L-arginine, a nitric oxide precursor, reduces dapsone-induced methemoglobinemia in rats

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Dapsone use is frequently associated to hematological side effects such as methemoglobinemia and hemolytic anemia, which are related to N-hydroxylation mediated by the P450 enzyme system. The aim of the present study was to evaluate the influence of L-arginine supplementation, a precursor for the synthesis of nitric oxide, as single or multiple dose regimens on dapsone-induced methemoglobinemia. Male Wistar rats were treated with L-arginine at 5, 15, 30, 60 and 180 mg/kg doses (p.o., gavage) in single or multiple dose regimens 2 hours prior to dapsone administration (40 mg/kg, i.p.). The effect of the nitric oxide synthase inhibitor L-NAME was investigated by treatment with multiple doses of 30 mg/kg (p.o., gavage) 2 hours before dapsone administration. Blood samples were collected 2 hours after dapsone administration. Erythrocytic methemoglobin levels were assayed by spectrophotometry. The results showed that multiple dose supplementations with 5 and 15 mg/kg L-arginine reduced dapsone-induced methemoglobin levels. This effect is mediated by nitric oxide formation, since the reduction in methemoglobin levels by L-arginine is blocked by simultaneous administration with L-NAME, a nitric oxide synthase inhibitor.

Uniterms: Dapsone. Methemoglobinemia. L-arginine. Nitric oxide. L-NAME.

O uso da dapsona é frequentemente associado a efeitos adversos hematológicos, como a metemoglobinemia e anemia hemolítica, ambos relacionados com a *N*-hidroxilação mediada pelo sistema P450. O objetivo do estudo foi avaliar a influência da suplementação de L-arginina, um precursor da síntese de óxido nítrico, administrado em regime de dose única ou múltipla na metemoglobinemia induzida pela dapsona. Ratos machos Wistar foram tratados com L-arginina (po, gavagem) em dose única ou múltipla de 5, 15, 30, 60 e 180 mg/kg 2 horas antes da administração de dapsona (40 mg/kg, ip). O efeito do L-NAME, um inibidor de óxido nítrico sintase (NOS), foi avaliado através do tratamento com doses múltiplas de 30 mg/kg. Amostras de sangue foram coletadas duas horas após a administração de dapsona. A concentração de metemoglobina eritrocitária foi analisada por espectrofotometria. Os resultados mostraram que a suplementação em dose múltipla de 5 e 15 mg/kg de L-arginina reduziu os níveis de metemoglobina induzida pela dapsona. Este efeito é mediado pela formação de óxido nítrico, uma vez que a redução nos níveis de metemoglobina pela L-arginina é bloqueada pela administração simultânea de L-NAME, um inibidor da óxido nítrico sintase.

Unitermos: Dapsona. Metemoglobinemia. L-arginina. Óxido nítrico. L-NAME.

INTRODUCTION

Dapsone (4,4'-diaminodiphenylsulfone, DDS) is a potent antibacterial and anti-inflammatory compound and has been clinically used in the treatment of leprosy as a component of a multidrug therapy that includes a combination of DDS, clofazimine and rifampicin (Katoch 2002; Walker, Lockwood 2007). The drug is also used for the treatment of malaria and *Pneumocystic carinii* pneumonia in patients with acquired immunodeficiency

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syndrome (Powell *et al*., 1967; Mills *et al*., 1988; Castro 1998; Tobin-D'Angelo *et al*., 2004; Nyunt, Plowe 2007).

The major metabolic pathway of DDS is acetylation, producing monoacetyldapsone (MADDS). DDS is also metabolized by *N*-hydroxylation mediated by cytochrome P450 isozymes CYP2C19, CYP2C9, CYP3A4 and CYP2E1 in man, and isozymes CYP2C6/11 and CYP3A1 in rats, producing DDS hydroxylamine (DDS-NOH) (Fleming *et al*., 1992; Vage, Svensson, 1994; Mitra *et al*., 1995; Gill *et al*., 1995; Ganesan *et al*., 2010). Glucuronidation of DDS and DDS-NOH is catalyzed by the enzyme UDP-glucuronosyltransferase (UGT) allowing its excretion in urine and bile (Coleman *et al*., 1996; Tingle *et al*., 1997).

The co-oxidation of DDS-NOH and hemoglobin produces nitroso derivatives and methemoglobin, thus causing methemoglobinemia and hemolysis (Tingle *et al*., 1990; Coleman, 1995), which are the major dose-dependent side effects of DDS chronic users (Kaluarachchi *et al*., 2001). MADDS-NOH has been shown to be a more potent methemoglobin (metHb) former than DDS-NOH in human erythrocytes *in vitro* (Coleman, Holden, 2004), whilst both metabolites present the same potency in rats and humans (Vage *et al*., 1994).

The reduction of xenobiotic-induced metHb formation and the mechanisms underlying this effect have been widely investigated in the last few years. The various attempts to reduce methemoglobinemia have included: preventing CYP-mediated oxidative metabolism of xenobiotics to hydroxylamines (Coleman *et al*., 1990; Malfará *et al*., 2002); biochemical attenuation of metHb formation with antioxidants (Prussick *et al*., 1992; Wright *et al*., 1996; Dötsch *et al*., 1998; Wright *et al*., 1998; Dötsch *et al*., 2000; Tanen *et al*., 2000; Matteuci *et al*., 2003; De Moraes *et al*., 2008; Jo *et al*., 2008); and reduction of metHb to hemoglobin by stimulating NADH diaphorase or NADPH diaphorase (Dötsch *et al*., 2000).

L-arginine (ARG), a semi-essential amino acid, is the nitrogenous precursor for the synthesis of nitric oxide (NO) by a NADPH-dependent NO synthase (NOS) and regulates vital metabolic pathways. NO is sufficiently non-polar to cross membranes without a carrier and is known to modulate vasorelaxation and exhibit antioxidant properties due to superoxide scavenger and heme oxygenase inductor activities (Wood *et al*., 2008).

In spite of all the evidence pointing to the importance of ARG in vital pathways, the role of ARG supplementation in DDS-induced methemoglobinemia has yet to be described in the literature. The aim of the present study was to evaluate the role of ARG in single and multiple dose regimens in DDS-induced methemoglobinemia. We also evaluated whether the effect of ARG on DDS-induced

methemoglobinemia can be modulated by pretreatment with *N*-nitro-L-arginine methyl ester (L-NAME), a non-specific NOS inhibitor.

MATERIAL AND METHODS

Dapsone was supplied by FURP (Fundação para o Remédio Popular; Guarulhos, Brazil) and L-(+)-arginine was supplied by Acros organics (Morris Plains, NJ, USA). L-NAME was purchased from Sigma-Aldrich (St. Louis, MO, USA). KCN was supplied by Merck (Darmstadt, Germany) and $K_3Fe(CN)_6$ was supplied by Merck (Rio de Janeiro, Brazil). Water was purified with the Milli-Q Plus system (Millipore, Bedford, MA, USA).

Experimental study

The experimental study was approved by the Ethics Committee for the Use of Animals of Ribeirão Preto Campus, University of São Paulo, Brazil, in accordance with the US National Institutes of Health *Guide for the Care and Use of Laboratory Animals* (Protocol number 06.1.461.53.6). Male Wistar rats (200 ± 20 g) were kept for 48 hours before the experiment in a room under controlled temperature (21-23 °C) and humidity (40-60%) and on a 12 h light:12 h dark cycle. The animals had free access to chow and water throughout the experiment. The animals (n = 8 per group) were treated in single or multiple dose regimens. ARG was administered orally (p.o. gavage, 200 µL), dissolved in sterile physiologic saline, whereas DDS was dissolved in dimethylsulphoxide (DMSO) and administered intraperitoneally (i.p., 200 µL). L-NAME at 30 mg/kg was administered using a multiple dose regimen in the same solution as ARG.

Single dose regimen

The control group received the vehicle of ARG (sterile physiological saline) p.o. by gavage two hours before the administration of the vehicle used to dissolve DDS (DMSO) i.p. The DDS group received 40 mg/kg DDS (i.p.) 2 hours after the administration of saline p.o. The groups DDS + 5 mg/kg ARG, DDS + 15 mg/kg ARG, DDS + 30 mg/kg ARG, DDS + 60 mg/kg ARG, DDS + 180 mg/kg ARG received ARG at 5, 15, 30, 60 and 180 mg/kg doses, respectively, 2 hours before the administration of 40 mg/kg DDS.

Multiple dose regimen

The control group received saline for five days p.o. (gavage). On the fifth day, the animals received DMSO i.p. 2 hours after saline administration. The DDS group

received saline for five days p.o. and 40 mg/kg DDS on the fifth day, 2 hours after saline administration. Groups DDS + 5 mg/kg ARG, DDS + 15 mg/kg ARG, DDS + 30 mg/kg ARG, DDS + 60 mg/kg ARG, DDS + 180 mg/kg ARG received ARG at 5, 15, 30, 60 and 180 mg/kg doses, respectively, for five days. On the fifth day, 2 hours after ARG administration, the animals received 40 mg/kg DDS.

Heparinized blood samples were collected two hours after DDS or DMSO administration, in both dose regimens (Liquemine 5000 IU, Roche, Rio de Janeiro, Brazil). Methemoglobin levels were determined immediately.

Methemoglobin assay

Methemoglobin levels relative to hemoglobin levels were determined according to the method described by Evelyn and Malloy (1938) (modified by Harrison and Jollow, 1986). Briefly, an aliquot (200 µL) of heparinized blood was added to 10 mL of 0.02 M phosphate buffer pH 7.8 with 0.05% triton X-100 and then shaken in a mixer for 30 seconds. The hemolysate was then fractionated into four tubes. Tube 1 (A_1) remained with hemolyzed blood. An aliquot (50 µL) of 20% $K_3Fe(CN)_6$ was added to tubes 3 (A_3) and 4 (A_4). An aliquot (50 µL) of 10% KCN was then added to tubes 2 (A_2) and 4. The absorbance of each tube was measured at 635 nm. Methemoglobin levels relative to hemoglobin levels were then calculated by the following equation:

$$\%Methemoglobin = \left(\frac{A_1 - A_2}{A_3 - A_4} \right) \times 100$$
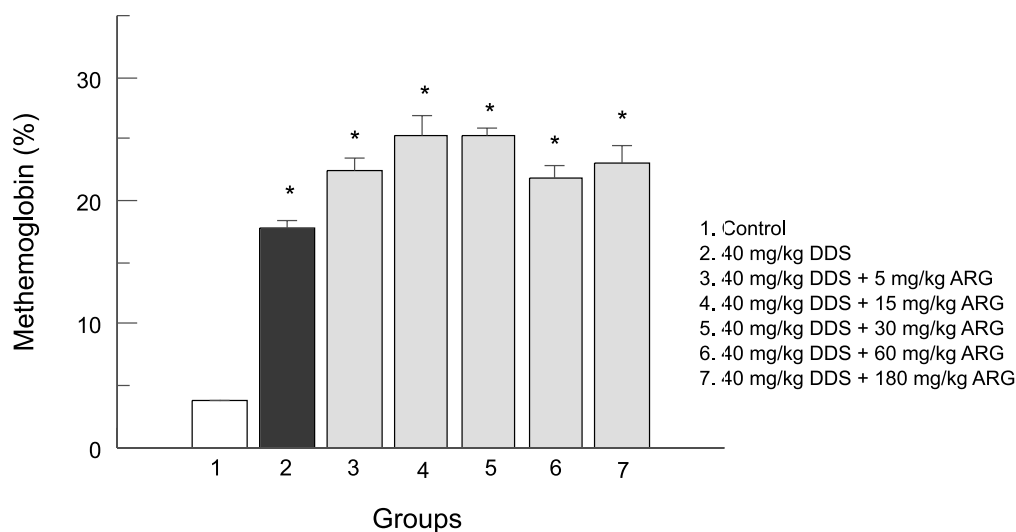
The initial blood measurement (A_1) is referent to MetHb and possible interferences. When blood is added to KCN (A_2), MetHb is converted to cyanomethemoglobin (CNMetHb) and then possible interferences are eliminated because CNMetHb does not absorb at 635 nm. When blood is added to $K_3Fe(CN)_6$ all hemoglobin is converted to MetHb, and this measurement refers to total MetHb (A_3). Finally, blood is added to $K_3Fe(CN)_6$ and KCN, with all hemoglobin converted to MetHb and then to CNMetHb (A_4).

Statistical analysis

GraphPad InStat® software (version 3.01) was used for the calculation of means ± standard deviation. ANOVA and the Tukey-Kramer post test for multiple comparisons ($p<0.05$) were used to compare groups.

RESULTS

As there is no reference value for methemoglobin levels in rats, some preliminary studies were conducted in order to evaluate whether ARG or the vehicles could produce methemoglobinemia. A single dose regimen control group was evaluated by administrating sterile physiological saline (p.o., 200 µL) and DMSO (i.p., 200 µL). The administration of these vehicles resulted in 3.77 ± 0.43% methemoglobin formation. In a pilot study, rats were treated with 5, 15, 30, 60 and 180 mg/kg of ARG (p.o., n = 8) by gavage. Methemoglobin levels were assayed in these groups, resulting in 1.70 ± 0.09%, 1.81 ± 0.08%,

FIGURE 1 - Effect of single dose ARG on methemoglobin levels (%). Animals were treated with ARG at 5, 15, 30, 60 and 180 mg/kg doses (p.o., gavage) 2 hours prior to the administration of 40 mg/kg of DDS (i.p.). Data expressed as means ± standard deviation. *p < 0.05 compared to control group.
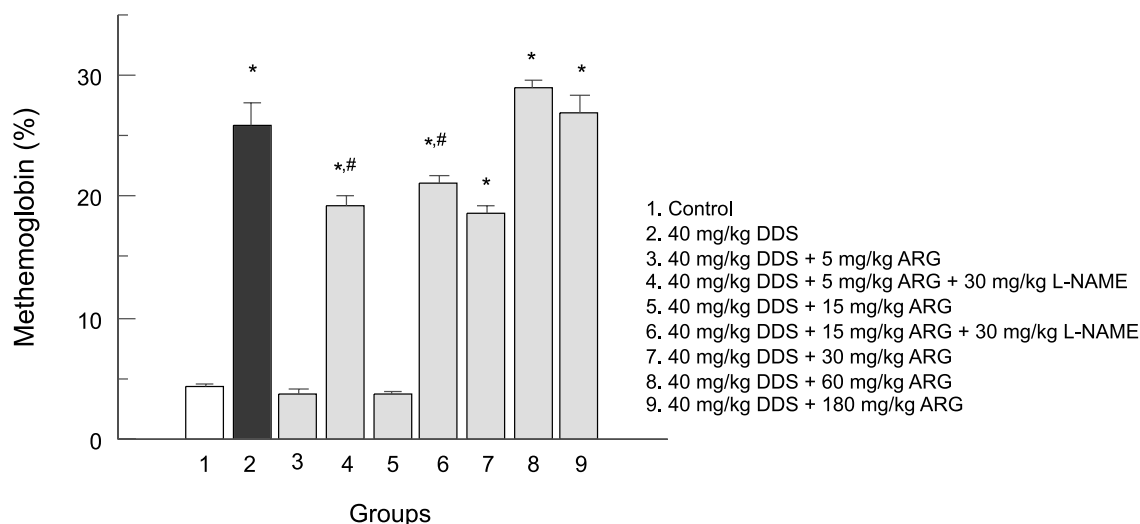
FIGURE 2 - Effect of multiple dose L-arginine (ARG) on methemoglobin level (%). Animals were treated with saline or ARG at 5, 15, 30, 60 and 180 mg/kg doses (p.o., gavage) or simultaneous gavage of ARG at 5 and 15 mg/kg doses and 30 mg/kg L-NAME for five days. On the fifth day, rats were treated with 40 mg/kg DDS (i.p.) 2 hours after ARG administration. Data expressed as means ± standard deviation. *$p < 0.001$ compared to the Control group; #$p<0.001$ when groups treated with L-NAME are compared to the respective group without L-NAME.

$1.59 \pm 0.05\%$, $1.96 \pm 0.08\%$ and $2.03 \pm 0.37\%$ of methemoglobin, respectively (data expressed as means ± standard deviation). These results showed that ARG alone did not produce methemoglobin.

Administration of 40 mg/kg DDS (i.p.) resulted in methemoglobin levels of $17.18 \pm 1.71\%$ (Figure 1). The dose of 40 mg/kg (i.p.) of DDS was known to produce methemoglobinemia in rats based on previous studies by our group (Malfara *et al.*, 2002; De Moraes *et al.*, 2008; Bergamaschi *et al.*, 2011). When ARG was administered in a single dose two hours prior to DDS (40 mg/kg) it failed to reduce DDS-induced methemoglobinemia (Figure 1).

ARG was also administered at doses 5, 15, 30, 60 and 180 mg/kg (po), in multiple dose regimens. Methemoglobin levels of groups treated with ARG only, in a multiple drug regimen, did not produce significant levels of methemoglobin ($1.88 \pm 0.68\%$; $2.00 \pm 0.13\%$; $2.56 \pm 0.44\%$; $2.43 \pm 0.43\%$ and $1.75 \pm 0.76\%$, respectively), as observed for a single dose regimen. Animals treated with 5 or 15 mg/kg ARG for 5 days prior to 40 mg/kg DDS administration showed a reduction in DDS-induced methemoglobinemia, with methemoglobin levels similar to the control group. However, higher doses of ARG (30, 60 and 180 mg/kg) in the multiple dose regimens did not inhibit methemoglobin formation (Figure 2).

The effect of L-NAME, a NOS inhibitor, was evaluated in order to understand the mechanisms related to the reduction of DDS-induced methemoglobinemia by ARG. L-NAME inhibited the reduction in DDS-induced methe-

moglobinemia promoted by ARG, leading to MetHb levels comparable to DDS administration alone (Figure 2).

DISCUSSION

The concentration of methemoglobin in erythrocytes is regulated by three systems: nicotinamide adenine dinucleotide (NADH), nicotinamide adenine dinucleotide phosphate (NADPH) and glutathione systems. Methemoglobin is converted to hemoglobin by the NADH system when sufficient NADH-methemoglobin reductase is available; it contributes to 95% of methemoglobin reduction to hemoglobin. The NADPH system reduces methemoglobin to hemoglobin through the enzyme NADPH-methemoglobin reductase and contributes to 5% of methemoglobin reduction. Finally, the conversion of reduced glutathione to glutathione influences methemoglobin levels by reducing oxidizing agents (Evelo *et al*., 1998; Ward, McCarthy, 1998; Umbreit, 2007).

The standard treatment for methemoglobinemia includes infusion with methylene blue, whose action depends on the availability of NADPH within the erythrocytes. This therapy requires glucose-6-phosphate dehydrogenase (G6PD) optimal activity to produce sufficient amounts of NADPH. In G6PD-deficient subjects, methylene blue therapy has been associated with hemolysis and methemoglobinemia (Rehman, 2001). Several other substances have been investigated as alternatives to methylene blue therapy. These have included ascorbic acid (Dötsch

et al., 1998), cimetidine (Coleman *et al.*, 1990; Malfará *et al.*, 2002), riboflavin (Dötsch *et al.*, 2000), α-lipoic acid (Coleman, Taylor, 2003), sodium thiosulfate (Matteucci *et al.*, 2003), ethyl pyruvate (Jo *et al.*, 2008) and *N*-acetylcysteine (Wright *et al.*, 1996; Wright *et al.*, 1998; Dötsch *et al.*, 2000; Tanen *et al.*, 2000; De Moraes *et al.*, 2008). *N*-acetylcysteine, a precursor of glutathione, used in combination with DDS in rats, has shown increased methemoglobin levels in these animals compared to rats treated with DDS alone. Some authors have suggested that glutathione can regenerate DDS-NOH from dapsone nitroso derivatives thus resulting in higher methemoglobin levels (De Moraes *et al.*, 2008).

Considering the potency of ARG and NO as antioxidant agents, it was proposed that the co-administration of ARG and DDS might reduce the methemoglobin levels associated to DDS use. The cationic amino acid ARG is the precursor for NO biosynthesis mediated by NO synthase. Three isoforms of NO synthase (NOS) occur in a number of tissues: neuronal NOS (nNOS); inducible NOS (iNOS) located in glia cells, and endothelial NOS (eNOS) located in endothelial cells (Palmer *et al.*, 1987; Thomas *et al.*, 2008). The iNOS can form much larger amounts of NO compared with other isoforms. In many cells and pathological conditions the supply of extracellular ARG is rate-limiting for NO production (Brunini *et al.*, 2007; Thomas *et al.*, 2008).

In the present study, L-NAME administration suppressed the reduction in DDS-induced methemoglobinemia mediated by 5 mg/kg and 15 mg/kg ARG in multiple dose regimens. Considering that L-NAME is a non-specific NOS inhibitor, our data suggest that the ARG effect on methemoglobin is mediated by NO. NO is considered a potent antioxidant agent *in vitro* and *in vivo*. Its antioxidant activity has been proven by suppressing iron-induced generation of hydroxyl radicals (OH) via the Fenton reaction, interrupting lipid peroxidation chain reaction, increasing the glutathione antioxidant potency and inhibiting cysteine proteases (Chiueh, 1999). On the other hand, increased methemoglobin levels are a known toxic effect of inhaled NO therapy, commonly used for hypoxic neonates. NO can combine with hemoglobin to produce nitrosylhemoglobin and thus form methemoglobin by oxidation (Weinberger *et al.*, 2001; Hamon *et al.*, 2010). Based on our observations, we can hypothesize that lower doses of ARG were beneficial to decrease methemoglobin levels because of the antioxidant properties of NO. However, higher doses of ARG do not decrease metHb levels because the antioxidant properties of NO are combined to its methemoglobinizant effect.

Excess ARG supplementation is also related with the production of N^G,N^G-dimethyl-L-arginine (ADMA) which is a NOS inhibitor (Masuda *et al.* 2002). This metabolite can convert NO to a superoxide generator (Thomas *et al.*, 2008). This may explain why NO is beneficial to DDS-induced methemoglobinemia at low ARG concentrations yet deleterious when excess ARG supplementation is administered to rats in multiple dose regimens.

NO also produces inhibitory effects in cytochrome P450 mediated drug metabolism. It is known that NO forms complexes with the catalytic center of P450 enzymes which results in a decrease in enzymatic activities of rat microsomes (Khatsenko *et al.* 1993). The inhibitory effects of NO are rapid, concentration-dependent and mainly in CYP 2C11 > 2B1/2 > 2E1 = 3A2 > 1A1/2 (Vuppugalla, Mehvar 2004a, 2004b). If NO had inhibited DDS oxidative metabolism, animals treated with higher ARG concentration would present lower metHb levels. However, our results showed that the reduction in MetHb levels by ARG is observed only when animals are treated with multiple doses of 5 and 15 mg/kg ARG. Thus, CYP inhibition by NO does not seem to explain the reduction in MetHb levels.

In conclusion, ARG reduces DDS-induced methemoglobinemia in rats when low doses (5 and 15 mg/kg) are administered as a multiple dose regimen. The effect can be blocked by the simultaneous administration of L-NAME (a NOS inhibitor). Thus, we can conclude that ARG supplementation can be an effective reducing agent for chronic treatment of DDS-induced methemoglobinemia and that its effect is mediated by NO.

ACKNOWLEDGMENTS

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