

## ANTIOXIDANT PROPERTIES OF AMINO GUANIDINE: A PARAMAGNETIC RESONANCE TEST

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### Abstract

The aim of these experiments was to investigate the radical scavenging properties of aminoguanidine, which can diminish advanced glycosylation of proteins and can be used to prevent the deleterious effects of chronic hyperglycemia. Electron Paramagnetic Resonance (EPR) was used to determine the scavenging abilities on enzymatically-produced superoxide anion and hydroxyl radicals with 5,5-dimethyl-1-pyrroline N-oxid (DMPO) used as a spin-trap. EPR studies showed that aminoguanidine did not express significant superoxide scavenging effects. Aminoguanidine exhibited significant hydroxyl radical scavenging properties characterised by an IC<sub>50</sub> value of 6.5% and these effects were concentration-dependent. These results suggest that aminoguanidine may be of clinical value for the prevention of chronic diabetic complications.

### Key words

Aminoguanidine, Antioxidant, Diabetes mellitus, Electron paramagnetic resonance, Free radicals

### INTRODUCTION

A chronically increased plasma concentration of glucose found in patients with diabetes mellitus initiates a series of biochemical reactions that are responsible for a number of secondary complications, especially in the microvascular bed (1). Persistent hyperglycaemia leads, among other things, to an imbalance between pro- and anti-oxidant factors, including an increase in non-enzymatic glycosylation of protein amino groups. This process is accompanied by production of reactive oxygen species (ROS) that may have highly toxic effects on cellular homeostasis. Aminoguanidine (AMG), a nucleophilic hydrazine compound, can reduce glycosylation of protein and has a therapeutic potential for preventing diabetic complications. Previous reports have described both pro- and anti-oxidant effects (2,3). The present study investigated the antioxidant effect of AMG by the technique of electron paramagnetic resonance (EPR) spectroscopy using spin-trapping techniques to assess the possible superoxide and hydroxyl radical scavenging effects of AMG.

## MATERIALS AND METHODS

### Chemicals

The spin-trap 5,5 - dimethyl - 1 - pyrroline - 1 - oxide (DMPO) was purified by double distillation. Xanthine oxidase activity was routinely assayed by spectrophotometry at 298 nm at the appropriate pH. All chemicals, including AMG, were provided by Sigma France.

The generation of superoxide and hydroxyl radicals was determined by EPR spin-trapping techniques using the spin-trap DMPO. EPR spectra were recorded in a flat-type quartz cell at 37 °C, using an IBM Bruker ESP 300E X-band spectrometer (Wissembourg, France), with a TM110 cavity at a modulation frequency of 100 kHz, a modulation amplitude of 0.8 G, a microwave power of 20 mW, a time constant of 0.16 sec and a scan rate of 1.25 G/sec. Results were expressed in arbitrary units (AU). Free radical generation was performed in 50 mM phosphate buffer adjusted to pH 7.4. The superoxide generating system consisted of xanthine-xanthine oxidase (50 μM - 500 mIU/ml), in the presence of catalase (500 IU/ml) and desferoxamine (1 mM), to avoid conversion of the superoxide anion to the hydroxyl radical. The spin-trap DMPO was used at a concentration of 50 mM. Hydroxyl radicals were also generated by the xanthine-xanthine oxidase system (25 μM - 2.5 mIU/ml) and by further addition of Fe-EDTA (5 μM - 10 μM) to promote conversion of the superoxide anion to the hydroxyl radical in the presence of 5 mM DMPO (Fig.1).

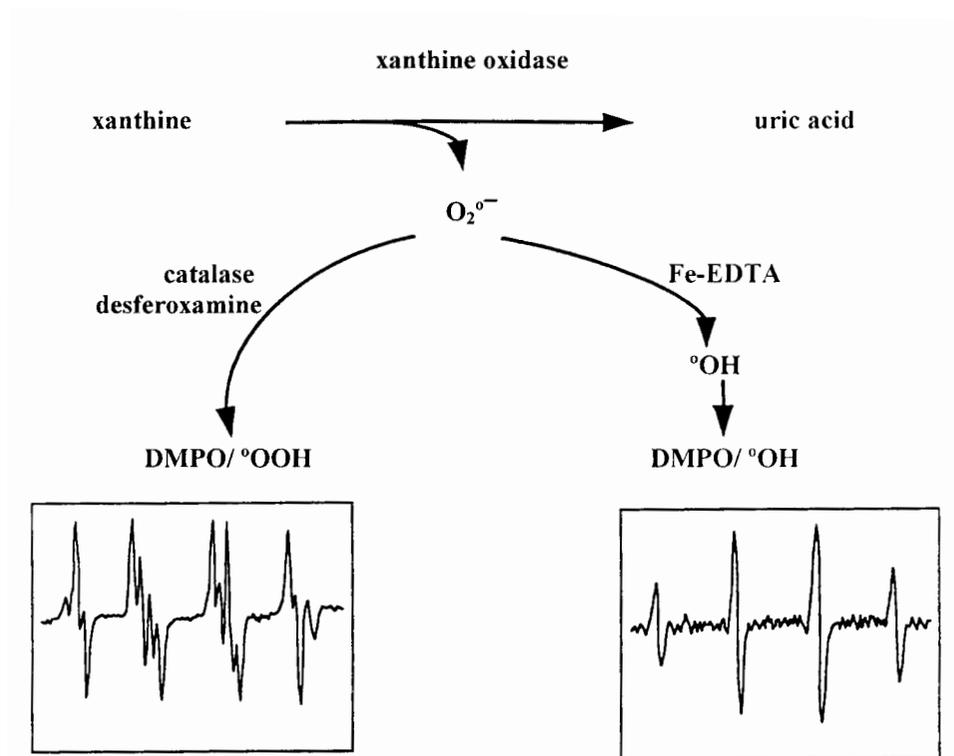
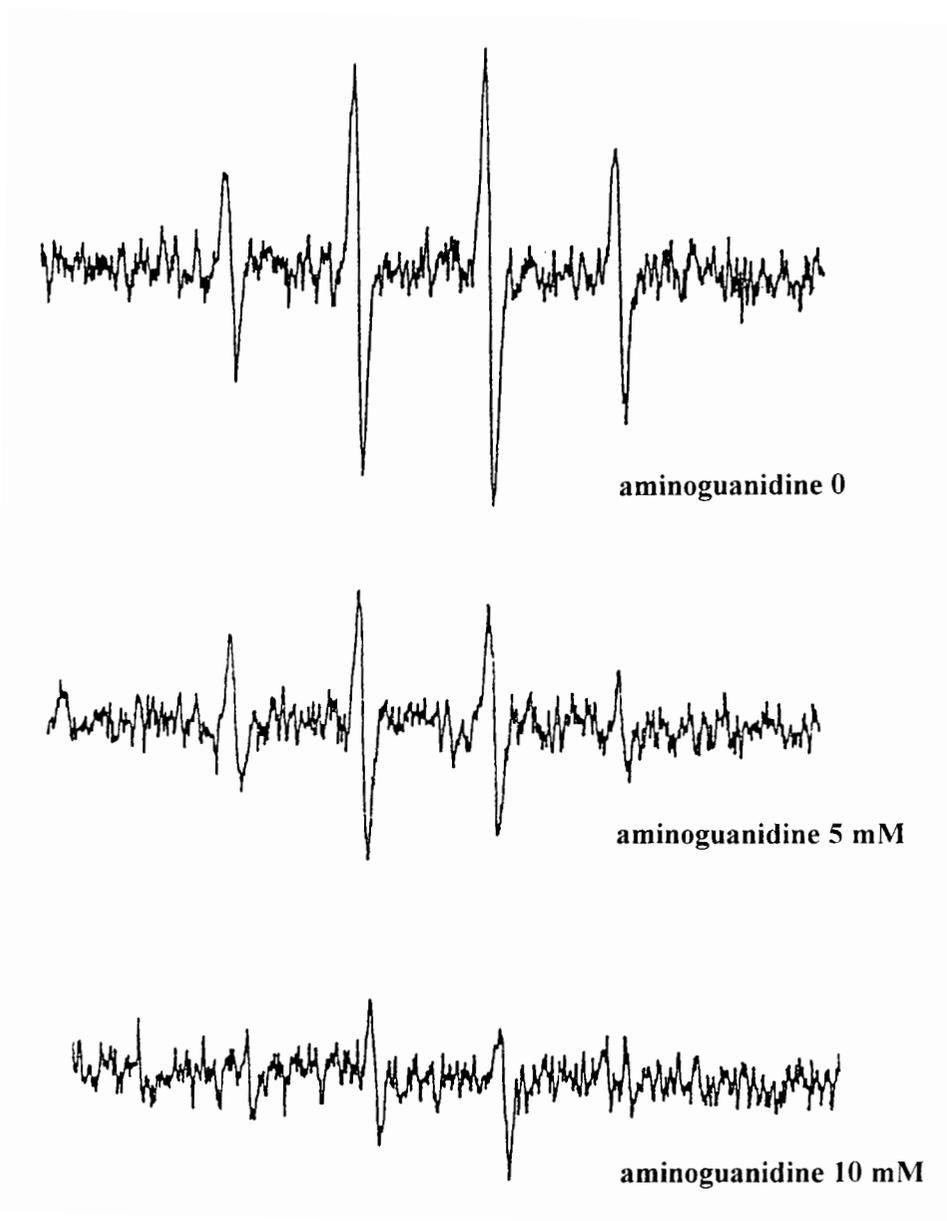


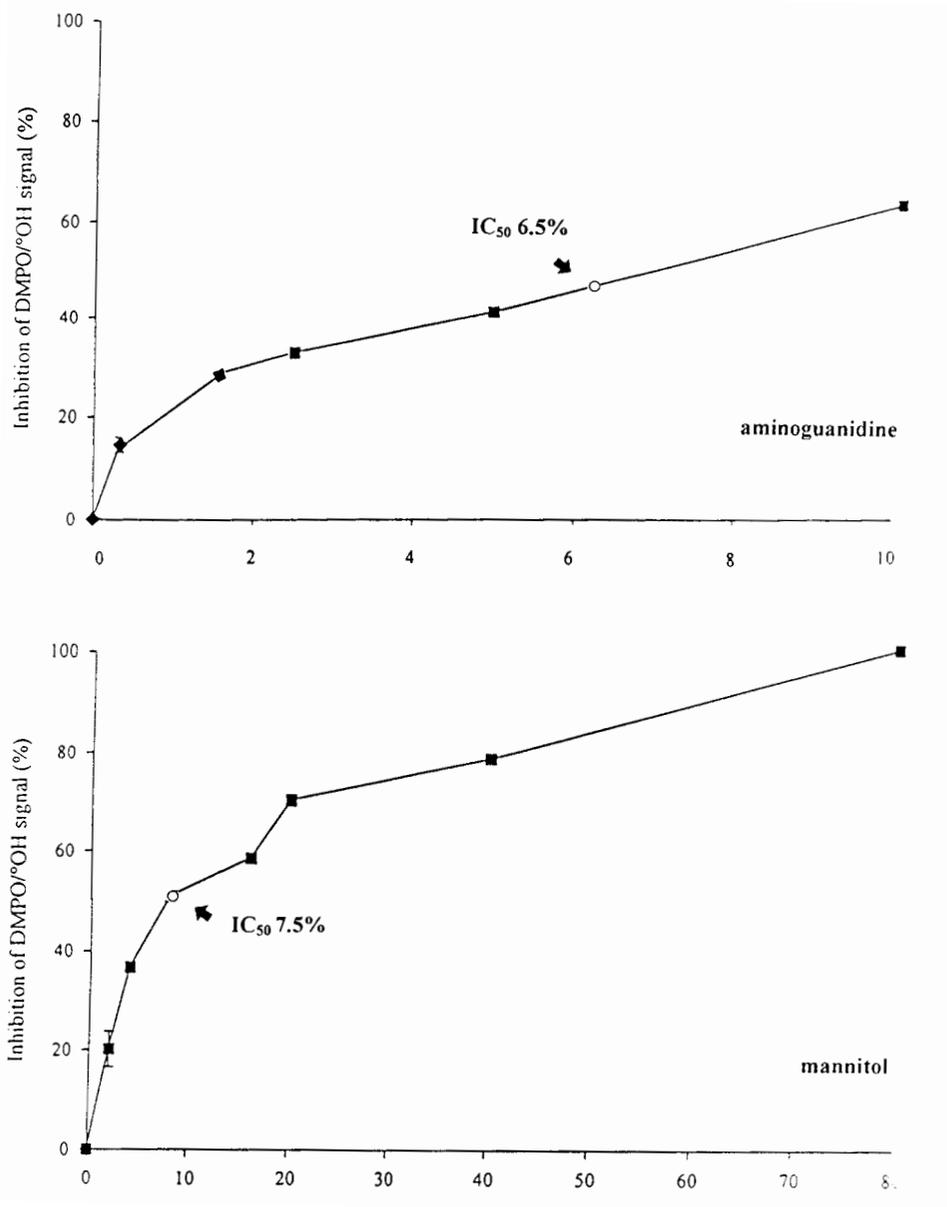
Fig. 1

Superoxide and hydroxyl radicals generated from the xanthine-xanthine oxidase system. The spin-adducts DMPO/ $^{\bullet}OOH$  and DMPO/ $^{\bullet}OH$  were detected by ESR technique.



*Fig. 2*

DMPO/ °OH EPR spectra showed decreased spin-adduct formation in the presence of aminoguanidine (5 and 10mM). Spectra intensity decreased by 41 and 65%.



*Fig. 3*

Hydroxyl scavenging effect of AMG (a) and mannitol (b) at pH 7.4 in phosphate buffer (50mM).

The drugs were solubilised in phosphate buffer at pH 7.0. AMG (0.1, 1, 2, 5 or 10 mM) was added to the medium before xanthine, which started the enzymatic reaction. The spectra for the superoxide-derived spin-adduct were recorded within the first 60 sec of the enzymatic reaction, and those for the hydroxyl-derived spin-adduct within the first 75 sec. Results were expressed by a curve representing the percent of inhibition of DMPO-adduct formation, and substances were characterised by the estimation of the 50% inhibitory concentration (IC<sub>50</sub>).

## RESULTS

In our experimental conditions, AMG expressed no significant superoxide scavenging effects. For example, 5 and 10 mM of AMG at pH 7.4 inhibited the formation of the DMPO/ °OOH adduct by 7.5 and 2.6%, respectively.

*Fig. 2* shows the spectra of the spin-adduct of DMPO with the hydroxyl radical: DMPO/ °OH. AMG exhibited hydroxyl radical scavenging properties characterised by an IC<sub>50</sub> value of approximately 6.5 mM at pH 7.4. Respectively, 41% and 65% of the hydroxyl radicals were trapped at concentrations of 5 mM and 10 mM. In comparison, mannitol, a well-known hydroxyl radical scavenger, is characterised by an IC<sub>50</sub> value of 7.5 mM, with all hydroxyl radicals being trapped at 80 mM (*Fig. 3*).

## DISCUSSION

This study shows that AMG displays antioxidant activity in *in vitro* conditions. Our results demonstrated that AMG was able to scavenge hydroxyl radicals. The scavenging properties were significant compared to the reference hydroxyl scavenger mannitol, and displayed a concentration-dependent degree of protection in the EPR experiments. Our results are in concordance with those of other authors who also showed that AMG had direct scavenging activities against hydroxyl radicals (4). Recently, *Giardino et al.* (5) demonstrated that AMG acted as an antioxidant *in vivo*, preventing ROS formation and lipid peroxidation in cells and tissues, and preventing oxidant-induced apoptosis. In diabetic diseases, the massive increase of ROS formation is attributed to the glycosylation reaction (6). Under diabetic conditions, glucose is converted into fructose through the polyol pathway, leading to an increase in the fructose level (7). Fructose has expressed a stronger reducing capacity than glucose and has induced a glycation reaction. The aldehyde groups of unbound sugars react with free amino groups of proteins, forming Schiff's bases which further undergo various rearrangements to generate advanced glycosylation end-products (8). *Wolff et al.* (9) have suggested that transition metals catalyse auto-oxidation of free sugars in glycosylation. This process, called auto-oxidative glycosylation, seems to be accelerated by transition metals and contributes to structural damage in diabetes. Copper levels have been found to be higher in diabetic patients than in the healthy population (10) and significant hydroxyl radical production has been demonstrated in the presence of glucose and copper (11).

It is concluded that the radical scavenging properties of AMG demonstrated in the present study may contribute to the development of effective prevention of secondary structural and functional alterations in diabetes mellitus.

## ANTIOXIDAČNÍ VLASTNOSTI AMINO GUANIDINU–TEST S PARAMAGNETICKOU REZONANCÍ

### S o u h r n

Cílem těchto experimentů byl výzkum antioxidačních vlastností aminoguanidinu, který snižuje předčasnou glykosylaci proteinů a který může být preventivně využit proti škodlivým účinkům chronické hyperglykémie. Elektronová paramagnetická rezonance (EPR) byla využita k určení antioxidačních schopností pomocí enzymatické produkce superoxidového aniontu a hydroxylového radikálu a s použitím tzv. spinové pasti 5,5-dimethyl-1-pyrrolin-oxid (DMPO). EPR studie neprokázala antioxidační efekt aminoguanidinu proti superoxidovému radikálu. Proti hydroxylovému radikálu aminoguanidin vykazuje signifikantní účinek charakterizovaný 50% inhibiční koncentrací (IC<sub>50</sub>) ve výši 6.5% a míra tohoto efektu je závislá na použité dávce. Tyto výsledky naznačují, že aminoguanidin by mohl mít klinické uplatnění v prevenci chronických diabetických komplikací.

### A c k n o w l e d g e m e n t s

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