1 min at room temperature, after which 600 µL of 55 mmol/L NaOH was added. The solution was stirred and centrifuged at 1,000 g for 3 min and the supernatant was recovered. For assay of urines, before the deproteinization process, 100 µL of urine was diluted with 900 µL of the glycine buffer. Total nitrite was quantitated by means of the Griess reaction after incubation of plasma or urine samples with *Escherichia coli* reductase to convert NO₃⁻ to NO₂⁻⁹. Griess reagent (1 ml, 1% sulfanilamide, 0.1% naphthylene diamine hydrochloride, and 2.5% phosphoric acid) (Sigma Chemical Co., St. Louis, MO, USA) was then added to 1 ml of plasma or urine specimens. Absorbance was read at 545 nm after a 30-minute incubation. Standard curves were prepared with known concentrations (1 to 100 µmol/L) of sodium nitrite.

Plasma and urinary adrenomedullin levels: After extraction and purification, plasma and urine samples were applied to supelcosil C18 columns (Cecil 100HPLC). Loaded material was eluted with 60% acetonitrile in 0.1% trifluoroacetic acid¹⁵. Rat AM (1-50) (Phoenix Pharmaceuticals, Inc.) was used as the standard in the determination of plasma and urinary AM levels. The urinary nitrite and AM levels were corrected using the urinary creatinine levels to avoid influence of the concentration of the urine itself.

Determination of other clinical parameters: BUN, creatinine, electrolytes, as well as urinary creatinine, electrolytes and urine osmolarity were determined by routine methods.

Fractional sodium (FE_{Na}), and potassium (FE_K) excretions, and tubular reabsorption of P (TRP) were calculated from formulas: FE_{Na} (%) = (U_{Na}X P_{cr}) / (P_{Na}X U_{cr})X100, FE_K (%) = (U_KX P_{cr}) / (P_KX U_{cr})X100, and TRP(%) =

[1- (U_PX P_{cr}) / (P_PX U_{cr})] X100¹⁶. Fractional excretions of total nitric oxide (FE_{NO}) and AM (FE_{AM}) were determined by the formulas:

FE_{NO} (%) = (U_{NO}X P_{cr}) / (P_{NO}X U_{cr})X100 [U_{NO} indicates urinary total nitrite concentration (µmol/L), P_{cr} plasma creatinine concentration (mg/dl), U_{cr} urinary creatinine concentration (mg/dl), and P_{NO} plasma total nitrite concentration (µmol/L)], and FE_{AM} (%) = (U_{AM}X P_{cr}) / (P_{AM}X U_{cr})X100 [U_{AM} indicates urinary AM concentration (pmol/ml), P_{AM} plasma AM concentration (pmol/ml)].

Results are given as mean±SD. Differences between before- and after-chemotherapy data were compared by paired samples test and correlation regression analysis. A level of p<0.05 was considered statistically significant.

Results

Plasma total nitrite level (µmol/L) was decreased after chemotherapy (78.73±47.28 vs. 46.69±13.89, p: 0.002, Fig. 1). A statistically significant difference was found between FE_{NO} before and after chemotherapy (25.89±23.11 vs. 51.74±40.01, p: 0.008, Fig. 2). The differences in plasma AM levels (pmol/ml) before (25.07±4.98) and after (30.20±1.39) chemotherapy were also statistically significant (p: 0.005, Fig. 3). FE_{AM} after chemotherapy (1.41±1.01) was found to be higher than before chemotherapy (0.64±0.43, p: 0.000, Fig. 4). FE_K (11.05±6.60 vs. 22.02±16.55) (p: 0.002) and FE_{Na} (1.06±0.57 vs. 1.91±1.33, p: 0.008) were increased after chemotherapy, while TRP was decreased (92.04±5.99 vs. 80.72±14.42, p: 0.001).

A positive correlation was found between FE_{AM} and FE_{NO} before chemotherapy (r: 0.005), but there was no correlation after chemotherapy (r: 0.235). There was a positive correlation between FE_K and FE_{Na} before and after chemotherapy (r: 0.018 and r: 0.004). A negative correlation was found between TRP and FE_K before (r: 0.002) and after (r: 0.000) chemotherapy.

Discussion

In this study, plasma total nitrite level, a stable product of NO, was found to be increased before chemotherapy and decreased after chemotherapy. In recent years, many evidences have shown that free radical species, NO or

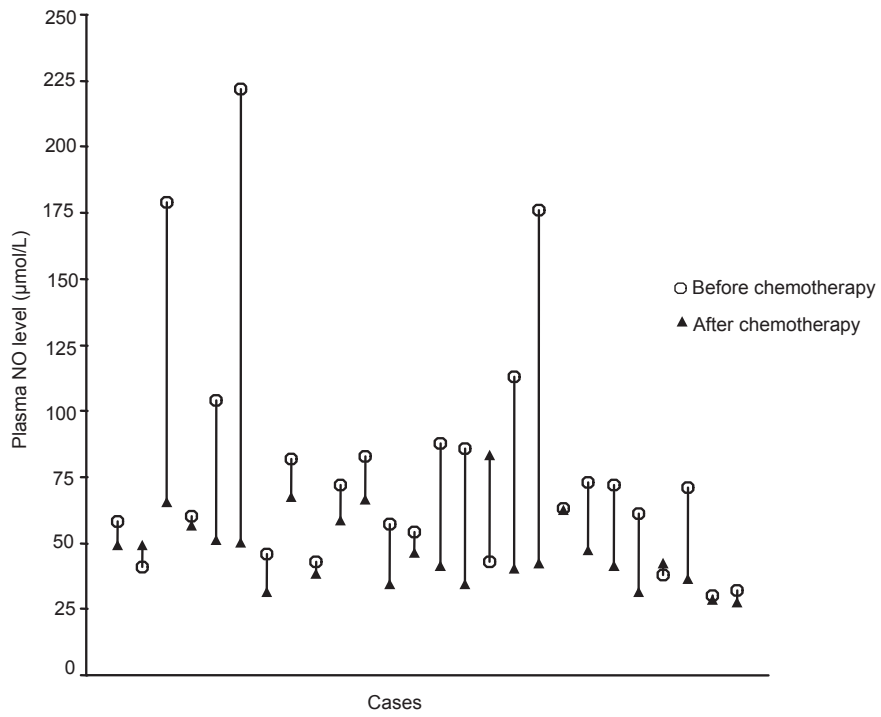


Fig. 1. Plasma nitric oxide (NO) levels of patients before and after chemotherapy.

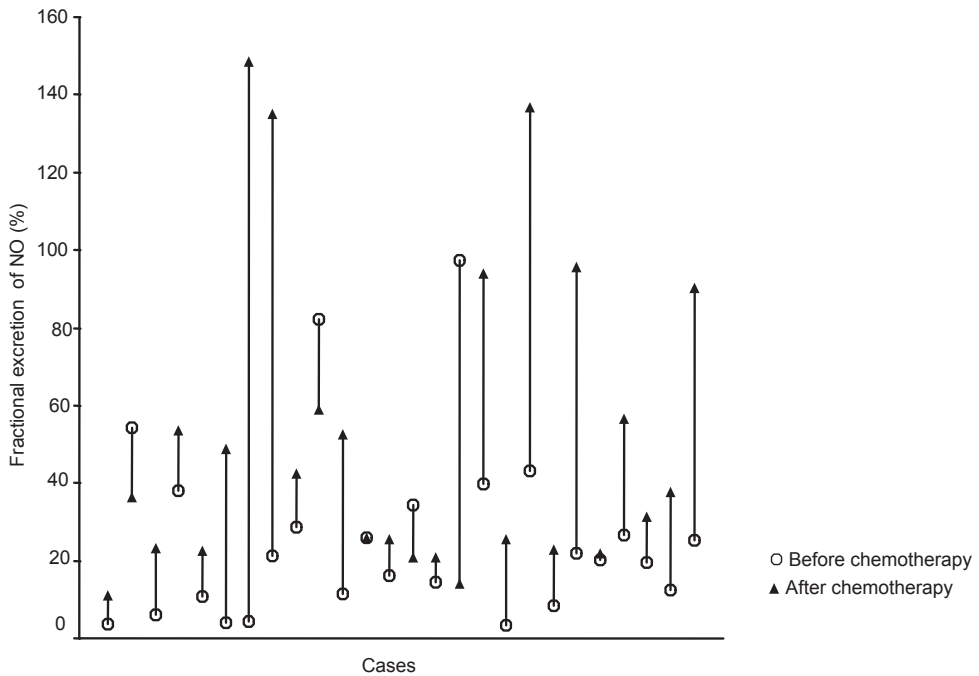


Fig. 2. Fractional excretion of nitric oxide (NO) in patients before and after chemotherapy.

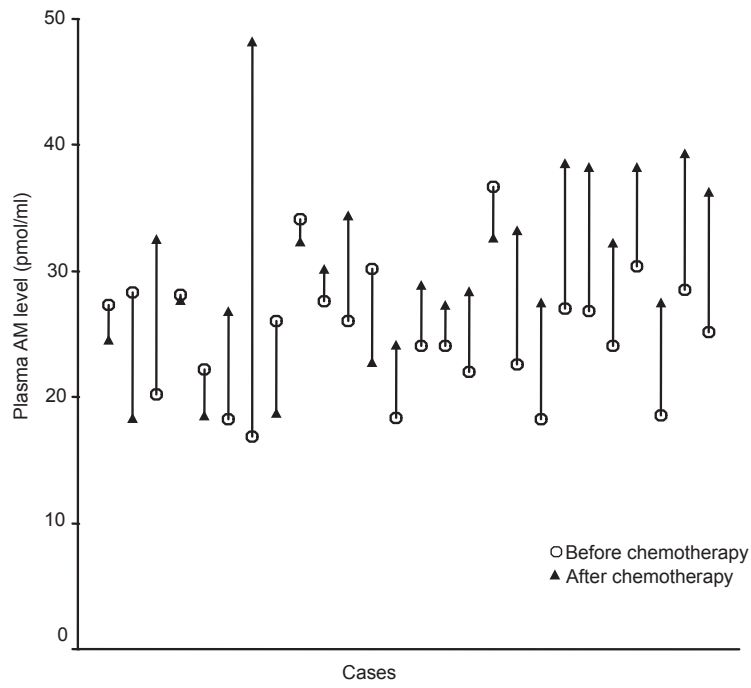


Fig. 3. Plasma adrenomedullin (AM) levels of patients before and after chemotherapy.

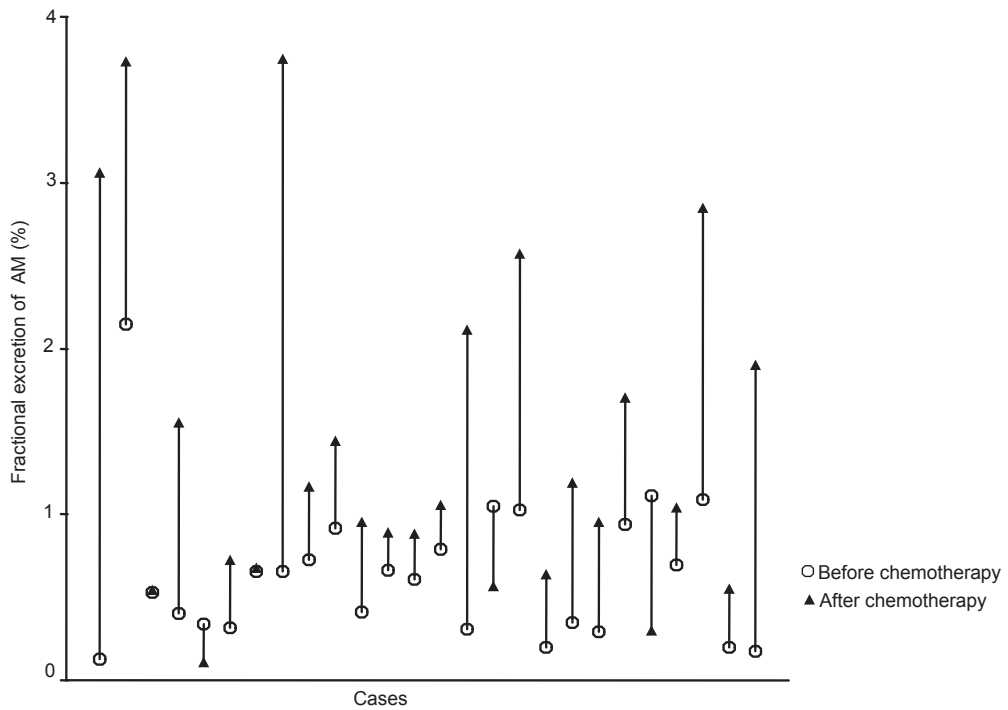


Fig. 4. Fractional excretion of adrenomedullin (AM) in patients before and after chemotherapy.

its derivatives, are the key denominators in carcinogenesis. These may effectively damage DNA, hence causing mutations, and probably are involved in multiple steps of carcinogenesis *in vivo*¹⁷. It has been demonstrated that NO functions as a vascular permeability factor, and most tumor tissue exhibits enhanced vascular permeability¹⁸⁻²⁰. Ziche et al.²¹ reported that vascular endothelial growth factor (VEGF) induces angiogenesis via formation of NO. Consequently, the angiogenic potential as well as the vascular permeability enhancing effect of NO may facilitate rapid growth of solid tumors. It is also known that the inhibition or scavenging of NO results in suppression of permeability and tumor growth¹⁸⁻²⁰. In light of these important findings in the literature, we suggest that plasma NO level was increased because of the tumors in our patients, and inhibited by chemotherapeutic agents. Two possible sources of increased FE_{NO} may be considered. One is glomerular filtration of circulating total nitrite, since plasma total nitrite levels are also increased. Increased FE_{NO} after chemotherapy may also suggest its renal synthesis and clearance. It may reflect enhanced NO production after chemotherapy by glomerular mesangial or renal tubular epithelial cells, which contain calmodulin and calcium-independent-inducible NOS^{22,23}. It could also derive from vascular endothelial cells through the endothelial, calcium-dependent isoforms of NOS²⁴. However, the cellular source(s) of this increased FE_{NO} cannot be determined from this study.

Although the pathophysiology of ifosfamide-induced nephrotoxicity has not been clarified, it is well known that ifosfamide may induce renal Fanconi syndrome in the vast majority of these patients. It was observed months or even years following cessation of chemotherapy²⁵. Ifosfamide-induced nephrotoxicity may be related to toxicity of the metabolite chloroacetaldehyde²⁶, to glutathione depletion of the renal proximal tubular cells by the ifosfamide-mesna combination²⁷, to direct toxic action of ifosfamide itself, or to metabolism of ifosfamide in the proximal tubular cell. It may interfere with proximal tubular energy supply²⁸. However, none of these possible pathways has been proven to be responsible for the nephrotoxicity seen in man. Younger age has been reported as a risk factor for

ifosfamide nephrotoxicity²⁹. Rossi et al.²⁸ also demonstrated that early impairment of TRP was highly predictive for the development of Fanconi syndrome, or generalized subclinical tubulopathy. High-dose MTX causes acute renal injury through a direct biochemical effect on tubular epithelial cells or directly through intratubular precipitation of the drug³⁰. Cisplatin has been reported to irreversibly damage restricted segments of the renal tubules³¹. Our study demonstrated that ifosfamide, MTX, and cisplatin administration are consistent with the development of renal tubular damage. The impairment of P, Na, and K reabsorption without acidosis or metabolic bone disease in our patients may suggest generalized subclinical tubulopathy. However, we cannot determine the exact site(s) of damage caused by these drugs from this study.

Plasma AM levels and FE_{AM} were found to be higher after chemotherapy. AM is expressed in a variety of tumors where it aggravates several of the molecular and physiological features of malignant cells. It has been shown to be a mitogenic factor stimulating growth in several cancer types³². Interestingly, in our study, we found increased AM levels and FE_{AM} only after chemotherapy, not before. This may be because of the release of AM produced by tumor cells after chemotherapy. It was previously established that AM is produced and secreted in the kidney³³. Increased FE_{AM} after chemotherapy may suggest glomerular filtration of circulating AM since plasma AM level is increased. Considering the reno-protective effect of AM, it may be acting by reducing the toxic effects of chemotherapeutics. Therefore, we suggest that increased FE_{AM} and FE_{NO} may also be used for the detection of subclinical acute tubular nephrotoxicity.

There was a negative correlation between TRP and FE_K . Since 50 to 70% of the filtered K^+ is reabsorbed by the proximal convoluted tubule, and since K^+ excretion is governed largely via the regulation of K secretion in the distal nephron¹⁶, this is an expected result.

Our results indicate that some chemotherapeutic agents (high-dose MTX, ifosfamide, and cisplatin) may cause renal tubular damage. FE_{AM} and FE_{NO} may be additionally used for the detection of subclinical acute tubular nephrotoxicity. However, further detailed

researches will be necessary to establish the certain roles of NO and AM in toxicities of chemotherapeutic agents.

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