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# Lung toxicity of nitrogen mustard may be mediated by nitric oxide and peroxynitrite in rats

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**Abstract**

Nitric oxide (NO) has previously been shown to be responsible for nitrogen mustard (NM)-induced tissue toxicity. Excessive amounts of NO are known to be able to produce peroxynitrite, an important reactive nitrogen compound, by reacting with superoxide. Previous studies reported that NO synthase inhibitors are able to prevent NM toxicity. The aim of this study was to evaluate whether peroxynitrite is also responsible for NM-induced lung tissue damage in rats. Wistar rats were divided into four groups. NM was injected intratracheally and was treated with the selective inducible nitric oxide synthase (iNOS) inhibitor aminoguanidine (AG) (intraperitoneal) or the peroxynitrite scavenger ebselen (intragastric). Control animals were exposed to saline only. NM injection caused both oxidative and nitrosative stress, reflected by dramatically increased levels of the lipid peroxidation end product malondialdehyde (MDA), iNOS activation and urine nitrite–nitrate (NOx) values. Histopathological evaluation demonstrated lung damage with NM exposure. AG blocked iNOS activation and decreased urine NOx levels, and resulted in less histopathological changes in the lung. Although the histopathological outcome was found to be similar to AG, ebselen did not change urinary NOx or lung iNOS levels. Furthermore, ebselen was more able than AG to protect against MDA accumulation. In conclusion, the ability of ebselen to prevent against lung damage without blocking NO synthesis suggests that peroxynitrites may have an important role in the pathogenesis of NM toxicity in addition to NO.


*Keywords:* Nitrogen mustard; Lung damage; Nitric oxide; Peroxynitrite

## 1. Introduction

Nitrogen mustard (NM) was the first non-hormonal agent used in cancer chemotherapy and a number of nitrogen mustard derivatives are valuable cytotoxic and radiomimetic agents for the treatment of cancer. However, therapeutic nitrogen mustards are themselves highly cytotoxic and induce severe side effects (Colvin, 1982). Furthermore, NM is a structural analogue of the sulfur mustard (SM), a strong chemical warfare agent, which induces severe injuries to the eyes, skin, and respiratory tract (Dacre and Goldman, 1996). Despite many years of research into these agents, the cytotoxic mechanisms induced by mustards and the initial events leading to cell death have not been fully elucidated. It is believed that the mechanism of the mustard injury is linked to alkylation of cellular targets (Sawyer et al., 1996).

Recently, it has been shown that increasing nitric oxide (NO) produced by nitric oxide synthase (NOS) may be responsible for the detrimental effects of mustards (Sawyer, 1998). This toxicity probably comes from overproduction of reactive nitrogen species (RNS), in particular peroxynitrite ($ONOO^-$), by the reaction of NO and superoxide ($\cdot O_2^-$) which appears abundantly in inflammatory areas (Virag et al., 2003). Cyclophosphamide (CP) is another

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alkylating antineoplastic chemotherapeutic agent in the nitrogen mustard group (Levine and Richie, 1989). Recently, the pathogenesis of CP-induced tissue toxicity has been extensively researched in our laboratory (Korkmaz et al., 2003, 2005; Oter et al., 2004; Ozcan et al., 2005; Topal et al., 2005; Yildirim et al., 2004). These studies strongly suggested that oxidative stress, inducible NOS (iNOS) induction leading to NO overproduction and peroxynitrite formation are responsible, at least in part, for toxic effects of the nitrogen mustard group alkylating agent CP.

In a recently published review, an activated ''devil's triangle'' in the targeted cell was suggested to be responsible for NM toxicity (Korkmaz et al., 2006). According to this mechanism, excess NO and excess $\cdot O_2^-$ both decrease the bioavailability of $ONOO^-$ and equimolar concentrations of the radicals are ideal for $ONOO^-$ formation. Peroxynitrite anion is in a pH-dependent protonation equilibrium with peroxynitrous acid (ONOOH). Homolysis of ONOOH gives rise to formation of the highly reactive hydroxyl radical ($^{\cdot}OH$) mediating molecular and tissue damage associated with peroxynitrite ($ONOO^-$) production (Radi et al., 2001). $ONOO^-$ is formed when NO and $\cdot O_2^-$ react in a near diffusion-limited reaction (Squadrito and Pryor, 1998).

The most powerful cellular antioxidant system protecting against the harmful effects of $\cdot O_2^-$ is embodied by the enzyme superoxide dismutase (SOD). However, it was shown that NO efficiently competes with SOD for superoxide and under conditions of increased NO production, NO can outcomplete SOD for $\cdot O_2^-$ resulting in $ONOO^-$ formation (Beckman and Koppenol, 1996). $ONOO^-$ is not a free radical but a short-lived and far more reactive species than its precursors. It can directly react with target biomolecules via one or two-electron oxidations (Alvarez and Radi, 2003). Higher concentrations and the uncontrolled generation of $ONOO^-$ may result in unwanted oxidation and consecutive destruction of host cellular constituents. $ONOO^-$ may oxidize and covalently modify all major types of biomolecules, such as membrane lipids, thiols, proteins and DNA (Virag et al., 2003). One of the most important mechanisms of $ONOO^-$-mediated cellular injury is an increase in DNA strand breakage, which triggers the activation of Poly(adenosine diphosphate-ribose) polymerase (PARP), a DNA repair enzyme. DNA damage causes PARP overactivation, resulting in the depletion of oxidized nicotinamide adenine dinucleotide (NAD) and adenosine triphosphate (ATP), and consequently result in necrotic cell death (Virag and Szabo, 2002).

Current knowledge makes it seem feasible that mustard toxicity comes from oxidative as well as nitrosative stress leading to lipid, protein and DNA damage in the target cell. We considered that lung toxicity of NM could also be mediated by both iNOS and peroxynitrite production. All possible molecular mechanisms should be taken into account in order to fight against such a high degree of toxicity. Thus, in this study we aimed to evaluate the effectiveness of the selective iNOS inhibitor aminoguanidine (AG) (Misko et al., 1993) and the peroxynitrite scavenger ebselen (2-phenyl-1,2-benzisoselenazol-3[2H]-on) (Klotz and Sies, 2003) against NM toxicity. Mechloretamine (2,2′-dichlorodiethylamine; $HN_2$), the prototype of NM class agents (Osterlund et al., 2005), was used to induce NM-exposed lung injury.

## 2. Materials and methods

### 2.1. Animals

A total of 40 male albino Wistar rats, with body weights of 220–250 g, were divided into four groups by the 'simple random sampling method' and given food and water ad libitum. The Afyon Kocatepe University Animal Care and Use Committee approved the experimental protocol.

### 2.2. Chemicals

All chemicals were obtained from Sigma–Aldrich Chemie GmbH (Taufkirchen, Germany) and all organic solvents from Merck KGaA (Darmstadt, Germany). All reagents were of analytical grade, were prepared each day (except the phosphate buffer) and stored in a refrigerator at +4 °C. The reagents were equilibrated at room temperature for 0.5 h before use when the analysis was initiated or reagent containers were refilled. Phosphate buffers were stable at +4 °C for 1 month.

### 2.3. Experimental design

The animals were given a toxic dose of 0.5 mg/kg $HN_2$ dissolved in 100 μl saline intratracheally (i.t.). Control animals were injected the same amount of saline only. 100 mg/kg/day AG (intraperitoneally, i.p.) and 20 mg/kg/day ebselen (intragastrically) were administered one hour before $HN_2$ application and continued every 12 h for a total of six doses. Ebselen is soluble in dimethyl sulfoxide (DMSO) or chloroform. Chloroform was used as solvent as DMSO has antioxidant properties. The control solution (vehicle) consisted of 0.25 ml of 99% chloroform. The treatment solutions were 20 mg/kg ebselen dissolved in 0.25 ml of vehicle.

### 2.4. Tissue preparation

After a period of 72 h, the animals were anesthetized with ketamine (85 mg/kg) and xylasine (12.5 mg/kg) and their chest was opened by median sternotomy. The lung parenchyma was flushed with 10 ml of ice-cold physiological saline via a right ventricular puncture to the heart to remove blood from tissues and to slow the metabolism of rats (Zamboni et al., 1996). The lungs were then removed immediately. One lung was fixed in 10% buffered formaldehyde for histopathologic evaluation and the other one washed with saline to remove residual blood, put into tubes, frozen with liquid nitrogen and stored at −70 °C.