Full Length Research Paper

In vitro* preventive effects of nitrate tolerance by a polyphenol-enriched extract of *Hibiscus sabdariffa

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Treatment failure or tolerance, which rapidly leads to a reduced hemodynamic effects and therapeutic efficacy is the major limitation of long-term use of nitrates, including nitroglycerin (NTG) in the treatment of coronary artery disease. These effects are most often associated with oxidative stress. Thus, in this work, we were interested in the prevention of nitrate tolerance by the antioxidant compounds from *Hibiscus sabdariffa* L. crude extract, a plant from the Senegalese Pharmacopoeia, rich in polyphenols. Thoracic aorta segments without endothelium were taken from rats and incubated in isolated organ chambers. The vessels were then pre-exposed with the *H. sabdariffa* polyphenolic extract (HSE, $5 \cdot 10^{-2}$ g/l) or antioxidants such as N-acetyl cysteine (NAC, 10^{-3} M) or vitamin C (VIT C, 10^{-2} M), taken as reference. After a 30 min treatment, aortic segments were exposed to NTG (50 μ M, 1 h) to induce tolerance state before being contracted to adrenaline (10^{-8} to 10^{-5} M), and then relaxed with NTG (10^{-9} to 10^{-5} M). Polyphenols from *H. sabdariffa* potentiated the relaxant response to NTG, whatever the state of vascular tolerance; the HSE partially corrected the *in vitro* nitrate tolerance. This work suggests interesting therapeutic perspectives by improving the response to treatment with nitrates in coronary patients.

Key words: Nitrate tolerance, antioxidant, vascular diseases, therapeutic agents, medicinal plants.

INTRODUCTION

Angina pectoris, usually due to coronary heart disease, is recognized as a transient retrosternal pain syndrome and

characteristic of myocardial ischemia. It occurs more readily after 50 years and more frequently favored by risk factors: hypertension, diabetes, dyslipidemia, smoking, obesity, heredity (Stritzke et al., 2009). Prevalence is difficult to quantify and varies by country. However, it is higher in industrialized countries where it affects about 2% of the population (Carevic et al., 2007). In the

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treatment of myocardial infarction, the first-line drugs consist of nitrates, including nitroglycerin. However, their long-term use is limited by a therapeutic escape or tolerance effect, which decreases the therapeutic efficacy of the drug, compromising the patient's prognosis (Munzel et al., 2005; Daiber et al., 2010a; b; Munzel, 2008).

In the mechanisms of tolerance to nitrates, two major phenomena are discussed:

(i) a pseudotolerance phenomenon (Munzel et al., 2005), linked to a neurohormonal activity, associated with volume expansion, both intended to limit the fall in blood pressure associated with a vasodilatory nitrates effect, and; (ii) a direct loss of vascular response called vascular tolerance (Wenzl et al., 2009; Fink and Bassenge, 2002; Wang et al., 2002b), whose mechanism is not fully elucidated. In the current state of knowledge, oxidative stress is a major cause of this phenomenon (Fadel et al., 2012; Oelze et al., 2010; Daiber et al., 2004; 2005; Mollnau et al., 2006).

Thus, our hypothesis derives from the antioxidant capacity of compounds to reduce or prevent vascular tolerance to nitrates, by reducing oxidative stress.

Antioxidant compounds have shown a growing interest to researchers. Indeed, studies have demonstrated their ability to prevent nitrate tolerance in animal models, including humans. These agents include natural antioxidants such as vitamin C, vitamin E and plant polyphenols. However, studies have shown that vitamins are able to produce free radicals with cytotoxic effects (Satoh et al., 1996; Kagan et al., 1994; Cai and Harrison, 2000), unlike the polyphenols. They are abundantly synthesized by plants, hence our interest in *H. sabdariffa* L., a plant from the Senegalese Pharmacopoeia, rich in polyphenols. Therefore, the objective of this work was to study the experimental conditions for the prevention of tolerance to nitroglycerin, and specifically demonstrate the *in vitro* antioxidant and preventive effects of *H. sabdariffa*.

MATERIALS AND METHODS

Drugs

Norepinephrine and Acetylcholine were purchased from Sigma Chemical Co; N-acetyl cysteine (Fluimucil® 5 g/25 ml, solution for infusion) and Vitamine C (VITAMIN C 10 Percent, AGUETTANT®, solution for injection, for infusion) was purchased from a local drugstore. Nitroglycerin (0.15 mg, Sublingual Tablets) was provided to us by the hospital pharmacy (Hôpital Principal) in Dakar (Senegal). All drugs were serially diluted in distilled water before each experiment. The concentration of the drugs is expressed as final molar concentration in the bath.

Hibiscus sabdariffa extract preparation

H. sabdariffa calyces was obtained from botanical laboratory of our

faculty and prepared as previously described (Sarr et al., 2009). In brief, calyces were dried during a week at room temperature. Dried and powdered calyx (Grinder RM-100, Retsch®) of *H. sabdariffa* (500 g) was extracted by maceration at room temperature for 2 h with 60% methanol. The hydroalcoholic extract was then filtered in vacuum conditions (Vacuum pump V-700, Büchi®) by means of the phial of Kitassato and evaporated on a rotary evaporator (Rotavapor R-210, Büchi®). Methanolic extract evaporation was realized during three successive days until the obtaining of a dry crude extract.

Organ chambers experiments

Vessels preparation

Experiments were conducted in accordance with the Guide for the Care and Use of Laboratory Animals as promulgated by the Senegalese Academic Bioethics Committee. Male Wistar rats weighing 150 to 200 g were procured from a local Institute. They were fed on standard rat feed and given free access to water. After anesthesia by intraperitoneal injection of pentothal (60 mg/kg body weight) for 10 min, rats were sacrificed and exsanguinated by cross section of the carotid. After supra-umbilical laparotomy, the thoracic aorta was removed carefully from the bottom up, transferred into a petri dish filled with Krebs solution of the following composition (in mM): (NaCl 119, KCl 4.7, KH₂PO₄ 1.18, MgSO₄ 1.18, CaCl₂ 1.25, NaHCO₃ 25 and D-glucose 11, pH 7.4, 37°C) and cleaned of adherent connective tissue. As indicated, the endothelium was removed by rubbing the intimal surface of rings with a pair of forceps and cut into 3 mm ring segments.

Protocol design

Dose response studies are typically conducted to assess concentration-response relationships in isolated rat thoracic aorta preparation which allow maintaining the integrity of the tissue for several hours in a temperature-controlled environment, while physiological measurements are performed. The rings were suspended between two wire hooks in organ chambers filled with 10 ml of Krebs solution (37°C, pH 7.40) aerated with O₂ 95%/CO₂ 5%. The upper hook was connected to a force transducer (Panlab-TRI 202P), and changes in isometric force were recorded (Labscribe® Iworx/118 Data Recording Software). After a resting tension (1 g) defined by preliminary studies, the rings were allowed to equilibrate for 45 min and were then precontracted with 60 mM potassium chloride to determine maximal contraction. The rings were washed twice with a fresh buffer solution and then precontracted with norepinephrine (10⁻⁶ M) to reach an optimal constriction (80% maximum). Pre-contracted rings were allowed to plateau and then acetylcholine (10⁻⁶ M) was added to assess the endothelial function. Only rings that exhibited less than 10% relaxation responses to acetylcholine were considered without endothelium and used in subsequent experiments. Rat aortic segments were then randomly assigned to one of the 8 groups: 1 - Control; 2 - Tolerant (Nitroglycerin (NTG)-treated); 3 - N-acetyl cysteine (NAC); 4 - Vitamin C (VIT C); 5 - *H. sabdariffa* extract (HSE), 6 - VIT C-tolerant; 7 - NAC-tolerant and 8 - HSE-tolerant. The concentration of HSE chosen (5.10⁻² mg/ml) was based on concentration-response data obtained in a previous study (Sarr et al., 2009).

Nitrate tolerance induction

To induce nitrate tolerance, thoracic aortic segments without

Figure 1. Concentration-effect curves of relaxant response to nitroglycerin in rat thoracic aorta without endothelium (Control) or pre-exposed (Tolerant) to nitroglycerin (NTG, 5.10^{-5} M, 60min). Results are presented as mean $\pm$ SEM of 7 experiments from 7 rats different. ** = $P < 0.01$ (2-way ANOVA).

Table 1. Relaxing responses of nitroglycerin expressed in percent and EC_{50} values determined by log-logit regression in rat thoracic aorta without endothelium or tolerant vessels pre-exposed to *H. sabdariffa* extract (HSE), N-acetylcysteine (NAC) or Vitamin C (VIT C).

Parameter	EC_{50} (M)	E_{max} (%)
Control	$1.01 \times 10^{-8} \pm 0.05$	81.16 ± 7.42
Tolerant	$9.69 \times 10^{-8} \pm 0.83$	31.33 ± 4.23
HSE	$4.68 \times 10^{-9} \pm 0.5$	111 ± 13.91
NAC	$1.16 \times 10^{-7} \pm 0.03$	119 ± 18.74
VIT C	$1.62 \times 10^{-8} \pm 0.09$	94.19 ± 11.32
HSE-tolerant	$4.75 \times 10^{-8} \pm 0.64$	66.71 ± 7.54
NAC-tolerant	$3.53 \times 10^{-8} \pm 0.08$	61.73 ± 9.26
VIT C-tolerant	$1.54 \times 10^{-7} \pm 0.12$	96.40 ± 21.13

endothelium were first exposed to NTG (50 μ M, 1 h) and were pre-contracted with cumulative concentrations of norepinephrine (10^{-8} to 10^{-5} M). When a plateau phase was obtained, cumulative concentration-response curves were obtained with NTG concentrations ranging from 10^{-9} to 10^{-5} M.

Prevention of nitroglycerin-induced nitrate tolerance

Before induction of tolerance state, the aortic segments were pre-exposed to one of the antioxidants taken as reference. To do this, they were treated with NAC (10^{-3} M), VIT C (10^{-2} M) or HSE (0.1 mg/ml). After 30 min exposition, they were pre-contracted with norepinephrine (10^{-8} to 10^{-5} M). When a plateau phase was

reached, cumulative concentration-response curves were obtained with NTG concentrations ranging from 10^{-9} to 10^{-5} M.

Statistical analysis

Values are expressed as mean $\pm$ standard error of mean (SEM). Concentration-response curves were compared by 2-way Analysis of Variance (ANOVA) or the multi-analysis of variance (MANOVA), as required. Values of $p < 0.05$ were considered statistically significant.

RESULTS

Nitrate tolerance induction

Results obtained in our study (Figure 1) clearly indicate a tolerance state in NTG-treated vessels. Indeed, after exposure of aorta rings with NTG, there was a significant reduction of at least 40% of the maximum relaxation to nitroglycerin (tolerant vessels: half maximal effective concentration (EC_{50}) = $9.695 \times 10^{-8} \pm 0.831$ M; E_{max} = $31.33 \pm 4.23\%$) compared to control (non-tolerant vessels: EC_{50} = $1.015 \times 10^{-8} \pm 0.054$ M; E_{max} = $81.16\% \pm 7.42$) as indicated in Table 1. This loss of the ability of relaxation observed in tolerant vessels highlights, in our model, the phenomenon of treatment failure associated with prolonged use of nitrates.

Potentiating effects of *H. sabdariffa* extract on the relaxant responses to nitroglycerin

In order to examine any potentiating effect on the NTG-induced relaxations (10^{-9} to 10^{-5} M) after HSE pre-exposure at a final concentration of 5.10^{-2} mg/ml during 30 min, we compare relaxations in HSE-exposed vessels with those obtained with control (not pre-exposed). Results obtained in Figure 2A showed in HSE-exposed vessels, a potentiating of the relaxant response to NTG (EC_{50} = $4.679 \times 10^{-9} \pm 0.498$ M; E_{max} = $111\% \pm 13.91$) compared to control vessels, for which the maximal effect is only about $81.16\% \pm 7.42$ (EC_{50} = $1.015 \times 10^{-8} \pm 0.054$ M). This demonstrates the ability of the *H. sabdariffa* polyphenolic extract to potentiate the pharmacological vasorelaxant effects of nitrates (Table 1).

In parallel, we studied the effects of two known antioxidants, namely NAC and VIT C on the relaxant responses to NTG in order to verify whether the potentiating effects of HSE can be due to its antioxidant character. For this, we also compare relaxations obtained after NAC or VIT C pre-exposure with those obtained with control not exposed. Our results as indicated in Figure 2B actually showed a potentiating effect of NAC only at high concentrations (10^{-5} M NTG) compared to the control vessels (EC_{50} : $1.015 \times 10^{-8} \pm 0.054$ M versus 1.163×10^{-7}

Figure 2. Concentration-effect curves of relaxant responses to nitroglycerin in rat thoracic aorta without endothelium (Control) or tolerant vessels (NTG, 5.10^{-5} M, 60 min), pre-exposed to (A) *H. sabdariffa* extract (HSE, 5.10^{-2} mg/ml), (B) N-acetylcysteine (NAC, 10^{-3} M) and (C) Vitamin C (VIT C, 10^{-2} M) during 30 min. Results are presented as mean $\pm$ SEM of 7 experiments from 7 different rats. ns = not significant, * = $p < 0.05$, ** = $p < 0.01$ (MANOVA) compared to control.

± 0.029 and $E_{\max}$: $81.16\% \pm 7.42$ versus $119\% \pm 18.74$; Control versus NAC, respectively). For Vit C, as indicated on Figure 2C, no significant improvements in relaxation responses were observed (EC_{50} : $1.015 \pm 0.054 \times 10^{-8}$ M versus $1.620 \pm 0.091 \times 10^{-8}$ and $E_{\max}$: $81.16\% \pm 7.42$ versus $94.19\% \pm 11.32$; Control versus VIT C, respectively). Thus, compared to NAC, the HSE better potentiates the vasorelaxant response to NTG, since this potentiating was observed for all doses in the range of relaxation. These results indicate a potential benefit role of HSE, while more potentiating the relaxant responses of NTG compared to other antioxidants NAC and VIT C.

Normalizing effects of *H. Sabdariffa* extract in tolerance conditions

Since some antioxidants potentiate the relaxant responses of NTG, we first sought to determine whether the responses in antioxidant-tolerant aortic segments were different to those obtained in control vessels (without any treatment). If it is the case, we can conclude a normalizing effect by the antioxidant. The results, as shown in Figure 2A, show a significant difference between the HSE-tolerant ($EC_{50} = 4.75 \times 10^{-8} \pm 0.64$; $E_{\max} = 66.71\% \pm 7.54$) and control vessels (EC_{50} : $1.015 \times 10^{-8} \pm 0.054$ M; $E_{\max}$: $81.16\% \pm 7.42$) demonstrating the non possibility of HSE to normalize the relaxant responses to NTG in tolerant vessels. Also, in comparison to the same untreated control, NAC-treated tolerant vessels did not normalize the relaxing effects of cumulatively administered NTG (Figure 2B), since a significant difference was observed between NAC-tolerant ($EC_{50} = 3.53 \times 10^{-8} \pm 0.08$ M; $E_{\max} = 61.73\% \pm 9.26$) and control vessels

(EC_{50} : $1.015 \times 10^{-8} \pm 0.054$ M; $E_{\max}$: $81.16\% \pm 7.42$).

However, this result contrasts with those obtained with VIT C, under the same conditions. Indeed, VIT C, without having potentiating effects on NTG-induced relaxation, normalized relaxation responses. Indeed, the results in Figure 2C showed no significant difference between VIT C-tolerant vessels ($EC_{50} = 1.62 \pm 0.09.10^{-8}$ M; $E_{\max} = 94.19\% \pm 11.32$) and non-tolerant controls (EC_{50} : $1.015 \pm 0.054 \times 10^{-8}$ M; $E_{\max}$: $81.16\% \pm 7.42$). It is interesting to note that VIT C has only potentiating effect in tolerant vessels.

Preventive role of *H. Sabdariffa* extract on the tolerance induction

In order to investigate the ability of HSE to prevent the development of tolerance, we first incubated aortic segments with the HSE before inducing the tolerance phenomenon. A preventive effect on tolerance induction was obtained when any significant difference was observed between relaxant responses in HSE-treated vessels with those of HSE-tolerant vessels. Figure 2A showed a significant difference in the relaxant responses between HSE ($EC_{50} = 4.68 \times 10^{-9} \pm 0.5$ M; $E_{\max} = 111\% \pm 13.91$) and HSE-tolerant vessels ($EC_{50} = 4.75 \times 10^{-8} \pm 0.64$; $E_{\max} = 66.71\% \pm 7.54$), showing a continuing state of tolerance. Thus, we conclude that HSE is not able to prevent nitrates tolerance, as well as NAC (Figure 2B). By contrast, VIT C completely reverse tolerance to nitroglycerin and any significant different were found between VIT-treated and VIT-tolerant vessels as shown in Figure 2C.

DISCUSSION

The aim of the present study was to evaluate the ability of a Senegalese and African Pharmacopoeia plant extract to prevent the development of nitrates tolerance. In light of all of our results, the polyphenol-rich extract of *H. sabdariffa* are able to potentiate the relaxant responses to nitroglycerin in non-tolerant and tolerant denuded-thoracic aortic rings; partially correcting the nitrate tolerance by normalizing the relaxant responses to nitroglycerin but can not prevent the development of this phenomenon.

Regarding the methodological approach, we used an *in vitro* nitrates tolerance induction model, which was already validated in numerous studies (Wang et al., 2002a; Van de Voorde et al., 1987, 1994; Stewart et al., 1989; Ratz et al., 2000; Otto et al., 2005; Chegaev et al., 2009; Bennett et al., 1988; Sarr et al., 2005). To do this, we used aortic denuded-rings in order to avoid possible interference between the exogenous nitrogen oxide (NO) from nitroglycerin and endothelium-derived one. The model is established when we observe a significant reduction in the relaxant response to nitroglycerin. This simple model allows overcoming the influence of neurohumoral counter-regulatory involved in vascular physiology.

Among factors involved in reducing the bioavailability of NO, recent work reported by researchers confirm that the development of tolerance to nitrates is associated with increased oxidative stress (Oelze et al., 2010; Daiber et al., 2009a; DiFabio et al., 2006; Muller et al., 2004; Parker, 2004; Gori et al., 2001; Laight et al., 1997, 1998). In addition, a key enzyme involvement of nitroglycerin and nitrates biotransformation, namely the mitochondrial aldehyde dehydrogenase (ALDH 2), is inhibited by reactive oxygen species (ROS) which in addition, can degrade nitric oxide (Wenzel et al., 2009a, 2009b; Daiber et al., 2004, 2009b; Sydow et al., 2004). Therefore, it would be interesting to study the benefit of antioxidants in preventing nitrate tolerance development.

Regarding the natural antioxidants, various therapeutic interventions have been considered and evaluated experimentally or clinically in order to improve endothelial function, the evolution of certain diseases affecting the vascular system and the prevention of tolerance to nitrates. These natural antioxidants include vitamin C, vitamin E and flavonoids. Moreover, the results of many clinical studies, particularly the Heart Outcomes Prevention Evaluation (HOPE) study (Yusuf et al., 2000) showed that vitamin E had no beneficial effect in patients with high cardiovascular risk. The Italian Group for the Study of Streptokinase nell'Infarto (GISSI) study (Hopper et al., 1999) meanwhile showed that in contrast to polyunsaturated fatty acids n-3 (or omega 3), vitamin E does not decrease significantly the incidence of

cardiovascular events among patients who have survived a myocardial infarction. These studies have also reported the limited effectiveness of these antioxidant vitamins, especially their low bioavailability following oral administration or some compartmentalization problems, and their ability to produce free radicals after reacting with oxidizing molecules. Thus it has been reported that vitamin E is capable of producing a tocopheroxyl radical and in the case of vitamin C, ascorbyl radical can be generated (Carr and Frei, 2000), to the extent that until now the results obtained with different classes of antioxidants, including vitamins, are not really satisfactory and further studies with other compounds remains justified.

Indeed, it is now accepted that polyphenols could prevent many diseases such as cancers (Yang et al., 2009; Korkina et al., 2009; Khan et al., 2009; Bracke et al., 2008; Franklin and McCubrey, 2007), cardiovascular (Madeira et al., 2009; Jones et al., 2011; Kumar et al., 2008; Agouni et al., 2009; Walter et al., 2008; Schini-Kerth et al., 2011; Sall Diallo et al., 2008; Sarr et al., 2006 ; 2009) and degenerative diseases (Mollnau et al., 2006; Chen et al., 2007; Berrino, 2002; Meydani, 2002; Meydani, 2001). Encouraging the consumption of fruits and vegetables is now a major public health recommendation. Moreover, among the plant antioxidants, polyphenols appear to be the most effective in their protective effects. The polyphenols in *H. sabdariffa* were identified by a study conducted in our laboratory (Sarr et al., 2009) and many classes were found: phenolic, chlorogenic and caffeic acids, anthocyanins and flavonoids. This same study also found that polyphenols have a good vasorelaxant activity *in vitro*. What about their efficacy on tolerance to nitrates? The main results obtained during the present study have clearly demonstrated the interest of polyphenols on nitrate tolerance.

One important result obtained in our study is the potentiation of the relaxant responses to nitroglycerin with the *H. sabdariffa* extract, whatever the type of vessels, tolerant or not tolerant. Similarly, potentiating effects were also found with N-acetyl cysteine. If potentiating response of nitroglycerin by antioxidants such as N-acetyl cysteine have been reported in numerous studies (Munzel et al., 1989; Pizzulli et al., 1997; Watanabe et al., 1998a, 1998b), few data relate those of plant polyphenols, and no data was reported for the polyphenols of *H. sabdariffa*. To our knowledge, such results linking these potentiating effects on relaxation responses to nitroglycerin and polyphenols from *H. sabdariffa* is an original result.

Another important result is that these potentiating responses of the *H. sabdariffa* extract on the responses of nitroglycerin are responsible for the continuing state of tolerance. This result is explained by the fact that the HSE potentiate all types of vessels relaxed with nitroglycerin, which persist the state of tolerance. This result is very interesting because it can be a possibility to

significantly reduce the therapeutic doses of nitroglycerin and delay the development of tolerance. Similar results were also found with NAC but not with VIT C. These results are consistent with those reported by Pizzuli et al. (1997), showing that NAC does not prevent tolerance during nitroglycerin infusion in patients with heart failure. However, the concomitant infusion of NAC and NTG in 48 h continuous infusion attenuates the development of nitrate tolerance in patients with normal left ventricular function. Regarding vitamin C, Watanabe et al. (1998b) have also shown that oral administration of this antioxidant may prevent nitrate tolerance during continuous administration of NTG in patients with ischemic heart disease.

Finally, data obtained with apocynin, a polyphenols structural analogue, reported by Fukatsu et al. (2007) indicated that this compound, pharmacologically known to be able to prevent the increase in protein expression of NADPH oxidase, a source of superoxide anions, could be a means of protection against nitrate tolerance.

Conclusion

In the long-term treatment of angina pectoris, the difficulty observed is the gradual reduction of coronary arteries relaxant responses to nitrates, despite increasing doses. Our work performed to assess the potential of *H. sabdariifia* polyphenols suggests interesting therapeutic prospects. This could improve the coronary relaxation responses by potentiating the relaxing effect of nitroglycerin.

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