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Este exemplar corresponde à versão final da Dissertação de Mestrado apresentada ao Programa de Pós-Graduação de Farmacologia da Faculdade de Ciências Médicas da UNICAMP, para obtenção do título de Mestre em Farmacologia, do aluno ***Lucas Cézar Pinheiro.***

Campinas, 01 de Agosto de 2011.

Prof. Dr. Jose Eduardo Tanus dos Santos

- Orientador –

"OMEPRAZOL ATENUA OS EFEITOS ANTI-HIPERTENSIVOS DO NITRITO DE SÓDIO EM RATOS"

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LUCAS CÉZAR PINHEIRO

Dissertação de Mestrado apresentada à Pós-Graduação da Faculdade de Ciências Médicas da Universidade Estadual de Campinas para a obtenção do título de Mestre em Farmacologia. Sob orientação do Prof. Dr. José Eduardo Tanus dos Santos

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Nietzsche

SUMÁRIO

L-NAME: hidrocloreto de N_{ω}-Nitro-L-arginina metil Ester.

PAM: Pressão arterial média

NO: Oxido nítrico

NO_2^-: Anion nitrito

NO_3^-: Anion nitrato

NOS: Óxido nítrico sintase

NOx: nitrito + nitrato

RESUMO

Resumo

O óxido nítrico (NO) regula diversos sistemas orgânicos. Disfunções na produção ou disponibilidade de NO podem comprometer sua atuação fisiológica. Além da produção de NO pelas óxido nítrico sintetases, outras vias de produção de NO são relatadas, entre elas a conversão de nitrito a NO. O nitrito é o produto inicial da oxidação do NO, sendo posteriormente oxidado a nitrato. Sabe-se que estas três moléculas formam um ciclo dentro do organismo. A conversão de nitrito a NO pode ocorrer de forma enzimática ou não enzimática. Como forma não enzimática, o nitrito é convertido a NO pela reação com H^+. Esta reação ocorre principalmente no estômago, todavia não se sabe se este NO formado tem efeito na pressão arterial sistêmica ou atua apenas localmente. A fim de verificar a influência do pH gástrico no efeito hipotensor do nitrito de sódio, utilizamos animais tratados agudamente com L-NAME e normotensos canulados acordados pré-tratados com omeprazol e, posteriormente, com nitrito de sódio 15mg/kg e 45mg/kg. Foi verificado que o nitrito de sódio reduz a pressão arterial média dos animais significativamente e de maneira dependente da dose. O pré-tratamento com omeprazol reduziu o efeito hipotensor do nitrito de sódio significativamente. Após, foram quantificados os níveis de nitrito e nitrato. Foi observado aumento em ambos após o tratamento com nitrito de sódio. A partir destes resultados podemos sugerir que o omeprazol atenua o efeito hipotensor do nitrito de sódio em ratos normotensos e hipertensos.

Palavras-chave: Nitrito de sódio, Omeprazol, hipertensão

ABSTRACT

Abstract

Many body systems are regulated by nitric oxide (NO). Dysfunctions in the production or availability of NO may impair it physiological roles. However, other routes of NO production are reported in addition to NO production by nitric oxide synthases, including the conversion of nitrite to NO. Nitrite is the initial product of oxidation of NO and is further oxidized to nitrate. It is known that these three molecules form a cycle within the body. The conversion of nitrite to NO can occur enzymatic or nonenzymatic. Non-enzymatic nitrite is converted to NO by reacting with H+. This reaction occurs mainly in the stomach, however it is unclear whether this NO affects blood pressure or simply acts locally. To study the influence of gastric pH on the hypotensive effect of sodium nitrite, we used hypertensive or normotensive cannulated animals pretreated with omeprazole and with sodium nitrite 15 mg/kg and 45 mg/kg. We found that sodium nitrite reduces mean arterial pressure of animals in a dose-dependent manner. Pretreatment with omeprazole reduced the hypotensive effect of sodium nitrite significantly. Thereafter, we quantified the levels of nitrite and nitrate. We found increase in both species after treatment with sodium nitrite. These results suggest that omeprazole attenuates the hypotensive effect of sodium nitrite in normotensive and hypertensive rats.

Keywords: Sodium nitrite, omeprazole, Hypertension

1 – INTRODUÇÃO

1.1 Óxido nítrico

O óxido nítrico (NO) é responsável pela regulação de importantes sistemas orgânicos, como o sistema circulatório (Kanematsu *et al.*, 2008). Neste sistema o NO é um mediador intracelular fundamental para o controle do tônus vascular (Moncada *et al.*, 1991). O comprometimento da produção desta molécula pode levar a diversas doenças (Tsuchiya *et al.*, 2005). Classicamente, a produção de NO é feita pelas enzimas óxido nítrico sintetases, subdivididas em três tipos: endotelial (eNOS), induzível (iNOS) e neuronial (nNOS) (Andrew *et al.*, 1999). Um importante mecanismo de regulação da vasodilatação e, conseqüentemente, controle da pressão arterial é mediado pelo NO produzido principalmente via eNOS (Moncada *et al.*, 1991).

A síntese enzimática do óxido nítrico necessita de L-arginina, FAD, NADPH, BH_4 e oxigênio. A ausência de qualquer um destes cofatores diminui a eficiência da enzima em produzir NO (Moncada *et al.*, 1991). Todavia, fontes alternativas de formação de NO independentes das óxido nítrico sintetases são relatadas, como por exemplo, a conversão do anion nitrito (Li *et al.*, 2003; Lundberg *et al.*, 1994a)

1.2 Nitrito pode gerar NO por vias enzimáticas e não enzimáticas

A formação de óxido nítrico a partir do ânion nitrito tem sido objeto de dezenas de estudos. Até recentemente o ânion nitrito era considerado apenas um metabólito da degradação do NO (Lauer *et al.*, 2001). Estudos iniciais com íon nitrito associaram esta molécula com a formação de compostos nitrosilados, capazes de levar a alterações no DNA, bem como interagir com

dezenas de drogas e levar a metabólitos mutagênicos (Brambilla *et al.*, 2007). Todavia, apesar de ter sido demonstrado em experimentos *in vitro* e *in vivo* a correlação de nitrito com formação de compostos nitrosilados, os estudos epidemiológicos não demonstraram a correlação entre ingestão de nitrito e ocorrência de câncer (Brambilla *et al.*, 2007; Tang *et al.*, 2011). O nitrito de sódio também foi associado à formação de metemoglobina (Tang *et al.*, 2011). Atualmente pesquisas mostraram que a administração de baixas doses nitrito intravenoso não produz níveis considerados clinicamente tóxicos de metemoglobina, (Hunault *et al.*, 2009a) bem como demonstram os efeitos benéficos do nitrito de sódio sobre o sistema cardiovascular (Dias-Junior *et al.*, 2006; Gilchrist *et al.*, 2011; Tsuchiya *et al.*, 2005). Estes trabalhos mostram que o nitrito leva a vasodilatação em humanos quando adiministrada por via intravenosa (Dejam *et al.*, 2007), protege o coração e outros órgãos contra isquemia reperfusão em diversos modelos animais (Duranski *et al.*, 2005). Recentemente nosso grupo de pesquisa mostrou que o nitrito por via oral em ratos leva a queda de pressão arterial e diminui o stress oxidativo na hipertensão já instaurada (Montenegro *et al.*, 2011).

Sugere-se que os efeitos benéficos do nitrito ao sistema cardiovascular são devido à conversão de nitrito a NO nos diversos leitos vasculares (van Faassen *et al.*, 2009). No que diz respeito ao mecanismo de conversão a NO, vias enzimáticas e não enzimáticas são apontadas como responsáveis (van Faassen *et al.*, 2009). Como vias enzimáticas, são centrais neste processo a deoxi-hemoglobina e outras heme proteínas, a xantina óxido redutase e enzimas do citocromo C mitocondrial (van Faassen *et al.*, 2009). Estudos

recentes sugerem que em condições de hipóxia o ânion nitrito é convertido a óxido nítrico, provavelmente na tentativa do organismo manter a homeostase durante baixas tensões de oxigênio (Cosby *et al.*, 2003). Atribui-se a conversão de nitrito a NO a proteínas que contém o grupo heme, principalmente a deoxi-hemoglobina (Alzawahra *et al.*, 2008), sendo grupo heme desta enzima responsável por essa conversão (Lundberg *et al.*, 2009). Adicionalmente dezenas de trabalhos na literatura, tanto *in vitro* quanto *in vivo,* demonstram a importância da enzima xantina óxido redutase (XOR) na conversão do nitrito a NO sob diversas situações, principalmente em nível tecidual (Lundberg *et al.*, 2008; Zweier *et al.*, 2010). Inclusive, é conhecido que esta via é finamente regulada pelas tensões de oxigênio, bem como pelo pH local (Li *et al.*, 2004). Outros trabalhos também demonstram que o nitrito atua diretamente em enzimas da família do citocromo P450 mitocondrial, regulando a atividade desta (van Faassen *et al.*, 2009). Como conversão não enzimática, temos a via através da reação com H^+, que leva a formação de compostos intermediários que formam NO.

1.3 Nitrito proveniente da dieta e o ciclo NO-nitrito-nitrato no organismo.

A principal fonte de nitrito e nitrato é através da dieta. Os vegetais e alguns tipos de carne tratadas para conserva, tem altos níveis destas moléculas (Hord *et al.*, 2009). Este nitrito, quando ingerido, já pode ser convertido a NO no estômago e o aumento do pH gástrico impede esta conversão (Lundberg *et al.*, 1994b). Recentemente foi sugerido que o nitrito e nitrato presentes nos vegetais participem dos efeitos benéficos da dieta

vegetariana (Larsen *et al.*, 2011). Assim é sugerido que o nitrito e o nitrato provenientes da ingestão de vegetais tenham uma importante participação nos efeitos cardiovasculares benéficos relacionados à dieta vegetariana (Lundberg *et al.*, 2006).

O nitrito é o metabólito intermediário do NO, posteriormente o nitrito é oxidado e convertido a nitrato (Lundberg *et al.*, 2008). Comumente todas estas oxidações são realizadas pela oxi-hemoglobina. O NO junto com estes dois ânions formam um ciclo dentro do organismo. O nitrato sangüíneo é ativamente secretado pelas glândulas salivares para a boca, levando a saliva a ter concentrações 10-20 vezes maior que a plasmática de nitrato (van Faassen *et al.*, 2009). Neste local, o nitrato é convertido a nitrito por bactérias (Lundberg *et al.*, 2004). Este nitrito é deglutido, podendo ser convertido a NO no estômago, ou sendo absorvido na forma de nitrito, atuando fisiologicamente. Todavia o NO posteriormente é oxidado a nitrito e este é oxidado a nitrato, reiniciando o ciclo. Este ciclo também é suprido pelo NO produzido pelas óxido nítrico sintetases, que entra no ciclo quando são oxidados a nitrito. Partindo deste conhecimento, foi demonstrada a importância do nitrito salivar no fluxo sangüíneo estomacal, efeito bactericida e aumento do muco gástrico (Björne *et al.*, 2006). Inclusive, foi demonstrado que o efeito protetor estomacal do nitrato se deve a conversão de nitrato a nitrito na boca (Petersson *et al.*, 2009).

1.4 Conversão não enzimática do nitrito a NO.

Em meados da década de 90, trabalhos realizados por grupos de pesquisa independentes, verificaram a formação de NO *in vivo* por uma via não

enzimática (Benjamin *et al.*, 1994; Lundberg *et al.*, 1994a). Observou-se que a administração oral de nitrito e nitrato levava a síntese de NO gástrico. Sugeriu-se que o fato se dava através da reação do nitrito presente no estômago com os prótons H^+ provenientes do suco gástrico. Inclusive foi verificada a relação direta entre o valor do pH gástrico e a formação de NO a partir de nitrito (Lundberg *et al.*, 1994a; McKnight *et al.*, 1997a). Ao decorrer da década foi verificada a importância do NO gástrico formado a partir do nitrito. Este NO é fundamental para matar bactérias hostis ao organismo (Björne *et al.*, 2006) bem como fundamental para manter um fluxo sanguíneo adequado no estômago (Petersson *et al.*, 2009). Adiciona-se que o NO leva ao aumento da camada de muco que recobre o estômago, diminuindo assim os efeitos deletérios da acidez gástrica (Petersson *et al.*, 2009).

Foi comprovado por estudos *in vitro* que o nitrito é convertido a NO em meio ácido, com o pKa da reação em 3,4 (McKnight *et al.*, 1997b). Este estudo corrobora os dados obtidos anteriormente *in vivo*, de formação de NO.

Anteriormente já haviam sido demonstradas as propriedades vasodilatadoras do nitrito, todavia apenas a partir de 2005 trabalhos sugeriram os efeitos hipotensores sistêmicos, contra a hipertensão, do nitrito por via oral. Também foi verificado que o óxido nítrico oriundo da via gástrica é importante para o sistema circulatório em nível sistêmico (Tsuchiya *et al.*, 2005). O mesmo autor verifica a diminuição da pressão arterial de ratos tratados com L-NAME após a administração de nitrito de sódio e verifica que a nitrosil hemoglobina plasmática após o tratamento é proveniente, em parte, do nitrito administrado

oralmente e sugere também efeitos renais deste (Tsuchiya *et al.*, 2005; Tsuchiya *et al.*, 2010). Logo sugerindo fortemente que o nitrito por via oral atua sistemicamente e em órgãos distantes do local de administração. Apesar destes avanços, apenas recentemente nosso grupo de pesquisa demonstrou em modelo animal o efeito hipotensor do nitrito em uma hipertensão já instaurada(Montenegro *et al.*, 2011). Apesar dos sugeridos mecanismos de ação do nitrito, nenhum estudo até o momento verificou a influência destes no efeito hipotensor sistêmico em animais hipertensos.

O mecanismo de conversão por via ácida do nitrito a NO é observado principalmente no estomago. Além de o pH gástrico(Benjamin *et al.*, 1994; Lundberg *et al.*, 1994a) ter sido demonstrado como um fator muito influente na conversão do ânion nitrito a NO, a presença de antioxidantes no estômago, tais como flavonóides (Rocha *et al.*, 2009), ou ácido ascórbico, aumenta a capacidade de formação do NO a partir do nitrito em meio ácido(McColl, 2009). Desta forma, acreditamos que ao aumentar o pH gástrico, através da utilização de um inibidor de bomba de prótons, como o omeprazol(Kirchheiner *et al.*, 2009), poderia reduzir a conversão acima descrita. Adiciona-se que recentemente foi verificada a ausência de efeito do omeprazol sobre a pressão arterial de ratos(Yenisehirli *et al.*, 2008), o que o torna aplicável ao nosso estudo. Trabalhos iniciais demonstraram que o aumento do pH gástrico devido à administração de omeprazol leva à diminuição de formação de óxido nítrico via nitrito proveniente de vegetais(Lundberg *et al.*, 1994a). Outros trabalhos verificaram que os níveis de nitrito estomacal são aumentados quando ocorre o tratamento com omeprazol sob determinadas condições(Mowat *et al.*, 1999).

Todavia, até o momento nenhum trabalho abordou a influência do tratamento com omeprazol sobre os efeitos cardiovasculares do nitrito.

Dessa forma, este trabalho visa compreender o mecanismo hipotensor do nitrito de sódio e a influência da possível via ácida, descrita acima, sobre o efeito hipotensor do nitrito.

2 - OBJETIVOS

Hipótese:

1) Imaginamos que o omeprazol, ao aumentar o pH gástrico, diminua o efeito hipotensor do nitrito de sódio administrado por via oral em ratos tratados agudamente com L-NAME.

Objetivos:

1) Avaliar o efeito hipotensor do nitrito de sódio agudamente em ratos normotensos e tratados agudamente com L-NAME.
2) Verificar se o aumento do pH gástrico leva a diminuição do efeito hipotensor do nitrito de sódio.
3) Verificar se o omeprazol altera as concentrações plasmáticas de nitrito e de nitrato.

3 - CAPÍTULO

Increase of gastric pH reduces hypotensive effect of oral sodium nitrite

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Running title: Increase of gastric pH reduce hypotensive effects of sodium nitrite

Key words: Nitrite, omeprazole, hypertension, L-NAME.

Summary:

Background and purpose: The new pathway nitrate-nitrite-NO has emerged as a physiological alternative to the classical enzymatic pathway for NO formation from L-arginine. Nitrate is converted to nitrite by commensal bacteria in the oral cavity and the nitrite formed is then swallowed and reduced to NO in the acidic conditions of the stomach. In this study, we tested the hypothesis that increases in gastric pH caused by omeprazole could decrease the hypotensive effect of oral sodium nitrite.

Experimental approach: We assessed the effects of omeprazole treatment on the acute hypotensive effects produced by sodium nitrite in normotensive and L-NAME hypertensive free-moving rats. In addition, we assessed the changes in gastric pH and plasma levels of nitrite and NOx (nitrate + nitrite) caused by treatments.

Key results: We found that the increases in gastric pH induced by omeprazole significantly reduced the hypotensive effects of sodium nitrite in both, normotensive and L-NAME hypertensive rats. This effect was associated with significant increases in gastric washing pH and plasma nitrite and NOx levels.

Conclusion and implications: These results suggest that part of hypotensive effects of oral sodium nitrite may be due to its conversion to NO in the acidified environment of the stomach. The increases in gastric pH induced by treatment with omeprazole blunts part of the beneficial cardiovascular effects of dietary nitrate and nitrite.

Key words: Nitrite, omeprazole, hypertension, L-NAME.

INTRODUCTION

The classical biological pathway for nitric oxide (NO) production includes nitric oxide synthases (NOS), which produce NO from L-arginine, oxygen, and cofactors (Andrew & Mayer, 1999). Part of formed NO rapidly reacts with oxygen to form NO_2, which in solution forms the anions nitrite (NO_2^-) and nitrate (NO_3^-) (Moncada *et al.*, 1991). *In vivo*, NO_2^- reacts which oxyhemoglobin within erythrocytes to form NO_3^- (Dejam *et al.*, 2004). Interestingly, a balance between these NO-related species explains the endogenous formation of NO_2^-/NO_3^- and these markers of NO formation have been used in clinical (Metzger *et al.*, 2006) and experimental studies to reflect endogenous NO (Kleinbongard *et al.*, 2006; Kleinbongard *et al.*, 2003; Lundberg, 2006). However, more recent research has shown that NO_2^- is more than a simple marker of NO formation and can be recycled back to NO as a physiological alternative to NO formation independently of NOS-related pathways (Gladwin *et al.*, 2005; Lundberg *et al.*, 2009; Lundberg *et al.*, 2008).

Several different pathways have been suggested to form NO from NO_2^-. These include enzymatic and nonenzymatic pathways including interactions with deoxyhemoglobin (Cosby *et al.*, 2003), myoglobin (Rassaf *et al.*, 2007; Shiva *et al.*, 2007), xanthine oxidase (Tripatara *et al.*, 2007; Webb *et al.*, 2004), mitochondrial cytochromes (Kozlov *et al.*, 1999) and particular conditions such as low pH, acidic conditions of the stomach (Benjamin *et al.*, 1994; McKnight *et al.*, 1997). In this respect, the first study showing nonenzymatic NO production was reported by Lundberg *et al.*, in 1994 (Lundberg *et al.*, 1994). In a very elegant study, they showed that intragastric NO production in humans was increased after ingestion of lettuce, which is an important source of nitrate/nitrite, and that NO generation was due the low pH of stomach (Lundberg *et al.*, 1994). In the same year of 1994, Benjamin *et al.* (Benjamin *et al.*, 1994) also proposed that NO formation in the stomach was an important defense mechanism against swallowed pathogenic microorganisms (Benjamin *et al.*, 1994). Although many enzymatic and nonenzymatic pathways have been proposed to explain NO production from NO_2^-, the relevance of each pathway to the hypotensive effects of sodium nitrite is still poorly understood (Montenegro *et al.*, 2011). In the present study, we evaluated the relevance of low gastric pH to the hypotensive effects associated with sodium nitrite. We aimed at evaluating whether pretreating animals with omeprazole, a proton pump inhibitor that increases gastric pH, could modify the hypotensive effects associated with sodium nitrite. We hypothesized that omeprazole could attenuate the antihypertensive effects exerted by sodium nitrite given orally, but not intravenously.

METHODS

Animals and treatments

This study was approved by the Institutional Animal Care and Use Committee of the Faculty of Medicine of Ribeirao Preto, University of Sao Paulo, and the animals were handled according to the guiding principles published by the National Institutes of Health. Male Wistar

rats (180-200 g) obtained from the colony at University of São Paulo (Ribeirao Preto Campus, Brazil) were maintained on a 12-h light/dark cycle at a room temperature (22-25°C) with free access to standard rat chow and water.

Assessment of mean arterial pressure (MAP) in unanesthetized free moving rats

One day before the experiments, the animals were anaesthetized with tribromoethanol (250mg/kg, i.p.) and the femoral artery was cannulated (2 cm segment of a PE-10 tube connected to 14 cm of a PE-50 tubing; Clay Adams, Parsippany, NJ, USA). The catheter was then tunnelled subcutaneously and exteriorized through the back of the neck. In some experiments, the femoral vein also was cannulated following the same procedures for drug infusions. After surgery, the nonsteroidal anti-inflammatory flunixine meglumine (2.5 mg/kg, s.c., Banamine®, Schering Plough, Brazil) was administered for post-operation analgesia. Following 12 hours of fasting, the arterial cannula was connected to a pressure transducer and the MAP in freely moving rats was recorded using a data acquisition system (MP150CE; Biopac Systems Inc. CA, USA) connected to a computer (Acknowledge 3.2, for Windows). When collecting data, we allowed at least 15 min of stabilization before or after each oral gavage.

Experimental protocols

This study was divided in 3 experimental protocols. In the protocol 1, we examined whether pretreatment with omeprazole modifies the acute effects of oral sodium nitrite on the MAP of normotensive rats. Therefore, after 12 hours of fasting, the rats received a single dose of vehicle (1 ml/kg of 2% Tween 80) or omeprazole (a proton pump inhibitor; 30 mg/Kg) by gavage. Two hours later, the rats received a single gavage of saline or sodium nitrite (15 or 45 mg/Kg) and the changes in MAP were evaluated. The nitrite doses used in the present study were chosen with basis on a previous study showing that similar doses lowered the systolic blood pressure by approximately 40 mmHg in 2 two-kidney one-clip hypertensive rats (Montenegro *et al.*, 2011). In addition, the dose of omeprazole used in the present study increased gastric pH to 5-6 (Campbell *et al.*, 2008).

In the protocol 2, we examined whether pretreatment with omeprazole modifies the acute effects of oral sodium nitrite on the MAP of rats with L-NAME-induced hypertension. Therefore, one hour after vehicle or omeprazole treatment (as described for protocol 1), the rats received a single gavage of N_{ω}-Nitro-L-arginine methyl ester hydrochloride (L-NAME; 100 mg/Kg) to increase the MAP. This dose of L-NAME was chosen with basis on pilot studies showing that it increases MAP by approximately 40 mmHg. One hour after L-NAME treatment, the rats were treated with saline or sodium nitrite (15 or 45 mg/Kg, by gavage) and the changes in MAP were evaluated.

In the protocol 3, we examined whether the effects of omeprazol on sodium nitrite-induced MAP reduction would be attenuated when sodium-nitrite is administered intravenously instead of being administered orally (as in protocol 2). Therefore, we compared the hypotensive effects of sodium nitrite (15 mg/Kg) administered intravenously to rats pretreated with omeprazol (or vehicle) and L-NAME exactly as described above for the

protocol 2. In the three protocols, the changes in MAP after vehicle or sodium nitrite treatments were assessed for 30 min.

At the end of experiments, arterial blood samples collected into tubes containing heparin and immediately centrifuged at 1000 x g for 3 min. Plasma aliquots were stored at − 70°C until analyzed for plasma nitrite and NOx levels determination. In addition, the gastric washing pH was measured as described below.

Measurement of gastric washing pH

The abdominal cavity was opened and the pyloric portion of the stomach was ligated. The lower portion of the esophagus was opened and a catheter was introduced with its tip placed into the stomach. A saline solution (1 ml, pH 4) was injected into the stomach via this catheter and the gastric washing (0.5 ml) was drawn, as previously described (Campbell *et al.*, 2008; Petersson *et al.*, 2007). The gastric washing samples were centrifuged at 6000 x g for 1 min to clean food debris excess and the pH of supernatant was assessed using a pH microelectrode coupled to *bench pHmeter* (Jenway, mod. 3510, *Stone, Staffordshire*, UK). To exclude the possibility of alteration of gastric pH due to blood collection, some experiments were performed without blood collection and the gastric washing pH was assessed following the same procedures. We found no significant differences on gastric washing pH between both approaches (data not shown).

Measurement of plasma nitrite concentrations

Plasma aliquots were analyzed in duplicate for their nitrite content using an ozone-based reductive chemiluminescence assay as previously described (Feelisch *et al.*, 2002; Montenegro *et al.*, 2010). Briefly, to measure nitrite concentrations in plasma, 300 µl of plasma samples were injected into a solution of acidified tri-iodide, purging with nitrogen in-line with a gas-phase chemiluminescence NO analyzer (Sievers Model 280 NO analyzer, Boulder, CO, USA). Approximately 8 ml of tri-iodide solution (2 g potassium iodide and 1.3 g iodine dissolved in 40 ml water with 140 ml acetic acid) was placed in the purge vessel into which plasma samples were injected. The tri-iodide solution reduces nitrites to NO gas, which is detected by the NO analyzer. The data were analyzed using the software Origin Lab 6.1.

Measurement of plasma NOx (nitrate + nitrite) concentrations

The plasma NOx concentrations were determined in duplicate by using the Griess reaction as described previously (Montenegro *et al.*, 2009). Briefly, 40 µl of plasma were incubated with the same volume of nitrate reductase buffer (0.1 M potassium phosphate, pH 7.5, containing 1 mM beta nicotinamide adenine dinucleotide phosphate, and 2 units of nitrate reductase/ml) in individual wells of a 96-well plate. Samples were allowed to incubate overnight at 37ºC in the dark. Eighty microliters of freshly prepared Griess reagent (1% sulphanilamide, 0.1% naphthylethylenediamine dihydrochloride in 5% phosphoric acid) were added to each well and the plate was incubated for an additional 15 min at room temperature.

A standard nitrate curve was obtained by incubating sodium nitrate (0.2–200 μM) with the same reductase buffer.

Drugs and solutions

All drugs and reagents were purchased from Sigma Chemical Co. (St Louis, MO, USA) and all solutions were prepared immediately before use.

Statistical analysis

The results are expressed as means ± S.E.M. The comparisons between groups were assessed by two way analysis of variance. The effects of nitrite infusions on MAP in each group were assessed by paired t test. A probability value <0.05 was considered significant.

RESULTS

Omeprazole reduces the acute hypotensive effect of sodium nitrite administered orally

In the normotensive rats, sodium nitrite at dose of 15 mg/Kg by gavage induced no significant change in MAP either in rats pretreated with vehicle or with omeprazole ($P>0.05$; Fig. 1, panels A and B). Conversely, sodium nitrite at dose of 45 mg/Kg by gavage significantly decreased the MAP by approximately 8 mmHg and pretreatment with omeprazole almost completely blunted this effect ($P<0.05$; Fig. 1, panels A and B). After obtaining these interesting results, we decided to investigate whether pretreatment with omeprazol would result in similar effects in hypertensive rats. Therefore we used L-NAME to induce hypertension in rats and repeated the same experiments using hypertensive rats. Interestingly, sodium nitrite used at 15 mg/kg or at 45 mg/kg by gavage exerted significant antihypertensive effects ($P<0.05$; Fig. 2, panels A and B), and pretreatment with omeprazole significantly attenuated these antihypertensive ffects ($P<0.05$, Fig. 2, panels A and B). Importantly, pretreatment with omeprazole itself induced no significant changes in MAP, either in normotensive rats ($P>0.05$; Fig. 1, panel A) or in L-NAME-treated hypertensive rats ($P>0.05$; Fig. 2, panel A).

Effects of treatments on gastric pH

To evaluate the effects of different treatments on gastric pH and to confirm that omeprazole really increased gastric pH, we measured gastric washing pH. While a single oral dose of omeprazole significantly increased the gastric washing pH in both normotensive and in L-NAME hypertensive rats (both $P<0.05$; Fig. 3), treatment with L-NAME induced no significant changes in gastric washing pH, as suggested by very similar gastric washing pH in normotensive and in L-NAME hypertensive rats ($P>0.05$, Fig. 3).

Effects of omeprazole on plasma nitrite and NOx levels

Because differences in the responses to sodium nitrite between rats pretreated with vehicle *versus* omeprazole could be due to differences in the circulating levels of nitrites, we

assessed the plasma levels of nitrite and NOx in the present study. As expected, higher plasma nitrite and NOx levels were found in rats treated with sodium nitrite (both in normotensive and L-NAME hypertensive) as compared to control rats (both $P<0.05$; Figs. 4 and 5, respectively). Interestingly, pretreatment with omeprazole had no effects on plasma nitrite and NOx levels, as revealed by similar nitrite and NOx levels in both vehicle and omeprazol pretreated rats ($P>0.05$; Fig. 4 and Fig. 5, respectively). However, lower increases in plasma nitrite levels were found in L-NAME hypertensive rats than in normotensive rats treated with sodium nitrite at 45 mg/Kg ($P<0.05$; Fig. 4), but not at 15 mg/kg.

Omeprazole does not modify the acute hypotensive effect of intravenously sodium nitrite

Given that omeprazole attenuated the hypotensive effect of sodium nitrite administered orally, we examined whether this attenuation would also occur when sodium-nitrite is administered intravenously instead of orally. Therefore, we repeated the same initial experiments with an important modification: sodium nitrite was injected intravenously. Curiously, the intravenous injection of sodium nitrite at 15 mg/kg induced very similar hypotensive effects in L-NAME hypertensive rats pretreated with vehicle and in rats pretreated with omeprazole ($P>0.05$; Fig. 6).

DISCUSSION

The main novel findings of this study were: (i) Oral sodium nitrite exerts rapid hypotensive effects both in normotensive and in hypertensive rats; (ii) Pretreatment *with* omeprazole attenuated the hypotensive effects of oral sodium nitrite, but not the hypotensive effects of sodium nitrite administered intravenously; (iii) The attenuation of the hypotensive effects exerted by sodium nitrite associated with omeprazole pretreatment is probably not due to differences in the increases in plasma nitrite or NOx levels achieved after sodium nitrite administration.

Several studies have reported vascular effects exerted by sodium nitrite, which are ascribed to its bioconversion to NO (Carlström *et al.*, 2011; Gladwin *et al.*, 2005; Lundberg *et al.*, 2009). However, the mechanisms involved in this bioconversion are not fully understood. While there is increasing evidence suggesting that several enzymes can convert nitrite to NO (Lundberg & Weitzberg, 2008), the excess of protons in the gastric juice has been shown as an important player in the conversion of nitrite to NO by nonenzymatic reduction (Lundberg *et al.*, 1994; McKnight *et al.*, 1997). Our findings further support this idea and show that the increases in gastric pH induced by omeprazole impair the antihypertensive effects of sodium nitrite, thus suggesting that a low stomach pH is very important for the beneficial vascular effects of sodium nitrite. Our findings may have important clinical implications, especially for patients taking omeprazole or other proton pump inhibitors for any medical reason.

The first report showing intragastric NO formation from nitrite associated this finding to host defense against pathogens swallowed with saliva and with a protective effect to the

integrity of the gastric mucosa (Benjamin *et al.*, 1994; Lundberg *et al.*, 1994). Our findings expand these previous observations and are consistent with the suggestion that intragastric NO formation at the low pH of the stomach may be important for the antihypertensive effects of sodium nitrite. Moreover, although we have not tested this hypothesis in the present study, it is possible that omeprazole may also decrease the lowering effects on blood pressure that have been shown in humans after a nitrate rich meal (Webb *et al.*, 2004), or with the used of oral inorganic nitrates (Kapil *et al.*, 2010).

We and others have shown the nitrite exerts antihypertensive effects in experimental models of hypertension (Beier *et al.*, 1995; Haas *et al.*, 1999; Kanematsu *et al.*, 2008; Montenegro *et al.*, 2011; Tsuchiya *et al.*, 2005). In this regard, it is reasonable to think that part of orally administered nitrite is reduced to NO in the stomach and part diffuses into the portal circulation, thus increasing the plasma nitrite levels (Webb *et al.*, 2008). Within the circulation, nitrite may be converted back to NO in the vascular resistance vessels and cause vasodilatation, as previously suggested (Webb *et al.*, 2008). Although unproven (because we have not directly measured intragastric NO formation in the present study), our results showing that increased gastric pH attenuates sodium nitrite-induced hypotension is consistent with the idea that orally administered sodium nitrite promotes intragastric NO formation, which results in hypotensive effects, which are attenuated when the gastric pH increases. Further supporting this suggestion, we found very similar hypotensive effects when sodium nitrite was administered intravenously to rats pretreated with vehicle or with omeprazole, thus showing no effects of omeprazole when nitrite is administered intravenously. Moreover, we found similar increases in plasma nitrite levels after sodium nitrite was administered orally to rats previously treated with omeprazole or vehicle, thus indicating that omeprazole had no effects on the increases in circulating nitrite levels after sodium nitrite administration. In other words, omeprazole had no effects on the circulating nitrite concentrations available to promote hypotensive effects. Together, these results clearly show that omeprazole modifies the hypotensive of sodium nitrite administered orally, but not intravenously, and strongly suggest that the pH of the stomach is relevant to the hypotensive effect exerted by sodium nitrite, and that omeprazole possibly blunts intragastric NO formation.

Interestingly, although omeprazole decreased the hypotensive effects of oral sodium nitrite, this proton pump inhibitor did not completely abolish the hypotensive effects, thus suggesting that other mechanisms are involved in nitrite-induced hypotension. While the administration of sodium nitrite increased plasma nitrites levels, most of it may have been rapidly converted to nitrate, as suggested by the significant increases in plasma NOx levels (which reflect mainly nitrates content). Indeed, while the low dose of sodium nitrite could result in increases in plasma nitrite levels to 100 µmol/l or higher levels, the plasma nitrite levels remained below 10 µmol/l. Similar results were found with the higher sodium nitrite dose. These results suggested that nitrites may be actively converted to nitrates, so that using much higher doses of sodium nitrite may be ineffective. Conversely, some studies reported showed that acute administration of nitrates results in increased plasma nitrites levels

(Carlstrom *et al.*, 2010; Kapil *et al.*, 2010). We believe that these issues deserve much more detailed studies.

Another interestingly observation is that we found lower increases in plasma nitrites levels in L-NAME hypertensive rats than those found in normotensive rats after treatment with oral sodium nitrite at 45 mg/kg, and this difference tended to be paralleled by NOx levels. The reason for this difference is not clear. Although it is possible that L-NAME may have reduced gastric blood flow, and therefore decreased gastric sodium nitrite absorption, other mechanisms may be involved.

In conclusion, our findings are consistent with the idea that part of the hypotensive effects of sodium nitrite given orally is due its conversion to NO in the acidified environment of the stomach. The increases in gastric pH induced by omeprazole clearly impair the hypotensive effects of sodium nitrite and this experimental finding may have important clinical implications, especially in patients taking proton pump inhibitors because these drugs may decrease the beneficial effects of dietary nitrites and nitrates.

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Legends for figures:

Fig. 1. Mean arterial pressure (MAP) in *unanesthetized free moving rats* that received a single gavage of saline or sodium nitrite (15 or 45 mg/kg) 2 hours after receiving vehicle or

omeprazole (30mg/Kg) by gavage. Panel A shows the MAP after each treatment and panel B shows the change elicited by each dose of nitrite.

Data are shown as mean ± S.E.M. (n = 8-12 per group).

*P<0.05

Fig. 2. Mean arterial pressure (MAP) in *unanesthetized free moving rats* that received a single gavage of saline or sodium nitrite (15 or 45 mg/kg) 2 hours after receiving vehicle or omeprazole (30mg/Kg) by gavage followed by L-NAME (100 mg/Kg). Panel A shows the MAP after each treatment and panel B shows the change elicited by each dose of nitrite.

Data are shown as mean ± S.E.M. (n = 8-12 per group).

*P<0.05

Fig. 3. Gastric washing pH in all experimental groups. After recording the hemodynamic data, the gastric washing pH was assessed using a *pH microelectrode.*

Data are shown as mean ± S.E.M. (n = 4-8 per group).

*P<0.05

Fig. 4. Plasma nitrite concentrations (µmol/l) in rats that received a single gavage of saline (control) or sodium nitrite (15 or 45 mg/kg) 2 hours after receiving vehicle or omeprazole (30mg/Kg) by gavage followed by L-NAME (100 mg/Kg) or vehicle.

Data are shown as mean ± S.E.M. (n = 6–8 per group).

*P<0.05 *versus* the control group.

#P<0.05 *versus* Nitrite 45 mg/Kg vehicle group.

Fig. 5. Plasma NOx (nitrate + nitrite; µmol/l) concentrations in rats that received a single gavage of saline (control) or sodium nitrite (15 or 45 mg/kg) 2 hours after receiving vehicle or omeprazole (30mg/Kg) by gavage followed by L-NAME (100 mg/Kg) or vehicle.

Data are shown as mean ± S.E.M. (n = 6–8 per group).

*P<0.05 *versus* the control group.

Fig. 6. Mean arterial pressure (MAP) in *unanesthetized free moving rats* that received a single intravenous injection of saline or sodium nitrite (15 mg/kg) 2 hours after receiving vehicle or omeprazole (30mg/Kg) by gavage followed by L-NAME (100 mg/Kg). Panel A shows the MAP after each treatment and panel B shows the change elicited by each dose of nitrite.

Data are shown as mean ± S.E.M. (n = 4 per group).

*P<0.05

N.S. = not significant

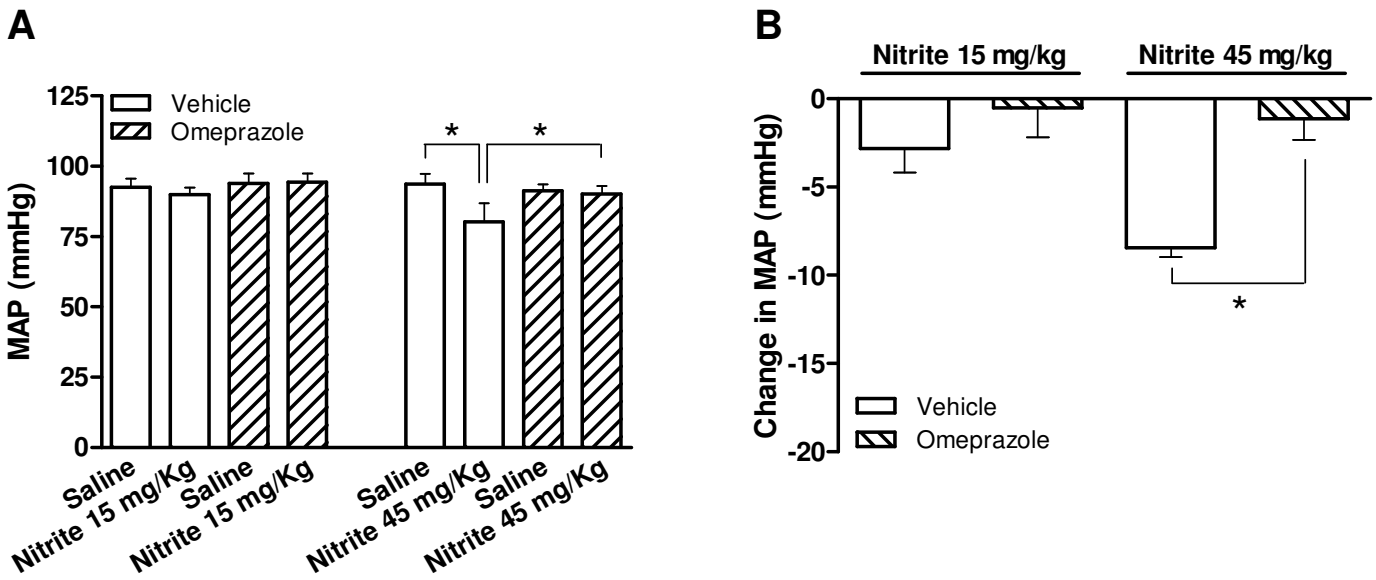
A
MAP (mmHg)
125
100
75
50
25
0
Vehicle
Omeprazole
*
*
Saline
Nitrite 15 mg/Kg
Saline
Nitrite 15 mg/Kg
Saline
Nitrite 45 mg/Kg
Saline
Nitrite 45 mg/Kg
B
Nitrite 15 mg/kg
Nitrite 45 mg/kg
Change in MAP (mmHg)
0
-5
-10
-15
-20
*
Vehicle
Omeprazole

Figure 1

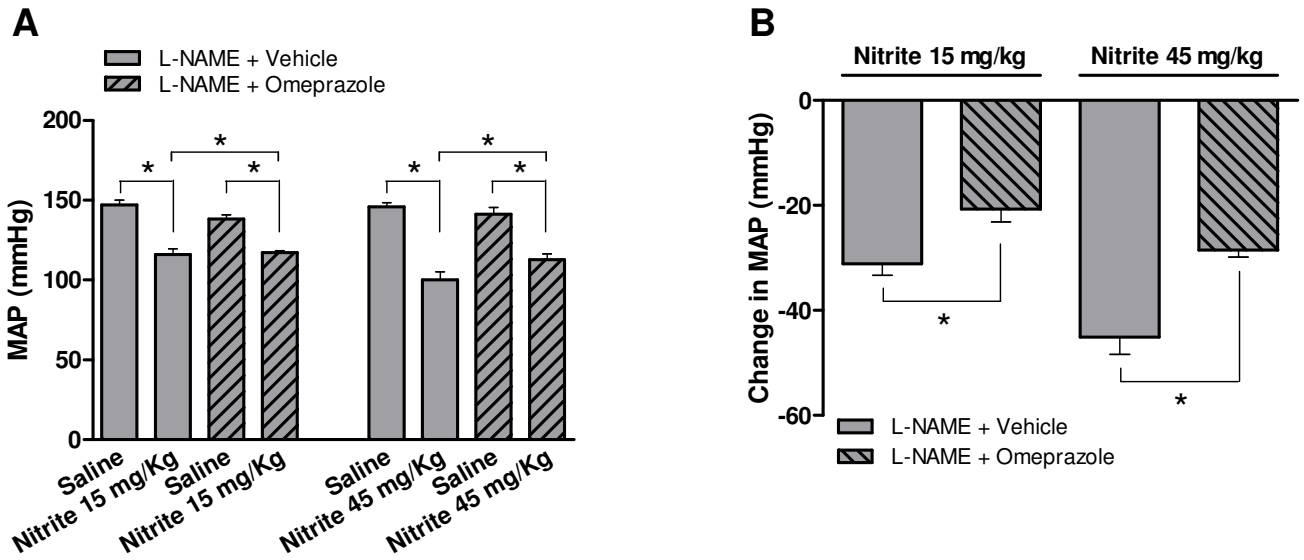
A
L-NAME + Vehicle
L-NAME + Omeprazole
MAP (mmHg)
200
150
100
50
0
*
Saline
Nitrite 15 mg/Kg
Saline
Nitrite 15 mg/Kg
Saline
Nitrite 45 mg/Kg
Saline
Nitrite 45 mg/Kg
B
Nitrite 15 mg/kg
Nitrite 45 mg/kg
Change in MAP (mmHg)
0
-20
-40
-60
L-NAME + Vehicle
L-NAME + Omeprazole

Figure 2

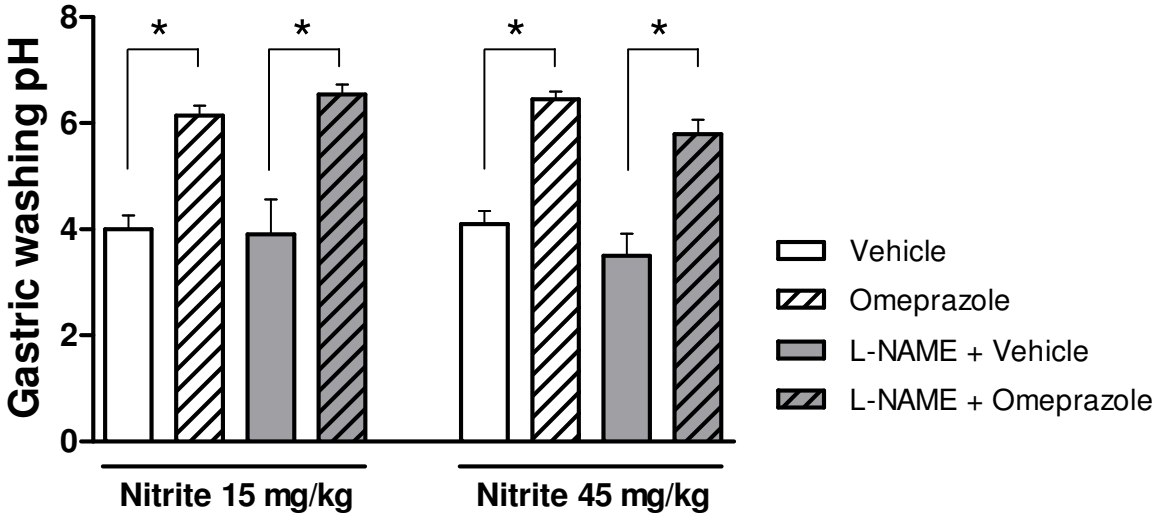
Gastric washing pH
8
6
4
2
0
*
*
*
*
Nitrite 15 mg/kg
Nitrite 45 mg/kg
Vehicle
Omeprazole
L-NAME + Vehicle
L-NAME + Omeprazole

Figure 3

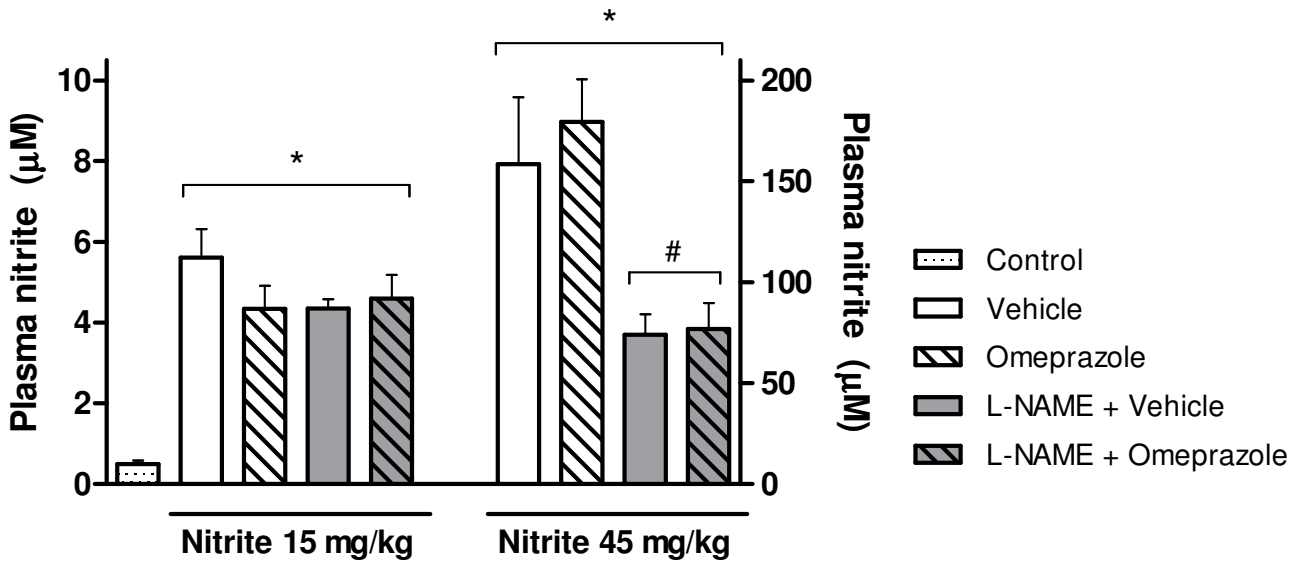
Plasma nitrite (µM)
10
8
6
4
2
0
200
150
100
50
0
Plasma nitrite (µM)
*
*
#
Nitrite 15 mg/kg
Nitrite 45 mg/kg
Control
Vehicle
Omeprazole
L-NAME + Vehicle
L-NAME + Omeprazole

Figure 4

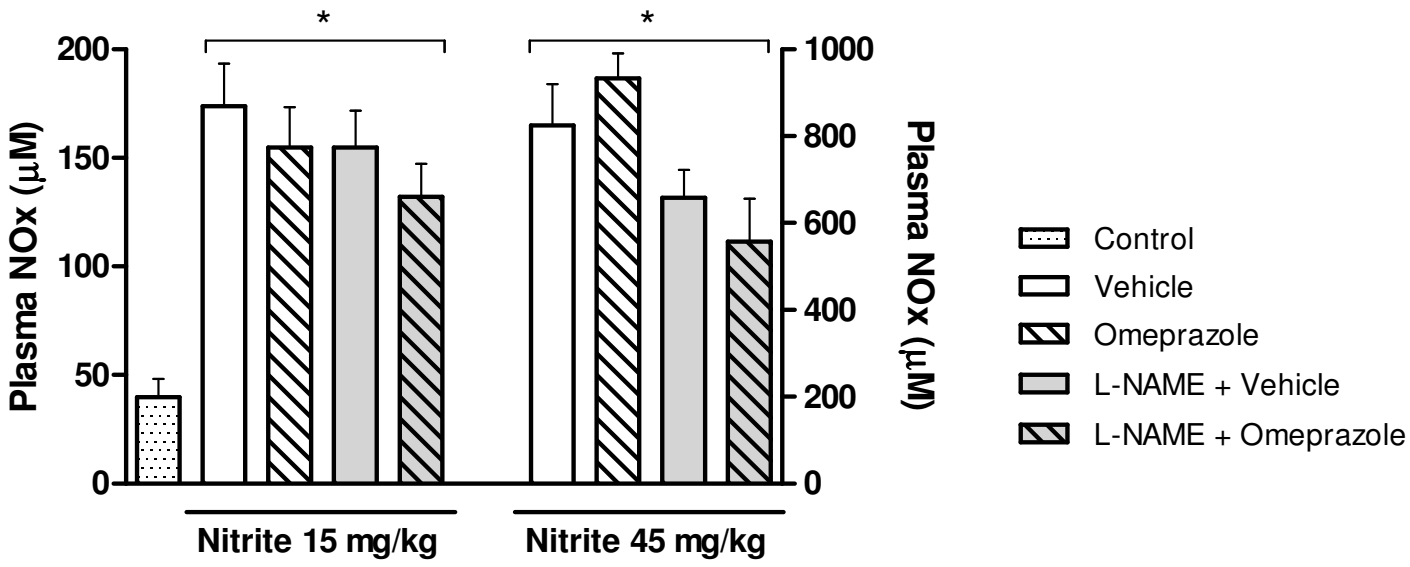
*
*
200
150
100
50
0
1000
800
600
400
200
0
Plasma NOx (μM)
Plasma NOx (μM)
Nitrite 15 mg/kg
Nitrite 45 mg/kg
Control
Vehicle
Omeprazole
L-NAME + Vehicle
L-NAME + Omeprazole

Figure 5

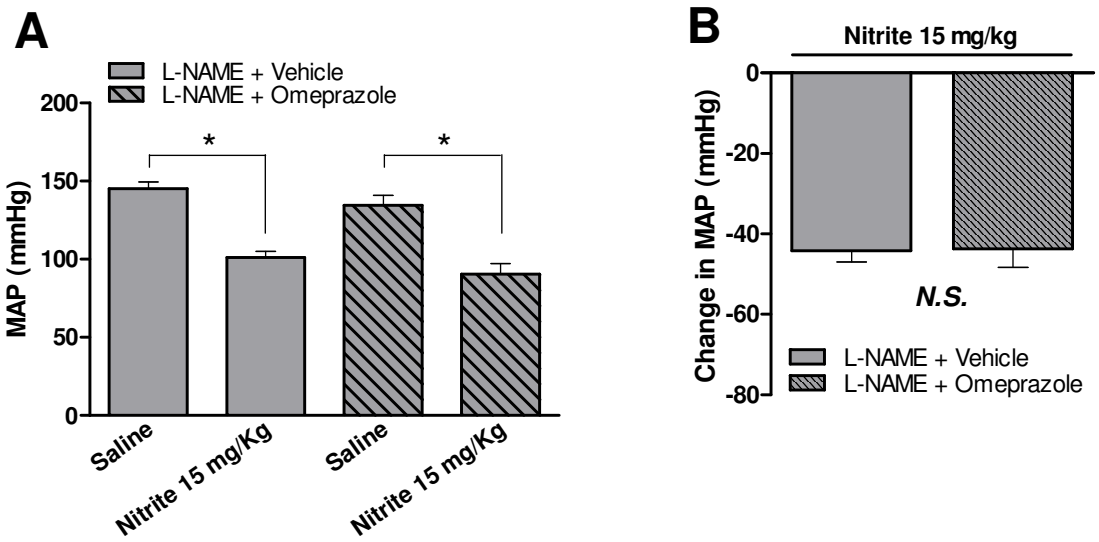
A
L-NAME + Vehicle
L-NAME + Omeprazole
MAP (mmHg)
200
150
100
50
0
*
*
Saline
Nitrite 15 mg/Kg
Saline
Nitrite 15 mg/Kg
B
Nitrite 15 mg/kg
Change in MAP (mmHg)
0
-20
-40
-60
-80
N.S.
L-NAME + Vehicle
L-NAME + Omeprazole

Figure 6

4 – DISCUSSÃO

De forma geral, nossos resultados permitem observar que o nitrito de sódio causa efeito hipotensor rápido em ratos normotensos e tratados agudamente com L-NAME. Adicionalmente, os animais pré-tratados com omeprazol, quando tratados com nitrito de sódio tiveram uma queda menor na pressão arterial média. Finalmente, os efeitos hipotensores são significativamente correlacionados com o aumento observado do pH. Também foi verificado que os níveis de nitrito e NO_x plasmático aumentam rapidamente após a administração de nitrito de sódio.

Diversos estudos demonstram os efeitos vasculares do nitrito de sódio e sugerem a bioconversão deste a óxido nítrico(Carlström *et al.*, 2011; Gladwin *et al.*, 2005; Lundberg *et al.*, 2009). Todavia os mecanismos que regem a conversão de nitrito a NO, apesar de estudados, não estão ainda completamente esclarecidos. Adicionalmente, ainda não é clara a influência das vias de conversão de nitrito a NO *in* vivo. Neste estudo observamos que a utilização de omeprazol, com conseqüente aumento do pH gástrico, reduz significativamente o efeito hipotensor do nitrito de sódio em ratos normotensos e agudamente tratados com L-NAME. Assim sugerimos que o pH gástrico contribui de forma relevante para o efeito hipotensivo sistêmico do nitrito por via oral.

O nitrito utilizado por via oral, proveniente de alimentos, saliva ou administrado como droga possui efeitos já conhecidos em outros aspectos sobre o estômago. Verifica-se na literatura que a formação de NO a partir do nitrito proveniente da saliva é associada à defesa contra patógenos e proteção

da mucosa gástrica (Benjamin *et al.*, 1994; Lundberg *et al.*, 1994a; Petersson *et al.*, 2009). Todavia, nossos dados sugerem que o nitrito por via oral tem efeitos hipotensores, assim podemos levantar a questão de que o nitrito deglutido, além da proteção gástrica, poderia ter um efeito benéfico ao sistema cardiovascular.

Acrescenta-se a tal sugestão que alimentos ricos em nitrito e nitrato são capazes de diminuir a pressão arterial em humanos (Webb *et al.*, 2008). Também já foi verificado que o nitrato inorgânico é hipotensor por via oral em ratos(Carlström *et al.*, 2011). Provavelmente, parte deste efeito é devida à formação de nitrito por bactérias bucais ao metabolizarem nitrato (Petersson *et al.*, 2009). Diversos trabalhos recentes demonstram que o nitrito tem efeito hipotensor por via oral ou endovenosa em diferentes modelos de hipertensão(Montenegro *et al.*, 2011; van Faassen *et al.*, 2009). Desta forma, considerando o nitrito como uma droga, é possível que este seja convertido a NO no estômago ou absorvido, aumentando os níveis plasmáticos de nitrito(Webb *et al.*, 2008). Na circulação, o nitrito é convertido a NO em diversos leitos vasculares e exerce seu efeito vasodilatador. Logo, nossos dados mostram que a conversão a NO, devido ao baixo pH gástrico, contribui para estes efeitos relatados acima do nitrito e nitrato de sódio, e o omeprazol diminui o efeito hipotensor. Atenta-se que, nos animais pré-tratados com omeprazol, ocorreu importante efeito hipotensor do nitrito de sódio. Este resultado sugere que a conversão ácida é apenas uma parte do efeito hipotensor, e que a maioria efeito é independente da acidez gástrica.

A fim de verificar uma possível interação entre omeprazol e nitrito, injetamos nitrito intravenoso em animais pré-tratados com omeprazol. Não foi encontrada qualquer diferença. Isto sugere que o omeprazol não altera significativamente os efeitos hipotensores do nitrito.

Verificou-se que a administração de nitrito levou a um rápido e significativo aumento dos níveis plasmáticos de nitrito. Todavia a maior parte deste foi convertido a nitrato em um curto espaço de tempo, visto o significativo aumento de NO_x plasmático. De maneira interessante, o aumento da dose em três vezes leva a um aumento de aproximadamente 40 vezes no nitrito plasmático. Provavelmente o fato ocorre devido à absorção do nitrito não ser linear com relação à dose. Este ponto ainda não foi devidamente estudado. Verifica-se também que ocorre aumento concomitante dos níveis de nitrato após a administração de nitrito. Tais dados estão de acordo com outros trabalhos na literatura(Hunault *et al.*, 2009b).

Observamos que o tratamento de animais hipertensos (após L-NAME) com nitrito de sódio na dose de 45mg/kg em levou a aumento dos níveis plasmáticos de nitrito significativamente menores do que aqueles observados em animais normotensos tratados com a mesma dose de nitrito. Também observamos a mesma tendência nos níveis de NO_x. O motivo desta diferença e a contribuição de cada via enzimática, aqui não estudadas, neste processo ainda não esta claro, mas é possível que o L-NAME tenha reduzido o fluxo sangüíneo gástrico, reduzindo assim a absorção de nitrito de sódio.

5 - CONCLUSÃO

A utilização de omeprazol pode levar a diminuição dos efeitos hipotensores do nitrito de sódio por via oral. Adicionalmente, nossos dados sugerem que a via de conversão ácida no estômago provavelmente é responsável por parte dos efeitos hipotensores do nitrito. Esta via, ao ser inibida pelo tratamento com omeprazol, diminui a formação de NO a partir do nitrito.

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7-ANEXOS

UNIVERSIDADE DE SÃO PAULO
FACULDADE DE MEDICINA DE RIBEIRÃO PRETO

— Comissão de Ética em Experimentação Animal —

CERTIFICADO

Certificamos que o Protocolo para Uso de Animais em Experimentação **nº 091/2009**, sobre o projeto intitulado "*Efeitos do nitrito de sódio na hipertensão experimentalmente induzida e na reatividade vascular de ratos hipertensos*", sob a responsabilidade do **Professor Doutor José Eduardo Tanus dos Santos** está de acordo com os Princípios Éticos na Experimentação Animal adotado pelo Colégio Brasileiro de Experimentação Animal (COBEA) e foi ***APROVADO*** em reunião de ***08 de junho de 2009***.

(We certify that the protocol ***nº 091/2009***, about ***"Effects of sodium nitrite on hypertension and vascular reactivity of hypertensive rats"***, agrees with the ETHICAL PRINCIPLES IN ANIMAL RESEARCH adopted by Brazilian College of Animal Experimentation (COBEA) and was approved in ***06/08/2009*** meeting.

Ribeirão Preto, 08 de junho de 2009.

Prof. Dr. Eduardo Melani Rocha
Presidente da Comissão de Ética em Experimentação Animal