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Original article

# Oral nitrite circumvents antiseptic mouthwash-induced disruption of enterosalivary circuit of nitrate and promotes nitrosation and blood pressure lowering effect

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ABSTRACT

The nitric oxide ($NO^{\bullet}$) metabolites nitrite and nitrate exert antihypertensive effects by mechanisms that involve gastric formation of S-nitrosothiols. However, while the use of antiseptic mouthwash (AM) is known to attenuate the responses to nitrate by disrupting its enterosalivary cycle, there is little information about whether AM attenuates the effects of orally administered nitrite. We hypothesized that the antihypertensive effects of orally administered nitrite would not be prevented by AM because, in contrast to oral nitrate, oral nitrite could promote S-nitrosothiols formation in the stomach without intereference by AM. Chronic effects of oral nitrite or nitrate were studied in two-kidney, one-clip (2K1C) hypertensive rats (and normotensive controls) treated with AM (or vehicle) once/day. We found that orally administered nitrite exerts antihypertensive effects that were not affected by AM. This finding contrasts with lack of antihypertensive responses to oral nitrate in 2K1C hypertensive rats treated with AM. Nitrite and nitrate treatments increased plasma nitrites, nitrates, and S-nitrosothiols concentrations. However, while treatment with AM attenuated the increases in plasma nitrite concentrations after both nitrite and nitrate treatments, AM attenuated the increases in S-nitrosothiols in nitrate-treated rats, but not in nitrite-treated rats. Moreover, AM attenuated vascular S-nitrosylation (detected by the SNO-RAC method) after nitrate, but not after nitrite treatment. Significant correlations were found between the hypotensive responses and S-nitrosothiols, and vascular S-nitrosylation levels. These results show for the first time that oral nitrite exerts antihypertensive effects notwithstanding the fact that antiseptic mouthwash disrupts the enterosalivary circulation of nitrate. Our results support a major role for S-nitrosothiols formation resulting in vascular S-nitrosylation as a key mechanism for the antihypertensive effects of both oral nitrite and nitrate.

## 1. Introduction

Evidence accumulated in the last two decades has changed our view of the nitric oxide ($NO^{\bullet}$) metabolites nitrite and nitrate [1–3]. Many experimental and clinical studies now clearly show that both anions activate biological mechanisms that protect the cardiovascular system against pathophysiological alterations of disease conditions including hypertension [1,4–8]. In this context, the effects of nitrate were intrinsically associated with its enterosalivary cycle, and a new NO synthase-independent pathway leading to $NO^{\bullet}$ formation has been acknowleged, the nitrate-nitrite-NO pathway [9,10]. The basic knowledge leading to the understanding of this new pathway has been established in studies showing that swallowed nitrite generates $NO^{\bullet}$ under the acidic conditions of the stomach [11,12]. Swallowed nitrite in saliva derives mostly from circulating nitrate, which is actively secreted by salivary glands and reduced to nitrite by oral commensal bacteria [13]. The critical role of oral microbiota in reducing nitrate to nitrite has been widely acknowledged and nitrite can be further reduced to NO by a variety of other enzymes under particular conditons [14,15]. Given the relevance of these findings, dietary nitrate supplementation has now been suggested as an effective therapeutic approach to lower blood pressure in hypertensive patients [6,8].

Recent studies, however, have shown that the use of antibacterial mouthwash may challenge the blood pressure lowering effects of nitrate. In fact, the use of antiseptic mouthwash increased blood pressure in healthy individuals [16] and abolished the blood pressure lowering effects of nitrate in rodents [17,18]. This deleterious effect associated with removal of the oral commensal bacteria has been

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attributed to disruption of nitrate reduction to nitrite by oral bacteria, thus preventing nitrite to achieve the stomach and attenuating the increases in plasma nitrite levels after a nitrate load [19]. While there is no doubt that circulating nitrite can promote arterial and venous dilation under normoxic conditions after nitrite is reduced to $NO^{\bullet}$ by deoxyhemoglobin, deoxymyoglobin or other enzymes with nitrite reductase activity [4,20,21], the mechanisms causing this effect are not fully understood [22,23]. In this respect, the effects of orally administered nitrite have recently been associated with increased gastric formation of S-nitrosothiols, independently of the concentrations of nitrite measured in the plasma [23]. In addition, very similar results were found with orally administered nitrate, thus suggesting that the antihypertensive effects of both nitrite and nitrate [23–25] critically involve the gastric formation of S-nitrosothiols [23], and are independent of increases in plasma nitrite concentrations [24].

In the present study, we hypothesized that treatment with oral nitrite circumvents antiseptic mouthwash-induced disruption of enterosalivary circuit of nitrate and therefore promotes blood pressure lowering effects in hypertensive rats. In addition, given that the use of antiseptic mouthwash severely reduces nitrite concentrations in swallowed saliva achiving the stomach in animals treated with nitrate, we hypothesized that the use of antiseptic mouthwash could impair the increases in plasma S-nitrosothiols after treatment with oral nitrate, but not with oral nitrite. We further examined tissue protein nitrosation to examine the consequences of alterations in S-nitrosothiols levels associated with the use of antiseptic mouthwash.

## 2. Material and methods

### 2.1. Animals and hypertension model

The animals used in the present study were handled according to the guiding principles published in the National Institutes of Health Guide for the Care and Use of Laboratory Animals and the study followed the guidelines of the Ribeirao Preto Medical School, University of Sao Paulo. Male Wistar rats (190–210 g) were obtained from the colony at University of São Paulo and maintained on a 12-h light/dark cycle at room temperature (22–25 °C) with free access to standard rat chow and water.

Two kidney, one clip (2K1C) hypertension was induced as previously described [26,27]. Briefly, the rats were anesthetized with tribromoethanol (250 mg/kg) and had their left renal artery clipped with a silver clip (0.2 mm). Sham-operated control rats underwent the same surgical procedure except for the clip placement. The nonsteroidal anti-inflammatory flunixinemeglumine (2.5 mg/kg, sc, Banamine; Schering Plough, Brazil) was administered after surgery. Systolic blood pressure (SBP) was assessed weekly by tail-cuff plethysmography [25], and the last assessment was carried out approximately 18 h after the last dose of nitrite or nitrate treatments. To minimize the effects of stress induced by this method on blood pressure measurement, the animals were trained for a week before surgery.

To confirm blood pressure measurements with the tail-cuff method, invasive mean arterial pressure (MAP) was evaluated at the end of study period, approximately 6 h after the last dose of nitrite or nitrate treatment. The animals were anesthetized with tribromoethanol (250 mg/kg, i.p.) and had their femoral artery 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 tunneled subcutaneously and exteriorized through the back of the neck. After surgery, the nonsteroidal anti-inflammatory flunixinemeglumine (2.5 mg/kg, s.c., Banamine®, Schering Plough, Brazil) was administered for post-operation analgesia. After 6 h of rest, the arterial cannula was connected to a pressure transducer and the MAP was recorded in freely moving rats using a data acquisition system (MP150CE; Biopac Systems Inc., CA, USA) connected to a computer (Acknowledge 3.2, for Windows). Before collecting data, we allowed at least 15 min of stabilization [28].

### 2.2. Nitrite or nitrate treatments and the use of antiseptic mouthwash

A first study was designed to examine the effects of antiseptic mouthwash on the antihypertensive effects of sodium nitrite. After two weeks of 2K1C hypertension, both hypertensive and Sham-operated received sodium nitrite 15 mg/kg (or vehicle) daily, by gavage [23,28,29], and had their mouths cleaned with a swab containing a commercial antiseptic mouthwash (Periogard®, chlorhexidine 0.12% or saline) once a day [17]. Nitrite and mouthwash (or respective vehicles) treatments were maintained for additional four weeks in the eight groups of animals (N=10 rats/group). Six hours after the last nitrite (or vehicle) administration, the rats were anesthetized with tribromoethanol (250 mg/kg) and arterial blood samples were collected into heparin containing tubes and immediately centrifuged at 1000*g* for 4 min. Plasma aliquots were mixed with a solution containing N-ethylmaleimide (10 mmol/L) and diethylenetriaminepentaacetic acid (2 mmol/L) to preserve S-nitrosothiols and stored at −70 °C until used to analyze nitrite, nitrate, and S-nitrosothiols concentrations. The aortas were dissected and stored at −70 °C until used to quantify protein nitrosylation.

A second study was designed to examine the effects of antiseptic mouthwash on the antihypertensive effects of sodium nitrate. This study was carried out using the same procedures used in the first study, except that sodium nitrate 140 mg/kg [17,23] was used to replace sodium nitrite (eight groups of animals; N=10 rats/group).

The daily doses of nitrite and nitrate used in the present study were chosen with basis on a series of previous studies by our group showing that these doses significantly reduce blood pressure in 2K1C [5,23,28], L-NAME [30], and DOCA-salt [31] hypertension models. In addition, the doses of both anions used here are the same doses used in a previous study by another group and correspond to pharmacological doses with effects on blood pressure that were prevented by antiseptic mouthwash in the case of nitrate [17]. Both nitrite and nitrate were administered by gavage (1.5 ml/kg of 10 mg/ml and 93 mg/mg, respectively) because we wanted to make sure that all rats received exactly the same dose/body weight.

### 2.3. Assessment of oral bacterial concentrations

A tongue swab was collected at the end of the study period to assess the concentration of oral bacteria [18]. This number was estimated with basis on the number of colony-forming units (CFU) counted after the bacteria from the swab were smeared on an agar plate and incubated for 18 h before counting the number of colonies.

### 2.4. Measurement of plasma nitrate, nitrite, and S-nitrosothiols concentrations

Plasma aliquots were analyzed in duplicate for their nitrite and S-nitrosothiols contents using an ozone-based reductive chemiluminescence assay as previously described [28,32]. Briefly, to measure nitrite concentrations in plasma, 50 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). To measure nitroso compounds (RSNO) concentrations, 500 µl of plasma samples were treated with acid sulfanilamide (5% sulfanilamide in HCl 1 mol/L) for 5 min before injection into the solution of acidified tri-iodide purged with nitrogen in line with the NO analyzer.

The plasma nitrate+nitrite (NOx) concentrations were determined in duplicate by using the Griess reaction as previously described [28], and plasma nitrate concentrations were calculated by subtracting plasma nitrite concentrations from NOx. Briefly, 40 µl of plasma were incubated with the same volume of nitrate reductase buffer (0.1 mol/L