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# TOTAL ANTIOXIDANT STATUS IN THE BLOOD SERUM OF RATS EXPOSED TO N-NITROSO COMPOUNDS AND NITRIC OXIDE SYNTHASE INHIBITORS

## CAŁKOWITY STATUS ANTYOKSYDACYJNY SUROWICY KRWI SZCZURÓW NARAŻONYCH NA ZWIĄZKI N-NITROZOWE I INHIBITORY SYNTAZY TLENKU AZOTU

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*Nitric oxide synthase (NOS) inhibitor, Nw-nitro-L-arginine methyl ester (L-NAME) was found to mitigate total anti-oxidant status (TAS) in the blood serum of rats pretreated with N-nitrosodiethylamine (NDEA) and N-methyl-N-nitrosourea (NMU). No such changes were found in animals dosed with L-NAME only nor even with L-NAME and spermidine, respectively. Since spermidine, also known as an inhibitor of iNOS synthesis, elevated TAS in rats dosed with L-NAME and NDEA/NMU, the polyamine was suggested to modify the NOS/NO origin to serve the physiological level of the total anti-oxidant status in rat blood serum.*

**Key words:** N-nitroso compounds, nitri oxide synthase (NOS), polyamines, total anti-oxidant status (TAS)
**Słowa kluczowe:** związki N-nitrozowe, syntaza tlenku azotu (NOS), poliaminy, całkowity status anty-oksydacyjny (TAS)

## INTRODUCTION

Nitric oxide (NO) produced from L-arginine by nitric oxide synthase (NOS) has been shown to elucidate anti-oxidant properties, however it was also found to enhance pro-oxidant shift(s) due to reaction with superoxide anion ($O_2^{\cdot -}$) to yield peroxynitrite ($ONOO^-$) [17, 25]. More recently, peroxynitrite and nitric dioxide ($NO_2^{\cdot}$) radical production has been evidenced in nitrite-enhanced mieloperoxidase/hydrogen peroxide ($MPO/H_2O_2$) systems, and it plausibly involved hypochlorus acid (HOCl) from hydrogen peroxide and chloride

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ion ($Cl^-$) [6, 30]. Interestingly, peroxynitrite was also produced by the action of xanthine oxidoreductase in the presence of inorganic nitrite ($NO2^-$), molecular oxygen, and reducing agent, such as pterin [8]. Since NO and peroxynitrite was found to elucidate opposite direction towards lipid peroxidation in cellular components, reactive oxygen and nitrogen species (RONS) from the endogenous NOS/NO origin have been critically evaluated in some pro-inflammatory and/or carcinogenic processes [9, 11, 22-24].

In recent years, a large body of interest has been devoted to study a potent role of the inducible form of nitric oxide synthase (iNOS) in promoting pro-oxidant shift(s) in animals treated with carcinogenic *N*-nitroso compounds. For example, *N*-nitrosodiethylamine (NDEA) has been found to induce iNOS and 3-nitrotyrosine (3-NT), a peroxynitrite tracer and/or biomarker in peroxynitrite-linked protein nitration processes, and it also increased lipid peroxidation in murine liver tissues [1, 12]. Interestingly, NOS inhibition with Nω-nitro-L-arginine methyl ester (L-NAME) has been found to mitigate lipid peroxidation in rat liver treated with NDEA and *N*-methyl-*N*-nitrosourea (NMU) [12, 13], but it was not evidenced in spleen and kidney, respectively [12, 13]. In contrast, the L-NAME inhibitor elevated lipid peroxidation in the spleen tissue of NMU-treated animals, elucidating a dual role of NOS inhibition in pro- and anti-oxidative processes [13]. Although NDEA is thought to be primary metabolized by CYP2E1 in the liver of laboratory rodents [4], more recent studies also evidenced that NDEA is decomposed to form NO and nitrite/nitrate intermediates by non-enzymatic reactions with the use of Fenton's reagents or UV light exposures [15, 16]. To data, NO-donating agents have been also recognized as well-known medicines, such as nitroglycerine and molsidomine [21], but more recently NO releasing agents were recognized among some environmental carcinogenes, such as *N*-methyl-*N*-nitrosourea, also known as a moiety of the streptozotocin's chemical structure, of the glucosamine-nitrosourea compound from the soil microorganism *Streptomyces achromogenes*, which is frequently used in experimentally-induced diabetes in rats [19, 29].

In an attempt to further understand whether or not NO plays a pivotal role in pro- and/or anti-oxidant shift(s), the total anti-oxidant status (TAS) in the blood serum of rats pretreated with NOS inhibitors and N-nitroso compounds (NDEA, NMU) was examined.

## MATERIALS AND METHODS

Male Wistar rats (220 ± 20 g) were used in the studies. Before the experiments, the animals were acclimatized for one week under standard conditions (ambient temperature 22 ± 2°C, air humidity 40-70%, light-darkness cycle 12/12 h). Throughout the experiment, the rats were given standard laboratory chow (Murigran pellet) and water *ad libitum*. The animals were divided into 6 groups of 10 animals in each group, and they were treated per os with either normal saline (control), or spermidine, SPR (10 mg/kg b.w.), N-nitrosodiethylamine, NDEA (0.1 mg/kg b.w.), *N*-methyl-*N*-nitrosourea, NMU (0.1 mg/kg b.w.), SPR (10 mg/kg b.w.) plus NDEA (0.1 mg/kg b.w.), and SPR (10 mg/kg b.w.) plus NMU (0.1 mg/kg b.w.) daily for 30 days. In the experiment, SPR was applied only for 21 days, and it was introduced at 3-4 hours after pretreatment with saline, NDEA, or NMU, respectively. On day 22$^{nd}$ of the experiment, the half of randomly selected rats in each group was additionally treated per os with a single daily dose of *Nw*-nitro-L-arginine methyl ester, L-NAME (10 mg/kg b.w.) for 3 days. At 24 hours after the post-dosing period (day 31st), the animals were sacrificed by cervical dislocation, and used for blood analysis. The total anti-oxidant status (TAS) in the blood serum of rats was determined using a diagnostic assay kit from Randox Laboratories Ltd., (Antrim,

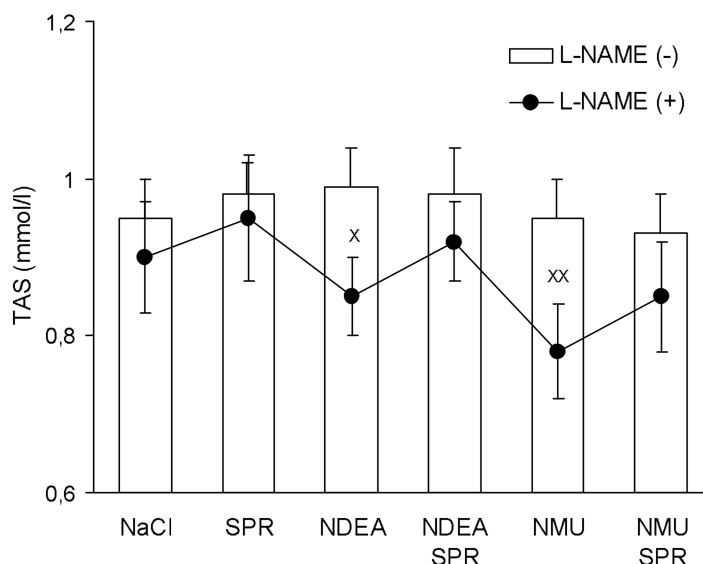
UK), as described previously by *Grudziński* and *Frankiewicz-Jóżko* [10]. Briefly, the azo-compound 2,2'-azino-di-[3-ethylbenzthiazoline sulphonate (ABTS) was incubated with a metmyoglobin and hydrogen peroxide to produce a radical cation $ABTS^{+}$, of which a stable blue-green color was measured spectrophotometrically (600 nm). Anti-oxidants in the analyzed sample of the rat blood serum caused suppression of this color to a degree, which was proportional to their concentrations. In the assays, 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid was used as a standard [10].

The statistical significance of the differences was determined by using *Student's* t-test for comparison between the repeated measures analysis of variance (ANOVA) in two groups and *Dunnet's* tests for multiple comparison where appropriate. Differences were considered significant when probability (p) values were less than 0.05.

## RESULTS AND DISCUSSION

In the present studies, spermidine did not have any effect(s) on the total anti-oxidant status (TAS) in the blood serum of rats (Fig. 1). This data clearly show that polyamine, as introduced for a multiple dosage regiment in the experiment, is not able to change(s) a stable anti-oxidant status in the blood serum of tested animals. In opposite to spermidine, a first-step polyamine from L-arginine/L-ornithine pathway, called putrescine, has been recently found to elevate the total anti-oxidant status of blood serum in rats treated with nitrite [10], and it also decreased the endogenous production of nitric oxide (NO) in cultured J774.2 macrophages stimulated with bacterial endotoxin (lipopolysaccharide; LPS) or with *gamma* interferon (INF) [28]. Since putrescine and spermidyne have been also identified as NOS inhibitors in animals [3, 28], a number of recent studies have been carried out to elucidate the pro- and/or anti-oxidant role of endogenous polyamines, including putrescine, spermidine and spermine in animals and/or tissues samples. For example, putrescine has been shown to decrease nitrite-induced lipid peroxidation in rats [10], and it also normalized lipid peroxidation in the gastric mucosa of nitrite-treated animals [11]. To data, spermidine and spermine have been also found to decrease malondialdehyde release in human red blood cells exposed to hydrogen peroxide in vitro [7], and the polyamines mitigated pro-oxidant shift(s) in DNA exposed to gamma radiation from a 60-cobalt source in vitro [5]. Both spermidine and spermine lowered lipid peroxidation processes in animals pretreated with paraquat and they also decreased pro-oxidant shift(s) in rats treated with some environmental carcinogens, including NDEA and NMU [12, 13, 18].

Over the last years, a great deal of attention has been devoted to study NDEA and its metabolites in lipid peroxidation processes [2, 14]. Although CYP2E1 and CYP2E6 isozymes have been mainly involved in NDEA biotransformation to induce pro-oxidant nucleophiles [4], a recent finding by *Hiramoto* and associates [16] also evidenced a non-enzymatic pathway of NDEA decomposition to form nitric oxide (NO) in vitro. It should be noted that malondialdehyde and NO elevation was also recognized for other chemical agents, including some xenobiotics, such as *N*-methyl-*N*-nitrosourea (NMU) [20]. Since endogenous NO from L-arginine was mainly recognized as a chain-breaking anti-oxidant molecule, which protects cells against the detrimental effects of reactive oxygen species and lipid peroxidation [17], a potent role of NOS inhibition in changing the balance between the pro- and anti-oxidant shift(s) has been examined in NDEA- and NMU-treated animals. As shown in figure 1, pretreatment of rats with L-NAME, a competitive inhibitor of nitric oxide synthase (NOS) did not affect(s) the total level of anti-oxidant status in murine blood serum,




Rats were treated *per os* with N-nitrosodiethylamine (NDEA) (0.1 mg/kg b.w./day) or N-methyl-N-nitrosourea (NMU) (0.1 mg/kg b.w./day) for 30 days. Normal saline was used as control. In the NDEA/NMU - or saline-treated rats, spermidine (SPR) (10 mg/kg b.w./day) was dosed *per os* for a first 21 days only. On day 22nd of the experiment, the animals were also treated per os with $N\omega$-nitro-L-arginine methyl ester (L-NAME) (0.1 mg/kg b.w./day) for 3 days. TAS levels were assayed in blood serum at 24 hrs after the last NDEA/NMU or saline dosage.

Values are mean ± SD, n=10, x $P<0.05$, *vs.* NDEA, xx $P<0.05$, *vs.* NMU, (+) and (-) represent groups with or without L-NAME.

Fig. 1. Total anti-oxidant status (TAS) in the blood serum of rats pretreated with N-nitroso compounds and iNOS inhibitors.

and the agent was found to have no further effect(s) in animals pretreated with spermidine (Fig. 1). In contrast, the NOS inhibitor (L-NAME) lowered the TAS level in animals pretreated with N-nitrosodiethylamine, and it also diminished the anti-oxidant status in the blood serum of animals pretreated with NMU (Fig. 1). In the studies, no effects of NDEA or even NMU, as dosed alone, was found to change(s) the total anti-oxidant status of blood serum in animals (Fig. 1). Although L-NAME was noted to decrease TAS levels in the blood serum of rats dosed with N-nitroso agents, it was previously shown that NOS inhibition with L-NAME also decreased lipid peroxidation in the liver and small intestinal mucosa of NDEA/NMU-treated animals [12, 13]. Results from our experiments were found in accordance with those reported by *Seven* and associates [27] who evidenced that L-NAME mitigated sreptozotocin/(NMU)-induced lipid peroxidation, and it diminished serum nitrite/nitrate levels in diabetic animals. It should be also noted that L-NAME decreased the elevated lipid peroxidation after experimental sciatic nerve ischemia-reperfusion in rats [26], providing further experimental evidences that excessive NO formation accelerates lipid peroxidation in cells. Present data in animals also supported other results obtained from *Mabrouk* et al. [20] who found that NMU-enhanced malondialdehyde and lipid peroxidation is asso-

ciated with nitric oxide (NO) origin from the murine sera. Interestingly, in the present studies, spermidine did not affect(s) the total anti-oxidant status of murine blood in animals dosed with or without N-nitrosamines (Fig. 1), but it also elevated TAS levels in NDEA/NMU-treated and L-NAME-dosed animals. It should be noted that spermine, spermidine, and putrescine inhibited LPS-induced nitric oxide synthase activity [3], supporting previous finding by *Szabo* et al. [28] who discovered that spermidine is enable to inhibit the endogenous production of NO in cultured macrophages stimulated with LPS and/or gamma interferon. It is of interested to note that neither spermidine, nor its metabolites, interferes with the production of NO from L-arginine or act as scavengers of NO, but the polyamines basically are recognized as inhibitors of the induction of iNOS [28]. Since both NDEA- and/or NMU-induced iNOS protein and lipid peroxidation in murine tissues [1, 12, 20], it seems plausible that spermidine could also acts as a terminator of pro-inflammatory mediators in nitrosamine-treated animals. Since we did not measure mRNA iNOS gene and protein expressions in the experiment, a potent biochemical mechanism(s) of spermidine action in nitrosamine-treated animals should be further tested in details.

In summary, it was found that nitric oxide synthase inhibitor (L-NAME) mitigated the total anti-oxidant status (TAS) of rat blood serum in NDEA- and NMU-treated rats. In the present study, spermidine, a simple polyamine obtained from L-arginine/L-ornithine pathway, normalized TAS levels in nitrosamine/L-NAME-dosed animals.

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### Summary

In this study, the total antioxidant status (TAS) was assayed in the blood serum of rats pretreated *per os* with either *N*-nitrosodiethylamine (NDEA) (0.1 mg/kg b.w./day) or *N*-methyl-*N*-nitrosourea (NMU) (0.1 mg/kg b.w./day) for 30 days. The animals were also dosed per os with spermidine (SPR) (10 mg/kg b.w./day) for a first 21 day period, and *Nω*-nitro-L-arginine methyl ester (L-NAME) (10 mg/kg b.w./day) given to animals for 3 days (days 22-24), respectively. Nitric oxide synthase (NOS) inhibitor, L-NAME was found to mitigate TAS levels in the blood serum of rats pretreated with NDEA and NMU. No such changes were found in animals dosed with L-NAME only nor even with L-NAME and spermidine, respectively. Since spermidine, also known as an inhibitor of iNOS synthesis, elevated TAS levels in rats dosed with L-NAME and NDEA/NMU, the polyamine was suggested to modify the NOS/NO origin to serve the physiological level of the total anti-oxidant status in rat blood serum.

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Streszczenie

W badaniach oznaczano całkowity status antyoksydacyjny (TAS) surowicy krwi szczurów narażonych *per os* przez okres 30 dni na N-nitrozodietyloaminę (NDEA) (0.1 mg/kg m.c./dzień) i N-metylo-N-nitrozmocznik (NMU) (0.1 mg/kg m.c./dzień). Zwierzęta otrzymywały również *per os* spermidynę (SPR) (10 mg/kg m.c./dzień), którą podawano przez pierwsze 21 dni eksperymentu oraz ester metylowy *Nω*-nitro-L-argininy (L-NAME) (10 mg/kg m.c./day), który podawano per os przez 3 dni w dniach 22-24. Inhibitor indukowalnej syntazy tlenku azotu (iNOS), L-NAME obniżał poziom TAS w surowicy krwi szczurów narażonych na NDEA i NMU. Nie odnotowano takiego efektu u zwierząt narażonych wyłącznie na L-NAME lub L-NAME i spermidynę. Ponieważ spermidyna (znany inhibitor biosyntezy iNOS) zwiększała poziom TAS u szczurów otrzymujących L-NAME i NDEA/NMU, sugerowano, że poliamina może modyfikować endogenną produkcję NO przy udziale iNOS, wpływając przy tym na utrzymanie fizjologicznego poziomu całkowitego statusu antyoksydacyjnego w surowicy krwi szczurów.

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**SZKOŁA GŁÓWNA GOSPODARSTWA WIEJSKIEGO W WARSZAWIE**
**WYDZIAŁ NAUK O ŻYWIENIU CZŁOWIEKA I KONSUMPCJI**

**ogłasza nabór od października 2005 na zaoczne**
**PODYPLOMOWE STUDIA PORADNICTWA ŻYWIENIOWEGO i DIETETYCZNEGO**

Studia są przeznaczone przede wszystkim dla absolwentów posiadających dyplom ukończenia studiów wyższych na kierunkach: technologii żywności i żywienia człowieka, lekarskim, pielęgniarskim, oraz innych związanych z ochroną zdrowia oraz nauczycieli specjalizujących się w propedeutyce żywienia człowieka.

Celem studiów jest wykształcenie specjalistów z zakresu żywienia człowieka i przygotowanie do samodzielnego udzielania porad żywieniowo – dietetycznych oraz nauczania podstaw nauki o żywieniu człowieka w szkołach na poziomie podstawowym i średnim.

Studia trwają trzy semestry w zakresie Poradnictwa Żywieniowego i Dietetycznego z możliwością ukończenia po zaliczeniu dwóch semestrów w zakresie Poradnictwa Dietetycznego. Studia są odpłatne.

Program studiów dwu semestralnych realizowany jest w ramach 20 zjazdów ( 272 godziny zajęć), natomiast trzy semestralnych – 27 zjazdów (356 godzin zajęć).

Zajęcia dydaktyczne odbywają się w SGGW na Wydziale Nauk o Żywieniu Człowieka i Konsumpcji , ul. Nowoursynowska 159C, w piątki po południu oraz w soboty.

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Po zaliczeniu wszystkich semestrów słuchacze otrzymują świadectwo ukończenia Studiów Podyplomowych według wzoru MENiS.

O przyjęciu na Studia Podyplomowe decyduje kolejność zgłoszeń.

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