Biological role of nitrite and nitric oxide in the stomach: from diet to protein tyrosine nitration with implications for gastric physiology

Bárbara S Rocha, Coimbra 2012

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Biological role of nitrite and nitric oxide in the stomach: from diet to protein tyrosine nitration with implications for gastric physiology

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I have found in my works something which I can devote myself to, heart and soul, and which inspires me and gives a meaning to life.

Vincent Van Gogh

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The potent vasodilator ethyl nitrite is formed upon reaction of nitrite and ethanol under gastric conditions

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RESUMO

O óxido nítrico (•NO) é um radical livre com funções de sinalização celular reconhecidas nos sistemas cardiovascular, nervoso e imunitário. A sua síntese *in vivo* é altamente regulada, sendo levada a cabo pelas •NO sintases. Uma vez produzido, o •NO participa numa panóplia diversificada de reacções químicas com consequências importantes para a fisiologia celular. Apesar de não ser um radical livre particularmente reactivo, os seus efeitos biológicos devem-se em grande parte às suas propriedades físico-químicas, nomeadamente à sua capacidade de difusão através das membranas celulares. Desta forma, não existe complementaridade estrutural entre este radical e um receptor específico, sendo as suas acções devidas a interacções aleatórias com moléculas-alvo, nomeadamente a guanilato ciclase solúvel. A dinâmica entre a sua síntese e, posteriormente, a sua difusão e reacções com alvos biomoleculares – e aqui incluem-se não só as interacções bioquímicas com a guanilato ciclase mas outras com implicações funcionais, nomeadamente com a hemoglobina, citocromo c oxidase, aconitase ou outros radicais –determina os efeitos biológicos do •NO. Na sua esfera de difusão, e em função do estado redox do microambiente, o •NO pode ser oxidado a nitrito e, posteriormente, a nitrato. Este paradigma, corolado pela *L-aginine-nitric oxide pathway*, sofreu recentemente uma reviravolta dado que foi demonstrado que os produtos da oxidação do •NO, nitrato e nitrito, podiam ser reduzidos *in vivo* de forma a produzir •NO num processo independentemente das NOS. Esta outra via foi por isso designada *nitrate-nitrite-nitric*

oxide pathway. O nitrito presente na dieta (vegetais, carnes curadas e água) é incorporado na saliva e posteriormente transportado para o estômago. No lúmen gástrico, devido ao pH extremamente baixo, o nitrito é reduzido a $^{\bullet}NO$ e outros óxidos de nitrogénio com funções sinalizadoras.

Com esta mudança de paradigma, a dieta passou a ter um papel crucial na biologia do $^{\bullet}NO$. Os polifenóis, particularmente abundantes em alimentos da Dieta Mediterrânica, reduzem univalentemente o nitrito a $^{\bullet}NO$ no ambiente acídico do estômago. Inicialmente, no trabalho apresentado nesta tese é estabelecida a prova de conceito de que a ingestão de alimentos com alto teor em polifenóis potencia a produção de $^{\bullet}NO$ no estômago humano a partir do nitrito. Uma vez que o $^{\bullet}NO$ derivado do nitrito desempenha funções gastroprotectoras, esta observação tem óbvias consequências para a fisiologia gastrointestinal. Tal como supracitado, o $^{\bullet}NO$ é uma molécula altamente difusível e, como tal, a sua capacidade de difusão no estômago foi também abordada neste estudo. Foi observado que o $^{\bullet}NO$ produzido no lúmen gástrico difunde para a mucosa e é, inclusivamente, detectado na camada serosa, num modelo *ex vivo*. Este resultado sugere que na ausência de uma circulação sanguínea funcional, o $^{\bullet}NO$ difunde todas as camadas histológicas da parede do estômago. A mesma experiência realizada *in vivo*, em ratos anestesiados, demonstrou que é impossível detectar $^{\bullet}NO$ na serosa, provavelmente devido à rápida reacção com a hemogblobina circulante. Em conjunto, estes resultados indicam que uma vez produzido no lúmen gástrico a partir do nitrito com origem na dieta, o $^{\bullet}NO$ difunde até aos vasos sanguíneos da mucosa modulando o tónus vascular local.

Tal como já foi referido, o nitrito é reduzido a $^{\bullet}NO$ no meio ácido do estômago, mas pode também originar outros óxidos de nitrogénio capazes de exercer funções de sinalização celular, nomeadamente através de modificações pós-tradução de proteinas. A nitração é uma modificação pós-tradução que se caracteriza pela ligação covalente de um grupo nitro à posição *orto* de um anel aromático, tipicamente de um resíduo de tirosina. Além de ser um processo selectivo, em algumas circunstâncias esta modificação bioquímica pode alterar a função da proteina, induzindo perda ou ganho de função. Neste sentido, a nitração de proteinas relevantes para a fisiologia gástrica, mediada pelo nitrito da dieta, foi também abordada neste trabalho.

A pepsina é uma endopeptidase secretada na forma de zimogénio, pepsinogénio, pelas células principais da mucosa gástrica. Uma vez no lúmen, devido ao pH ácido, o pepsinogénio é activado a pepsina. Os dados experimentais aqui apresentados demonstram que a pepsina pode ser nitrada pelo nitrito a pH ácido. Acresce ainda referir que, como seria expectável devido à capacidade de difusão do $^{\bullet}NO$, a nitração do pepsinogénio foi também observada ao pH fisiológico da mucosa. Provavelmente, o mecanismo envolvido inclui reacções secundárias do $^{\bullet}NO$ com outros radicais, culminando na formação de espécies nitrantes. O impacto da nitração na função proteolítica da pepsina foi também avaliado *in vitro,* observando-se uma dimunição de função para quatro substratos distintos. Por outro lado, *in vivo,* estes resultados são traduzidos na prevenção do desenvolvimento de úlceras secretagogas. Não obstante ser a protease mais abundante no estômago, a pepsina é responsável pela digestão de cerca de 15% das

proteinas da dieta. Tem, no entanto, um reconhecido papel no desenvolvimento de úlceras gástricas devido à sua capacidade de erodir a mucosa. Neste trabalho observou-se que a dimunição de função proteolítca da pepsina evita o desenvolvimento de úlceras gástricas provavelmente por inibição da digestão de proteinas da mucosa. Do ponto de vista mecanístico, ensaios realizados com urato, um sequestrador do $^{\bullet}NO_2$, inibiram quer a nitração da pepsina, quer o efeito preventivo de úlceras, sugerindo que este radical é, pelo menos um dos agentes nitrantes derivados do nitrito. Além disso, usando um modelo de ratinhos *germ-free*, também é demonstrado que o $^{\bullet}NO$ modula a ocorrência de reacções de nitratção na mucosa gástrica.

O trabalho apresentado nesta tese acrescenta novos dados à complexa biologia do nitrito no contexto gástrico. É estabelecida a prova de conceito que a ingestão de alimentos ricos em polifenóis potencia a formação de $^{\bullet}NO$ a partir do nitrito no estômago e que uma vez produzido, o $^{\bullet}NO$ difunde induzindo relaxamento da musculatura lisa e provavelmente modulando directamente o fluxo sanguíneo da mucosa. Além disso, é introduzido um conceito inovador que envolve a modificação da função de proteínas gástricas através de modificações pós-tradução induzidas pelo eixo nitrito-$^{\bullet}NO$ proveniente da dieta. Em particular, é demonstrado que o nitrito induz a nitração de proteinas gástricas com consequente dimunição de função, nomeadamente na prevenção da úlcera gástrica. Estas observações podem ter aplicações terapêuticas importantes uma vez que estes efeitos são modulados pela dieta.

ABSTRACT

Nitric oxide (•NO) is a ubiquitous signaling molecule that regulates several cell and tissue functions. Critical physiological mechanisms such as regulation of vascular tone, immune response and neuromodulation rely primarily on the dynamics of •NO synthesis by the family of NO synthases and the chemistry upon diffusion in the biological milieu. The biological fate of •NO depends on different conditions, including the availability of kinetic-favorable targets, from which reactions with functional outcomes result, and also on the redox microenvironment that may drive its oxidation to nitrite and nitrate. Until recently, nitrite has been considered either a stable metabolite of •NO metabolism or a noxious product that, in the stomach, could give rise to carcinogenic nitrosamines. This view is now rapidly changing and not only modulatory actions have been assigned to nitrite but it may represent the largest •NO reservoir in the body. Inorganic nitrite, derived from the reduction of nitrate in saliva, has recently emerged as a protagonist in •NO biology as it can be univalently reduced to this free radical, in the healthy human stomach. This reaction takes advantage of the high nitrite concentrations (achieved through the reduction of dietary nitrate by oral bacteria) and low pH. Despite the fact that several proteins (including enzymes) have been shown to reduce nitrite to •NO at low oxygen tensions, the highest yields of •NO *in vivo* are intragastric. Important physiological implications have been attributed to nitrite-derived •NO in the cardiovascular and gastrointestinal systems. In the

gastrointestinal tract, it has been shown to modulate host defense, blood flow, mucus formation and motility.

Dietary polyphenols, due to their redox potential and high availability in unmodified forms in the stomach, may promote the reduction of nitrite. These compounds univalently reduce nitrite to $^{\bullet}NO$ and simultaneously are oxidized to the respective semi-quinone radicals. Initially, in the work presented in this thesis it is established the proof of concept that the ingestion of foodstuffs rich in polyphenols enhance $^{\bullet}NO$ production from nitrite in the human stomach. Furthermore, due to its chemical properties (small, hydrophobic molecule), $^{\bullet}NO$ was shown to diffuse from its production site (gastric lumen) towards the mucosa. In *ex vivo* experiments $^{\bullet}NO$ was detected electrochemically within the gastric serosal layer, demonstrating that in the absence of a functional blood flow, it diffuses the entire gastric wall. On the contrary, using an *in vivo* rat model, $^{\bullet}NO$ was not detected, suggesting that circulating hemoglobin was acting as a sink for $^{\bullet}NO$. These results suggest that dietary nitrite-derived $^{\bullet}NO$ is produced in the stomach lumen and diffuses towards the mucosal blood vessel, likely modulating gastric blood flow and, as it is also shown, to induce smooth muscle relaxation.

Notwithstanding, at acidic pH, nitrite generates different nitrogen oxides depending on the local microenvironment, including $^{\bullet}NO$, $^{\bullet}NO_2$, dinitrogen trioxide (N_2O_3), and peroxynitrite ($ONOO^-$). Thus, the gastric environment is a significant source of nitrating and nitrosating agents. Therefore, in the second part of this work, the ability of dietary nitrite to nitrate specific gastric proteins was studied. Nitration, and in particular tyrosine nitration, is a post-

translational modification in which a nitro group is inserted in the *ortho* position of a tyrosine aromatic ring. Generally speaking, nitration may induce a gain or a loss of protein function. Following this rationale, intragastric protein nitration was tracked. Pepsin, the major gastric protease, and its zymogen, pepsinogen, were shown to be nitrated by nitrite in the rat stomach. Pepsinogen (at pH 7.4) and pepsin (at pH 2) nitration correlated with the improvement of both NSAIDs- and pentagastrin-induced ulcers. This observation is in accordance with data *in vitro*, showing that nitration decreases the proteolytic function of pepsin. In a scenario of peptic ulcers, the erosion of gastric mucosa by pepsin is inhibited when this protease is nitrated by dietary nitrite. Using a model of germ-free mice and a $^{\bullet}NO_2$ scavenger (urate) it is shown that gut microbiota, $^{\bullet}NO$ and $^{\bullet}NO_2$ are key players in intragastric nitration reactions.

The work presented herein establishes mechanistically, in humans, the reduction of dietary nitrite to $^{\bullet}NO$ by polyphenols in the stomach with different functional implications. Firstly, suggests that hence formed $^{\bullet}NO$ may induce local vasodilation and secondly adds a new concept to the field of nitrite biology by showing that dietary nitrite induces nitration of endogenous proteins with impact on the prognostics of gastric ulcers. From the biomedical viewpoint, this work contributes to open new therapeutic approaches whereby anti-ulcerogenic and smooth muscle relaxant compounds are dietary-driven but endogenously generated as a consequence of the rich nitrite:nitric oxide biochemistry at the acidic gastric pH.

CHAPTER 1

GENERAL INTRODUCTION

1. Nitric Oxide

1.1. Historical overview

Until the 1980's, nitric oxide (•NO) was known as an atmospheric pollutant with no relevance for human physiology. However, during the following decade, several independent groups made seminal observations that underpinned the important physiological functions of this free radical. Firstly, Furchgott and Zawadski observed that the vasodilatory effect of acetylcholine was dependent of either an intact endothelium or of a factor that was essential for muscular relaxation entitled *Endothelium Derived Relaxing Factor* (EDRF) [1]. In subsequent years, soluble guanylate clyclase (sGC) and cyclic guanylate monophosphate (cGMP) were identified as a target and an intermediate, respectively, of EDRF-dependent actions [2-3]. Still in the 80's, Moncada and Ignarro's groups showed that EDRF was •NO [4-5] and Palmer and co-workers demonstrated that L-arginine was the precursor of •NO in endothelial cells [6]. This observation, added to the discovery that glutamate induced an increase in cGMP levels [7] and that •NO activated cerebral sGC [8], led in 1989 to the understanding of the cascade whereby glutamate activates N-methyl-D-aspartate receptors (NMDAR) with subsequent production of •NO in the brain. Once formed, •NO is able to activate sGC that converts guanylate triphosphate (GTP) to cGMP, triggering downstream signaling cascades [9].

Thus, by the early 1990's strong evidence supported a physiological role for $^{\bullet}NO$ ranging from vasodilatation [2-3], neuromodulation [9] and immune response mediation [10-11].

1.2. Chemical and physical properties

Despite the copious amounts of studies devoted to its biochemistry and physiology, $^{\bullet}NO$ is still a puzzling molecule. At room temperature and atmospheric pressure $^{\bullet}NO$ is a colorless gas with modest solubility in water: at physiological temperature (37ºC) c.a. 1.63 mM can be measured [12]. Structurally, $^{\bullet}NO$ is a simple diatomic molecule capable of diffusing many cell diameters *in vivo* due to its hydrophobicity and small size. These characteristics make of $^{\bullet}NO$ a *sui generis* signaling molecule as it does not allow reactions on basis of structural complementary and, thus, does not interact specifically with membrane-bound receptors. Indeed, it diffuses in the lipidic phase of cell membranes and triggers signaling events intra or extracellularly (for a recent review see [13]). Furthermore, in addition to this peculiar features, $^{\bullet}NO$ is a free radical, meaning that its valence orbital is occupied by one single electron (it has 11 valence electrons with one unpaired electron in the antibonding π orbital) [12]. This unpaired electron often confers high reactivity but this is not quite the case for this molecule. In fact, important misconceptions regarding $^{\bullet}NO$ have been growing over time, as it is commonly referred as a highly reactive and short lived molecule. The reactivity of $^{\bullet}NO$ depends on the specific biological setting being investigated; for instance, inside a blood vessel its half life is c.a. 2 milliseconds while inside a cell can reach 2

seconds [14]. In the central nervous system the half-life on $^{\bullet}$NO has been estimated to be around 0.4-0.7s [15]. Thus, $^{\bullet}$NO is a molecule that conveys information associated to its concentration and its biological effects that depend on both, the rate and site of production (steady state concentration) but also on its consumption (availability of molecular targets, oxygen tension (pO_2) and diffusion). That is, $^{\bullet}$NO dynamics (the rate and pattern of $^{\bullet}$NO change in time), determines its biological effects and may be the answer to the already classical question: what determines the physiologic and pathophysiologic actions of $^{\bullet}$NO?

The dynamics of $^{\bullet}$NO production, reactivity and biological effects will now be briefly addressed.

1.3. Nitric oxide production: *nitric oxide synthases*

In the early 1990's it was demonstrated that $^{\bullet}$NO is produced endogenously by an enzyme, nitric oxide synthase (NOS), in a tightly regulated manner [16]. NOS catalyzes the oxidation of L-arginine to L-citrulline and $^{\bullet}$NO. In addition to L-arginine, the reaction requires several co-factors as well as oxygen and nicotinamide adenine nucleotide phosphate (NADPH) as co-substrates [17].

Three isoforms of NOS, product of distinct genes, have been described. Two of them are constitutive – endothelial-NOS (eNOS or NOS III) and neuronal-NOS (nNOS or NOS I) – and are Ca^{2+} and calmodulin dependent. These isoforms were first localized in endothelial cells and neurons, respectively, and are assumed to be responsible for the production of

low concentrations of $^{\bullet}NO$ (ranging from pM to nM) [16, 18]. The third isoform is the inducible-NOS (iNOS or NOS II), that requires cytokine- or endotoxin- dependent activation of immune cells, such as macrophages, monocytes and neutrophils to be activated [16]. In contrast to the constitutive isoforms, iNOS is Ca^{2+}- and calmodulin- independent and produces higher and longstanding $^{\bullet}NO$ concentrations as long as both L-arginine and co-factors are available [19]. This aspect is critical for the antimicrobial and antitumorigenic properties of iNOS-derived $^{\bullet}NO$.

Despite being the product of different genes and having different locations, catalytic properties and sensitivities to inhibitors, all NOS isoforms are expressed as homodimers. Each monomer consists of two domains: an oxygenase (N-terminal) and a reductase (C-terminal) domain, linked by a polypeptide which is the calmodulin binding site. The oxygenase domain contains the binding sites for iron protoporphyrin IX, tetrahydrobiopterin and L-arginine whereas the reductase moiety contains the binding sites for flavin adenine dinucleotide (FAD), flavin mononucleotide (FMN) and NADPH.

The binding of Ca^{2+}:calmodulin works as a “molecular trigger”, coupling the enzyme, that allows the electron flow from the reductase towards the heme. This facilitates the oxidation of L-arginine to citrulline and $^{\bullet}NO$ (reviewed in [20]).

1.4. Biological effects of nitric oxide

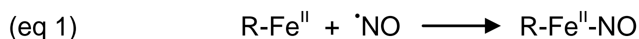
The regulation of $^{\bullet}NO$ actions encompasses two levels, the synthesis *via* the family of NOS and the chemistry upon diffusion in a biological milieu [21]. Regarding the chemistry, one may distinguish between direct and indirect effects. The former includes the interactions between $^{\bullet}NO$ and specific biomolecules fast enough to occur *in vivo*, usually at low $^{\bullet}NO$ fluxes (< 1 μM) whereas the indirect actions include the intermediary formation of products arising from the interaction of $^{\bullet}NO$ with oxygen or superoxide radical ($O_2^{\bullet-}$), associated with high $^{\bullet}NO$ fluxes (> 1 μM) [21]. This categorization allows taking into account two critical aspects of $^{\bullet}NO$ reactivity in a given biological setting: its steady state concentration and time of exposure of a certain target.

1.4.1. Direct effects

These reactions include those that occur between $^{\bullet}NO$ and specific biomolecules. Within the scope of these effects, the interactions with the following molecules are included:

- Metalloproteins and in particular hemeproteins (such as sGC and Cytochrome c Oxidase (CCO)). By far the most recognized signaling pathway for $^{\bullet}NO$ is its interaction with sGC. Nitric oxide binds to the ferrous (Fe^{2+}) heme of the protein forming a 5-coordinated nitrosyl complex by removal of a distal histidine residue, rendering the enzyme active [22-24] (for a general example see eq 1). This

conformational alteration allows the production of cGMP that through a cascade involving different protein kinases is responsible for the regulation of the vascular tone, platelet function and neuromodulation [25].


(eq 1) $R\text{-}Fe^{II} + {}^{\bullet}NO \longrightarrow R\text{-}Fe^{II}\text{-}NO$

- Oxygen metal complexes (such as oxyhemoglobin). The most important example is the reaction of ${}^{\bullet}NO$ with oxyhemoglobin (eq 2). Given the high rate of reaction between these two molecules and the high concentration of oxyhemoglobin, this is the principal route of ${}^{\bullet}NO$ removal. In this reaction, in addition to nitrate (NO_3^-), methemoglobin (ferric (Fe^{3+}) heme) is also produced [26-27].

(eq 2) $Hb\ (Fe^{II}\text{-}O_2) + {}^{\bullet}NO \longrightarrow metHb\ (Fe^{III}) + NO_3^-$

- Metallo-oxo complexes (such as catalase). These complexes, with high valence states, are powerful oxidants produced from the oxidation of metal complexes [28]. Nitric oxide has the ability to decrease their valence state, preventing further oxidative damage [29-30].
- Other radicals (radical-radical combination). An obvious example is the reaction of ${}^{\bullet}NO$ with alcoxyl ($LO^{\bullet}$) and peroxyl ($LOO^{\bullet}$) radicals produced during lipid

peroxidation (eq 3). Nitric oxide reacts with these radicals as soon as both species meet (k~10^9 $M^{-1}s^{-1}$) to terminate the propagation of lipid peroxidation [31].

(eq 3) $LOO^{\bullet} + {}^{\bullet}NO \longrightarrow LOONO$

1.4.2. Indirect effects

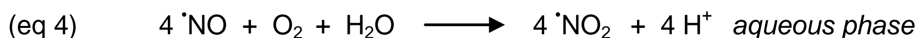
As stated above, these effects are not mediated by $^{\bullet}NO$ *per se* but instead by different Reactive Nitrogen Oxide Species (RNOS) produced from the reaction of $^{\bullet}NO$ with either oxygen or $O_2^{\bullet-}$. The corollary is the induction of post-translational modifications, including oxidation, nitration, nitrosation, nitrosylation and gluthatiolation of biomolecules. These modifications would be the outcome of RNOS-induced signaling cascades, impacting on the function of their targets, namely proteins and lipids.

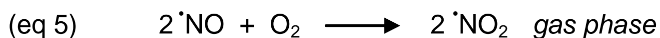
Both $^{\bullet}NO$ reactions, with oxygen and $O_2^{\bullet-}$, will be addressed separately.

- Nitric oxide interaction with molecular oxygen (auto-oxidation)

Nitric oxide oxidation involves a wide range of reactions not only between $^{\bullet}NO$ and oxygen but also with other RNOS. The principal is the trimolecular reaction between $^{\bullet}NO$ and oxygen, yielding nitrogen dioxide radical ($^{\bullet}NO_2$). This radical is formed both under gas or liquid phases, but whereas in the first it may dimerize to yield dinitrogen tetraoxide (N_2O_4,

that decomposes into NO_2^- and NO_3^-) in the second, this reaction is unlikely as $^{\bullet}NO_2$ is rather unstable in water (eq 4 – 5).


(eq 4) $4\,^{\bullet}NO + O_2 + H_2O \longrightarrow 4\,^{\bullet}NO_2 + 4\,H^+$ *aqueous phase*

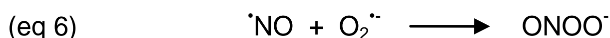

(eq 5) $2\,^{\bullet}NO + O_2 \longrightarrow 2\,^{\bullet}NO_2$ *gas phase*

Under these conditions, $^{\bullet}NO_2$ may combine with $^{\bullet}NO$ producing dinitrogen trioxide (N_2O_3, which upon hydrolysis yields NO_2^-). The rate of reaction is slow and it depends on the square concentration of $^{\bullet}NO$ and the concentration of oxygen, in an overall third order rate constant [32]. Moreover, oxygen has two unpaired electrons with the same spin in the valence orbitals (is a di-radical). In order to oxidize other molecules, oxygen would have to accept two electrons with a parallel spin to fit in the orbital otherwise one of the electrons would have to change spin. Since such a transition is not kinetically favorable, oxygen is not highly reactive [33]. For these reasons, $^{\bullet}NO$ auto-oxidation is probably not relevant *in vivo* unless the fluxes of both $^{\bullet}NO$ and oxygen increase above ordinary values. Curiously, this observation underpins one apparently paradoxical aspect on $^{\bullet}NO$ biochemistry: as the $^{\bullet}NO$ flux increases, its half life diminishes. For low levels, auto-oxidation is underappreciated, and $^{\bullet}NO$ can diffuse away from the site of production. On the contrary, for higher $^{\bullet}NO$ levels, auto-oxidation will become significant under biological settings and the production of RNOS will increase, giving protagonism to the indirect effects of $^{\bullet}NO$ (reviewed in [21]). In addition, it is important to note that since $^{\bullet}NO$ and oxygen are more

soluble in hydrophobic environments and that both can diffuse similarly in the same extent, $^{\bullet}NO$ oxidation is favored under hydrophobic conditions [34]. Therefore, lipid layers are the primary site for $^{\bullet}NO$ oxidation *in vivo*. One interesting aspect regarding this particular environment is the absence of water which will inhibit N_2O_3 hydrolysis and consequently stabilize this molecule which, as will be discussed below, is a mild oxidant (E^o= 0.8 V) but a powerful nitrosating agent [35].

- Nitric oxide interaction with superoxide radical

Nitric oxide combines by radical-radical interaction with $O_2^{\bullet -}$ (the product of the univalent reduction of oxygen) at a diffusion controlled rate to produce peroxynitrite anion ($ONOO^-$) [36-37] (eq 6); this is one of the fastest non-catalyzed reactions known (k= 16-20x10^9 $M^{-1}s^{-1}$ [38]) and it means that virtually every time that a molecule of $^{\bullet}NO$ encounters one of $O_2^{\bullet -}$, $ONOO^-$ is formed.



(eq 6) $$^{\bullet}NO + O_2^{\bullet -} \longrightarrow ONOO^-$$

The only species that can efficiently compete with $^{\bullet}NO$ for $O_2^{\bullet -}$ is superoxide dismutase (SOD, the enzyme that detoxifies/removes $O_2^{\bullet -}$), but with a slightly lower rate constant (k= 2x10^9 $M^{-1}s^{-1}$) which means that $ONOO^-$ is still formed in the presence of this enzyme (for a recent review see [39]). In addition to this kinetic constrain, in physiological conditions $O_2^{\bullet -}$ dismutation is probably less efficient than *in vitro* as chloride anions screen the

electrostatic field that attracts $O_2^{\bullet-}$ to the active site of the enzyme [40-41]. Consequently, the formation of $ONOO^-$ is even more likely *in vivo* than *in vitro*.

At physiological pH, $ONOO^-$ is unstable due to the equilibrium with peroxynitrous acid (ONOOH, pKa=6.8) which decomposes into hydroxyl radical-like species ($^\bullet OH$) and $^\bullet NO_2$ [42] (eq 7 – 8).

(eq 7) $ONOO^- + H^+ \longrightarrow ONOOH$

(eq 8) $ONOOH \longrightarrow {}^\bullet NO_2 + \text{“}^\bullet OH\text{”}$

In fact, the homolytic cleavage of the O-O bond seems unlikely and instead, ONOOH yields an intermediate with the reactivity of $^\bullet OH$ [43]. The formation of $^\bullet OH$ or an intermediate with its characteristics is one of the main issues related to ONOOH physiopathology. However, this species is too reactive to be highly toxic. That is to say, $^\bullet OH$ reacts randomly with every single molecule it encounters, being a critical or non-critical cellular component. Expectedly in a test tube, $^\bullet OH$ will damage the compound one adds (e.g. DNA, protein), but in more complex media (ranging from cell culture to a living tissue) $^\bullet OH$ behaves, in Beckman JS words, as a *promiscuous radical* reacting with whatever molecule. Therefore, there is a low probability to affect critical cell components [43]. This is an example of how highly reactive species are not necessarily highly toxic.

Nevertheless, the potent oxidant profile of $ONOO^-$ is well established and, essentially, in it relies the key aspects of $^\bullet NO$ indirect effects. At a molecular level, $ONOO^-$ oxidizes low

molecular weight compounds (cysteine, gluthatione, tyrosine, α-tocopherol, among others), proteins (such as albumin, myeloperoxidase, aconitase, SOD), polyunsaturated fatty acids and DNA. Furthermore, it also has an important impact at subcellular level (by inhibiting mitochondrial respiration, due to the inactivation of the electron transport chain components, and consequently decreasing ATP synthesis), cellular (as a cytotoxic agent) and organ levels (pulmonary emphysema, acute lung injury, atherogenesis and neuroexcitotoxicity) [21, 39, 42]. Indeed, peroxynitrite has been implicated in the etiology of disorders such as diabetes, cancer, inflammation, cardiac, vascular and neurodegenerative diseases [39, 42].

- Nitrosation and nitration reactions

Nitrosation results from the reaction of a nitrosonium ion (NO^+) with a nucleophile, such as thiols and amines. Nitrosothiols are found *in vivo,* exhibiting vasodilatory properties and having half-lives of c.a. 40 minutes [44]. It has been emphasized the potential interchange of $^{\bullet}NO$ with NO^+ which, by opposition with $^{\bullet}NO$, is able to nitrosate thiols. Physiologically, this is of particular interest as nitrosothiols are not *inactivated* by hemoglobin and therefore may "propagate" the biological effects of $^{\bullet}NO$ [44]. Indeed nitrosohemoglobin, nitrosoalbumin and nitrosogluthatione have been implicated as vasorelaxants, antimicrobial agents and key components of other redox signaling events [21, 45-47].

There are several pathways that may account for the formation of nitrosothiols: 1) reaction of $^{\bullet}NO$ with thyil radicals (eq 9), 2) transnitrosation (transfer of a NO^+ equivalent among low molecular weight thiols or thiols within proteins), 3) nitrosation by N_2O_3 (eq 10) and 4) transfer of a NO^+ group from metal nitrosyls. Although both the precise mechanisms of formation of nitrosothiols and $^{\bullet}NO$ signal transduction are still unclear, it is generally accepted that, at the bench, they are labile molecules as they can perish due to a wide range of factors, such as metal ions, enzymatic, thermic and photochemical decays [48].

(eq 9) $RS^{\bullet} + {}^{\bullet}NO \longrightarrow RSNO$

(eq 10) $N_2O_3 + RSH \longrightarrow RSNO + H^+ + NO_2^-$

In addition to nitrosation, the indirect effects of $^{\bullet}NO$ include also other post-translational modifications, being nitration one of most important in a biological setting. Since this modification occurs *in vivo*, in a physiological time scale and may have profound biochemical implications in the structure of both proteins and lipids (inducing gain or loss of function in the first case and producing anti-inflammatory signaling molecules in the second), with putative pathophysiological consequences [49-51], special attention will, from now on, be paid to such effect.

Nitrated proteins have been detected under physiological conditions but most importantly, in several diseases. The scope of disorders that exhibit increased levels of nitrated

proteins is fairly broad, ranging from cardiovascular and neurodegenerative to metabolic distresses [49, 52-53].

Protein nitration is a post-translational modification characterized by the insertion of a nitronium ion (NO_2^+) in the *ortho* position of a phenolic ring of aminoacids, typically tyrosine (Tyr) even though it may also occur in other aminoacids, such as tryptophan. This is a strong covalent bond that is established stepwisely: first, Tyr is univalently oxidized yielding tyrosil radical ($Tyr\text{-}O^{\bullet}$) and second, a NO_2^+ group is inserted in the ring. Several oxidants may oxidize Tyr to $Tyr\text{-}O^{\bullet}$, namely $^{\bullet}OH$, $CO_3^{\bullet -}$ (carbonate radical) and $^{\bullet}NO_2$ itself (reviewed in [52]). Studies in the early 1990s, demonstrated that $ONOO^-$ could nitrate tyrosine, due to its decomposition into several oxidizing and nitrating agents. Though, other pathways are known to induce Tyr nitration [49]. Figure 1.1 summarizes the biological effects of $^{\bullet}NO$.

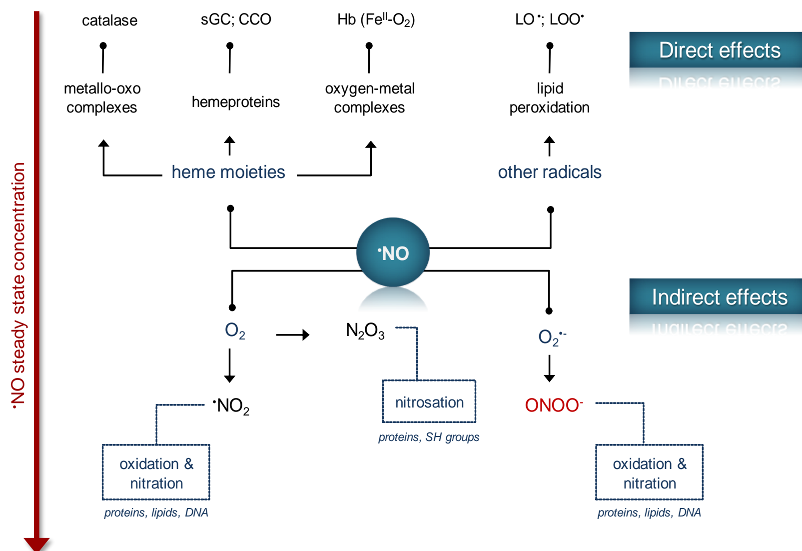


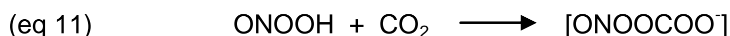
Figure 1.1 – Biological effects of $^{\bullet}NO$. Nitric oxide participates in both direct and indirect reactions depending on its concentration dynamics, target availability and O_2 concentration.

- Many roads to nitration *in vivo*

The diverse $^{\bullet}$NO-dependent pathways that can induce protein tyrosine nitration, strongly suggest that this modification is a crucial pathway in $^{\bullet}$NO signaling. In fact, nitrotyrosine (NT) has been detected in several disorders, including atherosclerosis, Alzheimer's, diabetes and inflammatory conditions [54-56]. Indeed, several nitrating pathways are thought to operate simultaneously or at different time points of disease progression. Therefore, the nitrating species will depend on aspects such as, the nitrogen oxides being produced, kinetics, compartmentalization, presence of inflammatory cells (that enhance nitration) and scavengers of nitrating agents (e.g. antioxidants) [57]. Due to this complexity, it is imperative to take into consideration that a wide range of species may contribute to nitration reactions.

- Tetranitromethane (TNM). It was the first compound used to nitrate Tyr residues *in vitro*, even before $ONOO^-$ was unraveled as a nitrating agent. TMN was used to nitrate Tyr residues in order to study the function of this aminoacid within proteins but lacks biological significance [58].
- Peroxynitrite. This anion, due to the decay in oxidizing and nitrating radicals, is the likely candidate to mediate protein nitration *in vivo*. It was a hitherto unrecognized cytotoxic molecule until the beginning of the 1990's when its strong oxidizing properties towards sulfhydryls were reported [59-60]. *In vivo*, in addition to $^{\bullet}$OH

and $^{\bullet}NO_2$, $ONOO^-$ reacts with CO_2 (present in mM range) yielding the intermediate nitrosoperoxycarbonate ($ONOOCOO^-$) (eq 11). Approximately 30% of $ONOOCOO^-$ decays in carbonate ($CO_3^{\bullet-}$) and $^{\bullet}NO_2$ radicals (eq 12). The former is an efficient oxidant of Tyr, thereby favoring the formation of Tyr-O$^{\bullet}$ and thus facilitating protein nitration (reviewed in [42]).

(eq 11) 

$$ONOOH + CO_2 \longrightarrow [ONOOCOO^-]$$

(eq 12)

$$[ONOOCOO^-] \longrightarrow CO_3^{\bullet-} + {}^{\bullet}NO_2$$

- Nitrogen dioxide radical ($^{\bullet}NO_2$). This is by far the first species that comes to one mind when thinking on protein nitration. The explanation relies on the fact that $^{\bullet}NO_2$ is both an oxidizing and a nitrating agent, that is to say, it can oxidize Tyr to Tyr-O$^{\bullet}$ and react with the latter yielding NT [52, 61]. However, *in vivo* this may not be that straightforward. Firstly, $^{\bullet}NO_2$ is not an efficient oxidant of Tyr (the rate limiting step on nitration) and secondly, both $^{\bullet}NO_2$ and Tyr-O$^{\bullet}$ may be easily scavenged (for instance, by ascorbate and cysteine) [49]. This pathway may be facilitated within hydrophobic environments because both $^{\bullet}NO$ and oxygen concentrate here and the steady state concentration of $^{\bullet}NO_2$ is likely to increase [34].

- Reaction of $^{\bullet}NO$ with Tyr-O$^{\bullet}$. The product of this reaction is nitrosotyrosine [62], a quite unstable nitrosophenol that nonetheless is able to be further oxidized to NT [63-64].
- Myeoloperoxidase/Hydrogen peroxide systems (MPO/H_2O_2). MPO is a peroxidase abundantly expressed in neutrophil granulocytes. It can utilize nitrite as substrate and, in the presence of H_2O_2, nitrate proteins. MPO is oxidized by H_2O_2 yielding compound I which is a strong oxidant. In turn, compound I may oxidize nitrite to $^{\bullet}NO_2$. In addition, chloride anion oxidation by MPO/H_2O_2 originates hypochlorous acid (HOCl) that upon reaction with nitrite produces nitryl chloride (NO_2Cl), a powerful nitrating agent. Furthermore, MPO/H_2O_2 system can also oxidize Tyr to Tyr-O$^{\bullet}$, thereby yielding not only the nitrating species ($^{\bullet}NO_2$, NO_2Cl) but also the targets for nitration (Tyr-O$^{\bullet}$) [65-66].
- Nitrite anion. The acidification of nitrite, resulting in the production of nitrous acid (HNO_2) also culminates in protein nitration [67]. Commonly, Tyr nitration by this pathway is accompanied by other modifications. In the case of albumin, lysine, arginine and histidine are lost during the exposure to acidified nitrite [68]. In addition, if the target protein is rich in cysteine residues, exposure to nitrite at low pH will induce the formation of nitrosocysteine [69]. Finally, and by contrast to $ONOO^-$, nitrite-dependent nitration is a kinetically slow process. In addition to these cons, the role of nitrite anion as a nitrating agent *in vivo* has been overlooked due to the need of a considerable drop in pH ($pH < 6$) to generate

nitrating agents, a condition that is not often observed under physiological conditions. An important exception however, is the human stomach, in which the pH can fall down to 0.8.

Given the diverse pathways for nitration, it seems nevertheless intriguing that such (apparently) random reactions can yield species able to nitrate specific proteins. Indeed protein nitration is a surprisingly selective modification and not only not all proteins are nitrated nor all Tyr residues suffer equally this modification. Studies report that only 1-5 out of 10 000 Tyr residues are nitrated *in vivo* [52, 70]. It is thus difficult to predict what protein is prone to nitration; but some features can let one make a rough prediction. The abundance of a certain protein or the amount of Tyr residues can give a hint on the probability of a protein to be nitrated. However, this is not a rule of a thumb since, for instance, albumin (the most abundant protein in plasma) is nitrated but at the same extent as other proteins that are much less abundant in this specific environment. Other factors that may modulate protein nitration are the protein folding (Tyr residues exposed to the solvent are more likely to be nitrated), the neighbor aminoacids (some aminoacids such as glutamate may enhance Tyr nitration) and the nitrating species itself (reviewed in [71]).
Regarding the degradation of nitrated proteins, some reports show that they can be ubiquitinated and degraded by the proteasome or activate the immune system, for antibodies against nitrated proteins (specifically nitrated α-synuclein) have been detected [72-73].

The first nitrated protein identified *in vivo* was MnSOD in 1994 by McMillan Crow [74]. This group demonstrated that this protein was nitrated in rejected human kidney allografts. This finding was remarkable as further studies showed that nitration of MnSOD decreased its function, that is to say, the dismutation of $O_2^{\bullet-}$ was impaired, increasing the steady state concentration of this radical. As consequence of such protein nitration, the production of $ONOO^-$ would be enhanced as the reaction of $O_2^{\bullet-}$ with $^{\bullet}NO$ is favored. Of note, the biological impact of the nitration of MnSOD is connected with the fact that it triggers an auto-catalytic cycle because as the concentration of $ONOO^-$ increases, so the rates of nitration increase, leading to further decrease of MnSOD function and more availability of $O_2^{\bullet-}$ for reaction with $^{\bullet}NO$ and formation of $ONOO^-$. This is a classical example as protein nitration may induce structural and functional changes in critical proteins, with impact on pathophysiology.

2. Nitrite

2.1. Historical overview

Despite all the recognized actions of •NO *in vivo,* the biological impact of this gaseous free radical is actually quite recent when compared with that of its oxidation product, nitrite. Nitrite has been known to underlie vasodilatory effects since 1880 [75], an activity that was later suggested to involve the interaction with sGC [76-77]. Nevertheless, due to the fact that the (high) concentrations needed to induce vasodilatation are hardly achievable *in vivo*, pharmacological rather than physiological properties were assigned to nitrite during the last century. Given this scenario, the interest in this anion has been unpretentious and beside its role as a metastable intermediate of •NO oxidation [78], a water pollutant as well as an undesirable fertilizer residue, nitrite has never attracted much attention in the biomedical community as a health-promoting molecule [79]. On the contrary, since the 1960's, nitrite consumption was associated with formation of carcinogenic N-nitrosamines [80] and it has been regarded as a malicious agent to human health ever since. However, although intense research has been dedicated to establish a causal relationship between nitrite consumption and intragastric N-nitrosamine formation with consequent gastric cancer development in humans, such a relation has not been proved yet [81-82].

In the figure 1.2 the major milestones on the uses of nitrite through ages are represented.

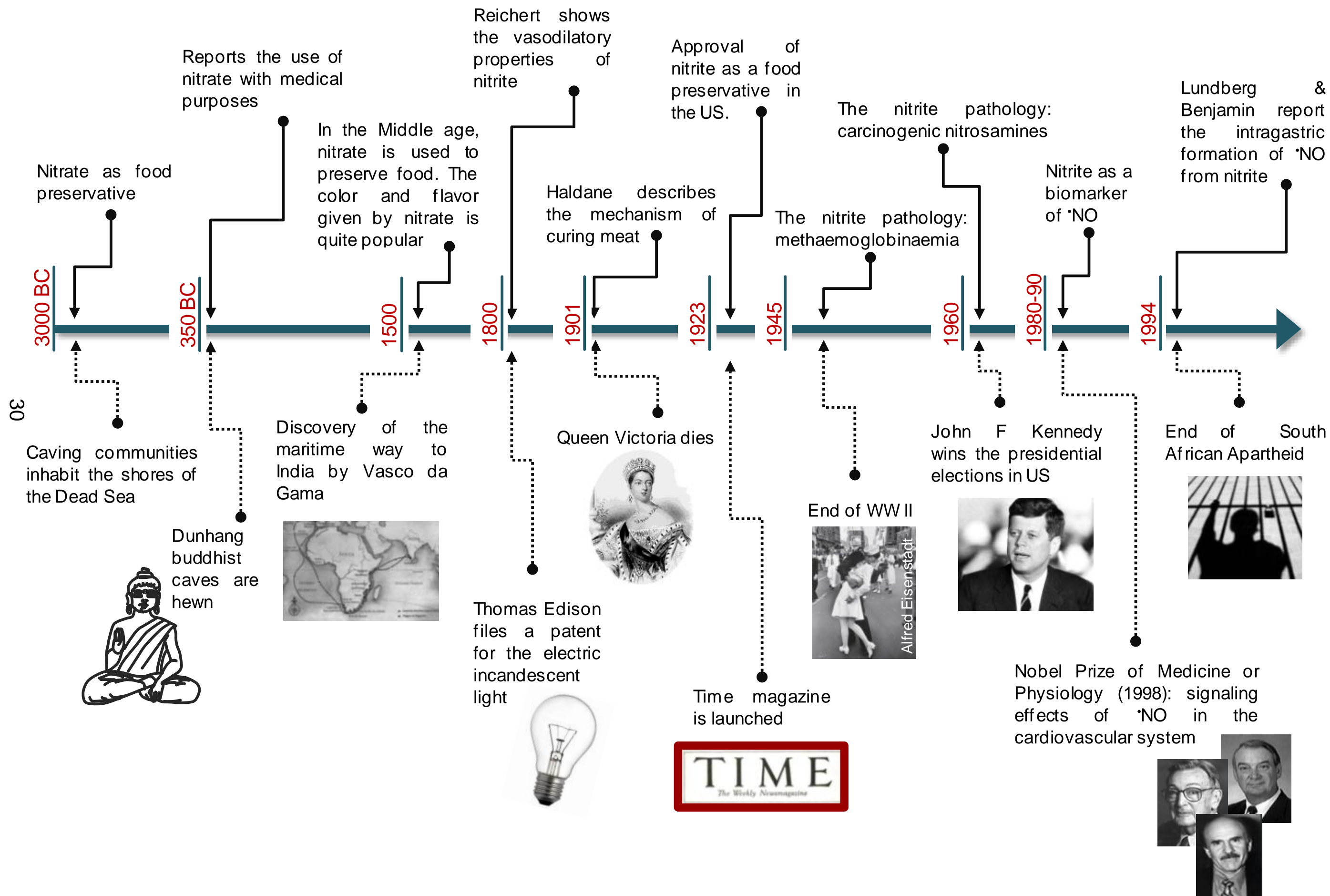

3000 BC
Nitrate as food preservative
Caving communities inhabit the shores of the Dead Sea
350 BC
Reports the use of nitrate with medical purposes
Dunhang buddhist caves are hewn
1500
In the Middle age, nitrate is used to preserve food. The color and flavor given by nitrate is quite popular
Discovery of the maritime way to India by Vasco da Gama
1800
Reichert shows the vasodilatory properties of nitrite
Thomas Edison files a patent for the electric incandescent light
1901
Haldane describes the mechanism of curing meat
Queen Victoria dies
1923
Approval of nitrite as a food preservative in the US.
Time magazine is launched
TIME
The Weekly Newsmagazine
1945
The nitrite pathology: methaemoglobinaemia
End of WW II
Alfred Eisenstadt
1960
The nitrite pathology: carcinogenic nitrosamines
John F Kennedy wins the presidential elections in US
1980-90
Nitrite as a biomarker of •NO
Nobel Prize of Medicine or Physiology (1998): signaling effects of •NO in the cardiovascular system
1994
Lundberg & Benjamin report the intragastric formation of •NO from nitrite
End of South African Apartheid

Figure 1.2 – Milestones of nitrite biology. The figure depicts a brief historical review on nitrite physiopathology, ranging from the times when it was used based on its salutary effects to the dark part of the history, when it was associated with serious distresses. The work shown in this thesis relies in the findings that came to light in 1994 and therefore, the contemporary facts on nitrite will be extensively addressed. The timeline is accompanied by key historical facts.

2.2. The biology of nitrite anion: from a noxious undesirable compound to a physiological messenger

It was not until 1994 that nitrite gained physiological relevance in relation to the gastric environment when two independent groups showed that nitrite-rich saliva generates $^{\bullet}$NO in the human stomach at a rate dependent on the pH and nitrite concentration [83-84]. In addition, nitrite-derived $^{\bullet}$NO was assigned with antimicrobial properties, thereby assigning physiological implications for this pathway.

Traditionally, nitrite has been viewed as a metastable intermediate of $^{\bullet}$NO oxidation to the more stable metabolite, nitrate. This pathway was thought to be irreversible under biological conditions, but a growing number of studies are uncovering an active recycle of nitrite to $^{\bullet}$NO along the oxygen gradient [85]. Indeed, several enzymes (such as deoxyhemoglobin, deoxymyoglobin, eNOS, xanthine oxidase, just to cite a few) have been shown to acquire a nitrite reductase activity and reduce nitrite to $^{\bullet}$NO under low oxygen tensions, precisely when the classical *L-arginine pathway* is failing [86-92]. For functioning as a storage pool for $^{\bullet}$NO, nitrite is now regarded as a critical player in hypoxic signaling [85]. It goes beyond the scope of this thesis to exploit the different pathways for $^{\bullet}$NO generation from nitrite. Instead, a special highlight will be made on the pathway that

unraveled nitrite as a $^{\bullet}NO$ donor, namely the acidic disproportionation of dietary nitrite in the stomach.

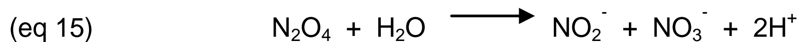
First, it urges to pinpoint the sources of nitrite in mammalian systems. There are three major sources of nitrite, namely 1) endogenous $^{\bullet}NO$ oxidation, 2) reduction of nitrate in the oral cavity by the local flora and 3) the diet [79, 93]. The latter is by far the major source of nitrite. Actually, green leafy vegetables (such as lettuce, broccoli and spinach) account for up to 80% of a daily ingestion of nitrate and only 16% of nitrite [94]. However, as it will be addressed bellow, dietary nitrate is reduced to nitrite by commensal oral bacteria [95-96], and therefore nitrite levels are intimately related with nitrate intake; in fact, this pathway contributes as much as 90% to the total intake of nitrite [97]. Cured meat and cereals also contribute significantly to the endogenous levels of nitrite [98]. The production of $^{\bullet}NO$ in the stomach encompasses nitrite protonation to HNO_2 and subsequent univalent reduction to $^{\bullet}NO$. Thus, this is a chemical rather than an enzymatic pathway and its description radically changed the paradigm that $^{\bullet}NO$ is solely produced enzymatically by NOS and is further oxidized to nitrite and nitrate.

It is now established that nitrite can be recycled back to $^{\bullet}NO$ and this pathway is maximized in the stomach, where surprisingly high concentrations of nitrite are achieved and indeed, the highest $^{\bullet}NO$ concentrations found in the living body occur in the stomach. Nevertheless, HNO_2 exists in a quite complex equilibrium with $^{\bullet}NO$, $^{\bullet}NO_2$ and N_2O_3 (eq 13 – 17) and the outcome of nitrite reduction will depend on different aspects such as, the redox microenvironment (*e.g.* presence of reductants such as ascorbate or glutathione,

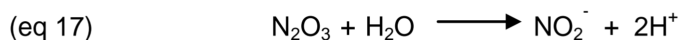
transition metal centres and oxygen levels), abundance of biotargets, pH and relative hydrophobicity of the milieu [99-100]. The biological chemistry of nitrite will be address in the topic *Biochemistry of intragastric nitrite*.

(eq 13) $$NO_2^- + H^+ \longrightarrow HNO_2$$

(eq 14) $$2\ {}^{\bullet}NO_2 \longrightarrow N_2O_4$$


(eq 15) $$N_2O_4 + H_2O \longrightarrow NO_2^- + NO_3^- + 2H^+$$

(eq 16) $${}^{\bullet}NO + {}^{\bullet}NO_2 \longrightarrow N_2O_3$$


(eq 17) $$N_2O_3 + H_2O \longrightarrow NO_2^- + 2H^+$$

Noteworthy, despite the implication of nitrite in diseases such as methemoglobinemia and gastric cancer (through the formation of N-nitrosamines), no link has been established between them. In addition, the potential beneficial effects of nitrite that have been being discovered over the past decade in different systems (depicted in figure 1.3) suggesting that this small anion could be indeed of therapeutic use.

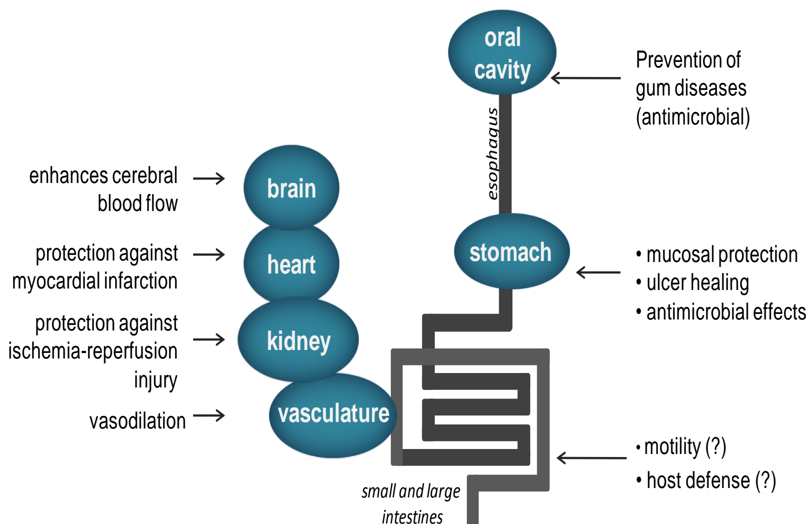


Figure 1.3 - Beneficial effects of nitrite-derived ˙NO that could support a therapeutic usage

Particularly in the stomach the chemical reduction of nitrite to ˙NO has been shown to exert several protective effects. Benjamin et al demonstrated that *Candida albicans* and *Escherichia coli* retain viability when exposed to acid alone (pH 3) but are killed by the combination of acid and nitrite at concentrations as low as 250 μM (nowadays known to be physiological) [83]. This pioneer work highlighted the importance of non-enzymatic ˙NO as a preventive agent of gastrointestinal infections. Moreover, in 1998 Dykhuizen and co-workers demonstrated that 1 mM of acidified nitrite eradicates *Helicobacter pylori* cultured from gastric biopsies after 30 minutes of incubation [101]. These results show that concentrations of nitrite easily achievable *in vivo* have antimicrobial effects in the stomach, affecting different strains of bacteria, including the ulcerogenic pathogen *H. pylori*.

Evidence suggests that intragastric $^{\bullet}$NO generation is involved also in other important physiological processes [102-104]. For instance, Petersson et al have observed that rats treated with nitrate exhibit increased gastric mucosal blood flow and mucus thickness [104]. In fact, the gastric mucus layer has long been implicated in mucosal protection since it became clear that NSAIDs and *Helicobacter pylori* inhibit mucin production and decrease mucus thickness [105-109]. Since a higher blood supply and mucus production have been shown to protect the gastric mucosa, nitrite-derived $^{\bullet}$NO is regarded as a gastroprotective molecule.

In line with such protective effects of ingested nitrate, it was recently shown that the gastroprotective and blood pressure lowering effects of dietary nitrate are abolished by an antiseptic mouthwash [110], revealing that the bioactivation of nitrate is dependent on oral nitrate reducing bacteria.

Recent reports have also attributed a proactive role for nitrite-derived $^{\bullet}$NO in the protection of gastric ulcers. In fact, rats ingesting nitrate on a daily basis showed not only an increase of $^{\bullet}$NO levels in the gastric headspace but also a reduction of acute gastric ulceration induced by diclofenac [111]. This study supports that the regular consumption of nitrite may prevent inflammatory processes in the gastric compartment.

Some of these effects are depicted in figure 1.4.

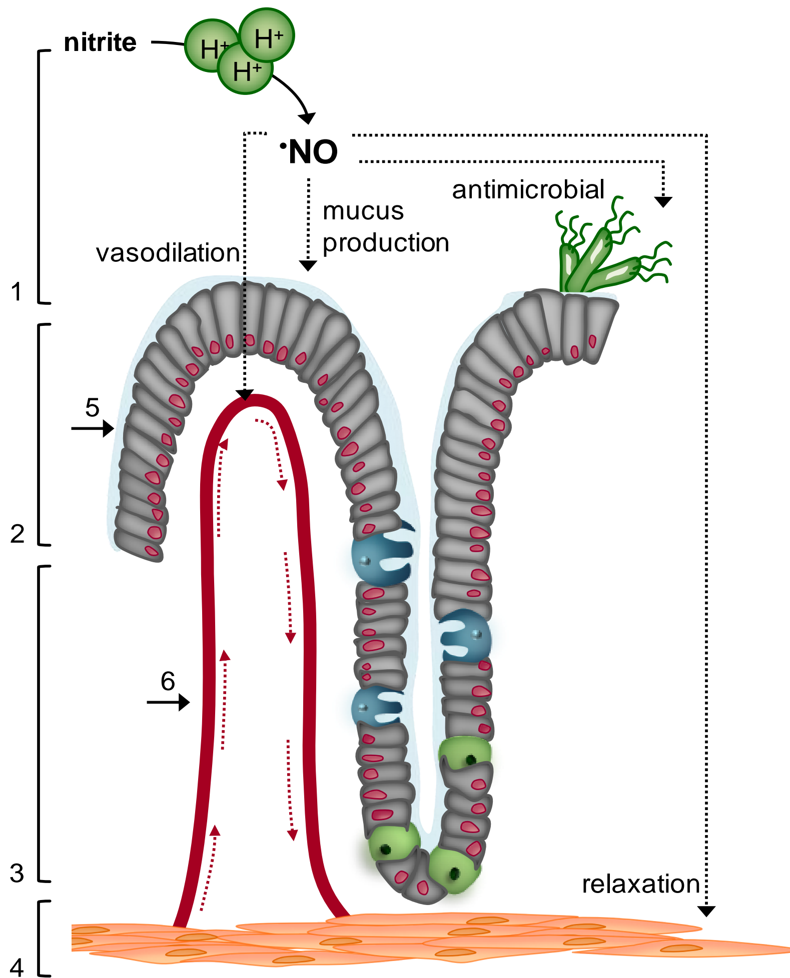


Figure 1.4 – Effects of nitrite-derived nitric oxide in the stomach. The cartoon is a simplified scheme of the gastric wall showing 1- gastric lumen, 2- epithelium and gastric pit, 3- gastric gland, 4- smooth muscle (muscularis mucosa), 5- mucus layer, 6- blood vessel. Adapted from [112].

2.3. Entero-salivary circulation of nitrate

It has been known for many years that nitrite and nitrate are present in human saliva [113]. Studies with $^{15}NO_3^-$ showed that, both in humans and rats, nitrate is absorbed in the upper small intestine and in the systemic circulation adds to the nitrate arising from the endogenous $^{\bullet}NO$ oxidation [114-115]. The increase in plasma nitrate is observed after a nitrate rich meal, peaking 30 minutes after the mealtime and being up for several hours [116]. Roughly, 75% of nitrate is excreted by the kidneys, but vestigial amounts can also be expelled in sweat, feces and exhaled breath [117]. The remaining nitrate, about 25%, is taken up by the salivary glands and secreted into the oral cavity by an active transport mechanism, not fully understood [95, 114-115]. For this reason, the concentration of nitrate in saliva is at least 10-times higher than in blood, reaching c.a. 2-10 mM [79, 118].

Since facultative anaerobic bacteria - that use nitrate instead of oxygen as an electron acceptor - are abundant in the posterior tongue clefts, about 20% of the oral nitrate is here efficiently reduced to nitrite [95-96]. Due to this mechanism, the salivary concentration of nitrite rises from 50-300 μM under fasting, to 1-2 mM after a nitrate load (reviewed in [93]). This is the so-called entero-salivary circulation of nitrate, depicted in figure 1.5.

Once nitrite-rich saliva is swallowed, it encounters the acidic gastric juice, and is readily protonated to form HNO_2 (pKa= 3.3 – 3.4). Nitrous acid can further decompose into $^{\bullet}NO$ and $^{\bullet}NO_2$ and, in turn, these two radicals may combine to form N_2O_3 [83, 119]. Through

secondary reactions, this complex chemistry may yield again nitrite and nitrate that propagate the entero-salivary dynamics of these anions [120].

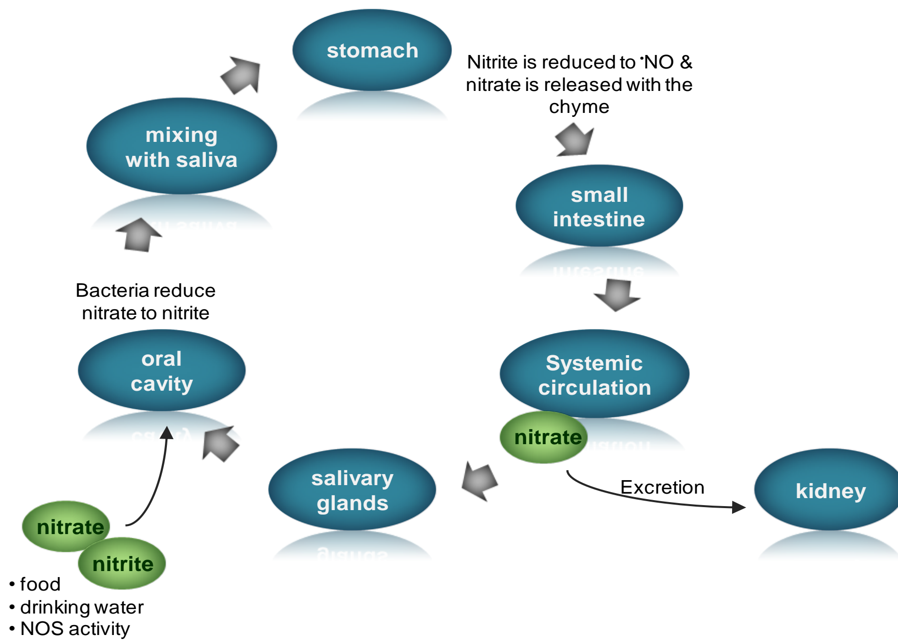


Figure 1.5 – The entero-salivary circulation of nitrate. Adapted from [93].

2.4. Biochemistry of intragastric nitrite

In 1930, long before $^{\bullet}NO$ was regarded as a signaling molecule, Abel et al described the decomposition equilibrium of HNO_2, involving the production of $^{\bullet}NO$, among other RNOS [121]. Also noteworthy, in 1938, Philpot and Small [122], elaborating on the nitroxide chemistry described by Abel, reported tyrosine nitration (at the present time recognized as such) in the presence of HNO_2. These are two extraordinary observations pointing not only to the potential presence of $^{\bullet}NO$ *in vivo* but to $^{\bullet}NO$-derived protein post-translational modifications, that have remained unappreciated in the scientific community for decades until the late 1980's, when $^{\bullet}NO$ was identified with the EDRF (as discussed earlier). Currently, it is known that the reaction conditions and biological processes that favour the formation of $^{\bullet}NO$ from nitrite may also result in the generation of secondary species, collectively known as RNOS, with the ability to induce post-translational modifications, such as protein tyrosine nitration [67-68].

Once protonated, nitrite yields HNO_2 that decomposes almost instantaneously into $^{\bullet}NO$ and $^{\bullet}NO_2$. In the presence of reductants, most of nitrite yields $^{\bullet}NO$ whereas in the absence of these species only 1% of the anion is converted to $^{\bullet}NO$, yielding $^{\bullet}NO_2$ as an important product. Nitric oxide and $^{\bullet}NO_2$ may combine to produce N_2O_3 that, in aqueous solutions hydrolyses to nitrite that can be once again protonated and converted to HNO_2, propagating this cycle of reactions [83, 99, 119, 123-125].

In the gastric compartment, given the high pO_2 (~70 torr [126]), $^{\bullet}NO$ auto-oxidation may also become relevant on the basis of nitrite biochemistry. It is easily conceivable that high fluxes of $^{\bullet}NO$ (as the ones obtained after a nitrate load, c.a. thousands ppb [84]) in the presence of such pO_2, would favour $^{\bullet}NO$ oxidation in both the aqueous and gas phases of the stomach. Finally, nitrite-dependent $^{\bullet}NO$ may also react with $O_2^{\bullet-}$ (both, the diet - and the activity of epithelial Duox [127] may account for $O_2^{\bullet-}$ production) yielding $ONOO^-$. At gastric acidic pH, ONOOH will be the predominant form and the CO_2 from the headspace, as well as the HCO_3^- from the mucus layer, favor the production of both oxidant and nitrating species.

Overall, the biochemistry of nitrite at acidic pH involves a complex equilibrium of several RNOS with intrinsic signaling properties, in which the outcome is dependent on different conditions, being the dietary intake the most surprising and perhaps, the most relevant.

As emphasized previously, the ability of acidified nitrite to induce post-translational modifications in endogenous proteins has already been forwarded from the biochemical but not from the physiological point of view. Furthermore, on the light of the knowledge at the time, such finding has remained largely obscure. Indeed, in 1938, Philpot and Small, on the quest for the specific activity of pepsin (the major gastric protease), noticed two fundamental aspects: they (…) *found that 1.7N $NaNO_2$ plus 1.7 N HCl added to a solution of crystalline pepsin (about 1 %) at 25º caused rapid formation of a pale yellow precipitate.* But more impressively, they further state that *very little* (peptic) *activity could be detected* and that (…) *the statement that pepsin is not inactivated by nitrous acid in a medium of*

hydrochloridric acid does not hold true for pure pepsin [128]. Nowadays, with the accumulating knowledge on RNOS biochemistry, pepsin nitration and inactivation by acidified nitrite can be foreseen.

3. Nitrite, nitric oxide & gastric physiology

3.1. Gastric physiology

Most text books and research papers dealing with gastric physiology begin by stating that "the stomach is a storage sac located between the esophagus and the duodenum" [129]. It is indeed located in the abdominal cavity, between the esophagus and the duodenum, and one of its functions is to store food, but is much more than that. The major *mechanical* functions are to store, churn and mix food with the gastric juice to produce the chyme. Also, through a complex system of muscle contraction and relaxation, it controls the release of this thick mixture into the duodenum and also has an important antimicrobial activity (due to the acidity of its juices). Besides, the stomach exhibits a highly specialized mucosa that secretes as many as twenty chemical messengers, including hormones, paracrine messengers and neurotransmitters [129]. The exocrine messengers, include those secreted into the gastric lumen, such as pepsin, and are collective known as gastric juices. The paracrine messengers are those produced and acting within the mucosa, being histamine one obvious example. The major endocrine secretion is gastrin that acts both locally in the stomach and systemically in the duodenum, pancreas and liver. These chemical messengers are produced by specialized cells of the gastric glands. The gastric glands arrange the oxyntic glandular region of the *fundus*, body (or *corpus*) and pyloric *antrum* (figure 1.6).

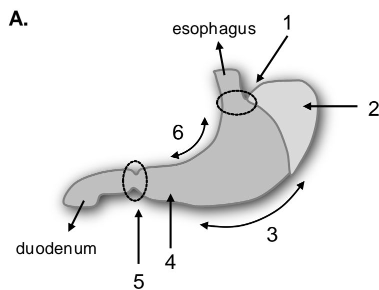
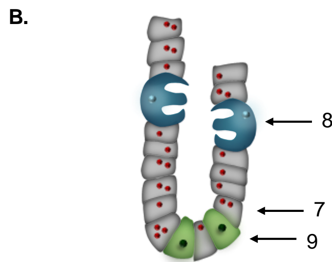


Figure 1.6 – Schematic anatomy of the stomach. A) Main gastric anatomic regions: 1- cardia, 2- fundus, 3- greater curvature, 4- pyloric antrum, 5- pyloric sphincter, 6- lesser curvature. B) Schematic representation of a gastric gland depicting 7- chief cells, 8- parietal cells and 9-enteroendocrine cells.



The gastric surface is characterized by the presence of rugae depending on the degree of distention. These rugae are lined by the gastric epithelium which invaginates, forming the gastric pits, at the bottom of which one or more gastric glands open. The glands comprise secretory cells responsible for the production of the aforementioned exocrine digestive juices (such as parietal and chief cells that produce acid and pepsinogen, respectively); these secretions are released to the gland duct and then conducted through the gastric pit to the stomach lumen. The gastric glands also produce other secretions such as histamine, produced by the enterochromaffin cells, somatostatin, produced by the D cells and gastrin produced by the G cells. The mediators released by these cells control many digestive functions including gastric secretion and motility. Details on gastric histology are shown in figure 1.7.

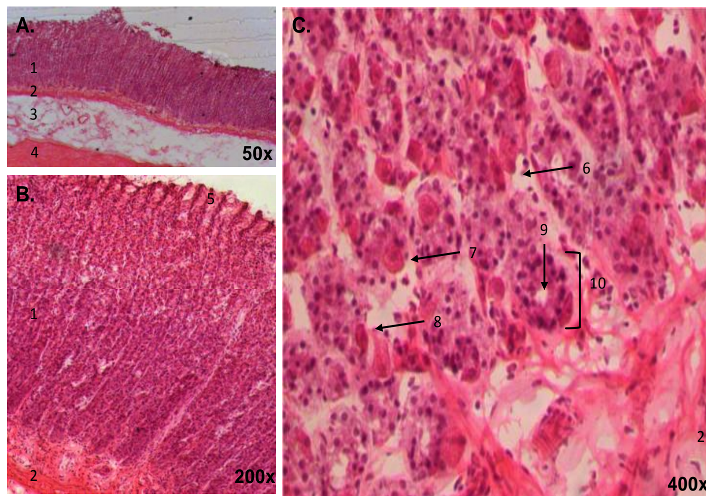


Figure 1.7 – Rat stomach histology (Hematoxylin & Eosin staining). A) Four histological layers can be distinguished: 1- mucosa, 2- muscularis mucosa, 3- sub-mucosa, 4- muscularis externa. B) Same picture as A) but with higher amplification. A well organized glandular mucosa (with glands in a longitudinal orientation) can be seen (1), as well as the gastric pits (5). C) Detail of the deep gastric mucosa; 6- extracellular matrix, 7- parietal cell, 8- enteroendocrine cell, 9- gland lumen, 10- gastric gland.

Interestingly, the stomach also has a pivotal role in bridging the gut functions to the enteric and central nervous systems. Two examples can be forwarded: 1) ghrelin, produced in the stomach, was the first *hunger hormone* identified. It acts in the hypothalamus. 2) gut microbiome; c.a. 9 out of 10 cells in the living body are microbial. Most of these prokaryotic bacteria inhabit the gut and are enrolled in the control of body weight and in the prevention of symptoms associated with inflammatory bowel disease [130].

3.2. Nitrite, nitric oxide and gastric mucosal defense

The constant exposure to foreign agents forces the gut, and the stomach in particular, to display a tightly regulated war chest against microbes, external toxins, foodstuffs of different osmolarities and pH. Gastric mucosal defense is divided in three stages: pre-

epithelial, epithelial and post-epithelial, being nitrite and •NO key players in all of them [112, 131-132]. Indeed, studies in animals have provided evidence that inhibition of endogenous production of •NO exacerbated the damage induced by acid or ethanol [133-134]. Besides, studies in humans have also proven the gastroprotective role of •NO [135].

The pre-epithelial defense comprises both the continuous coat of mucus that buffers the luminal acidic pH with HCO_3^- (produced by epithelial cells) and the gastric surfactant (that confers hydrophobicity to the gastric surface [136]). Nitric oxide donors [137] and salivary nitrite, through the reduction to •NO, have been shown to increase the production of mucus through a cGMP-dependent mechanism [102, 104].

The epithelial defense relies in an extremely tight epithelium almost impermeable to luminal components due to the attachment of the apical side of epithelial cells by tight junctions. These junctions are complex structures comprising different transmembrane proteins that are linked to the cellular cytoskeleton [138]. They render the paracellular transport exceptionally more selective [138-139]. Nitric oxide has a controversial role regarding tight junction function. Some studies show that it can increase the epithelial permeability whereas others demonstrate the opposite [132]. Also, Ara et al, have shown in an *in vitro* model that acidified nitrite decreases the epithelial resistance and forwarded occludin disarrangement as a putative underlying mechanism [140].

The post-epithelial defense comprises the mucosal microcirculation and innervation. The mucosal blood flow has an important impact on gastric defense, because it neutralizes and carries away toxic substances. It also transports oxygen, nutrients and gastric hormones to

their different final destinations [112]. In this context, •NO has been shown to increase mucosal blood flow, therefore exhibiting gastroprotective properties [102, 104]. Furthermore, when released from the non-cholinergic non-adrenergic terminals, •NO has been implicated in the regulation of gastric motility [141-142].

Moreover, •NO plays also other roles as modulating inflammatory responses [143-144], acting as an antimicrobial agent (mostly due to its indirect effects) [83, 101, 145] and preventing the development of gastric ulcers [104, 111].

3.3. Signalling properties of dietary polyphenols beyond their antioxidant activity

Polyphenols are plant secondary metabolites that have long been believed to exert beneficial effects on human health due to their well documented antioxidant properties *in vitro* [146-148]. Still, their relevance *in vivo* has been questioned on such a basis [149-150]. There are two main reasons that justify this skepticism. On one hand, polyphenols are extensively metabolized *in vivo* yielding derivatives without the chemical characteristics of the initial molecules, in particular the chemical details supporting the antioxidant activity observed *in vitro* are blocked following absorption [151-152] and, on the other, the concentration of flavonoids and their metabolites achieved in tissues is lower than that of endogenous antioxidants, such as ascorbate and α-tocopherol [149-150]. Moreover, the concept of polyphenols as “global antioxidants” is considered not appropriate to

understand the potential health benefits of such molecules, as supported by recent updates of the concept of oxidative stress, emphasizing the modulation of discreet redox pathways [153-154]. These findings brought into discussion the relevance of other possibilities to explain their beneficial effects [155-156]. For instance, resveratrol, at concentrations likely found *in vivo*, modulates several metabolic pathways including the activation of NAD^+-dependent deacetylases sirtuins, thereby preventing metabolic dysfunction [157-158].

In addition, several groups have recently unraveled previously unrecognized physiological effects of polyphenols in connection with nitrite and $^{\bullet}NO$ bioactivities in the stomach [159-162]. Dietary polyphenols, increase $^{\bullet}NO$ availability in the gastric lumen by favoring nitrite reduction at acidic pH. Together, these findings strongly suggest that polyphenols may have a positive impact on human health, but through mechanisms beyond their acute antioxidant activity.

3.4. Pepsin & digestion of exogenous and endogenous proteins

Pepsin, the major gastric protease, is normally responsible for less than 15% of exogenous protein digestion [129]. It is formed from an inactive precursor, pepsinogen, synthesized by the chief cells of the gastric mucosa. Pepsinogen is stored in cytoplasmic vesicles and after a meal it is released to the gland duct and further to the gastric lumen. Due to the acidic pH (optimum pH ~ 3.5), a 44-aminoacid pro-segment is hydrolyzed and active

pepsin is released. Further on, the activated enzyme will act autocatalytically to increase its rate of production. Pepsin is an endopeptidase that will cleave bounds where one of the aminoacids is aromatic, thereby degrading dietary proteins (for a recent review see [163]).

Gastric ulcers are erosions occurring mostly in the antrum, where the oxyntic mucosa meets the pyloric mucosa [164-165]. These lesions are characterized by the loss of part of the mucosa, being more or less pronounced and afftecing or not local blood vessels. In such cases, life-threatening situations such as intensive bleeding or even gastric perforations (the entire gastric wall is disrupted) may arise [129].

Even though as many as 70% of the actual gastric ulcers are provoked by *Helicobacter pylori*, there is still an important number of cases in which such cause is not observed. In this regard, it should be mentioned that the mucus layer does not form a continuous layer and the protection is largely given by the rate of acid production keeping pace with the buffering capacity of the food. Peptic ulcers develop when the stomach is no longer able to protect itself and to remove the damaged cells, being unable to withstand damage (that can be caused by several agents, such as NSAIDs [166]. In these cases, pepsin is implicated in ulceration [129, 167-168]. In an acidic environment, this enzyme digests mucosa from the esophagus, stomach and duodenum [168-169]. Disruption of the mucosal integrity provides the conditions for pepsin to penetrate the mucosa and erode the columnar epithelium. In addition, since both pepsinogen and acid are released to the glandular duct within the mucosa, in this microenvironment, the pH can be low enough to

activate pepsinogen and therefore, some active pepsin can be already obtained here. Therefore, under this scenario, pepsin potentiates, rather than triggers, gastric ulceration. Pepsin can then be regarded as a Janus-faced molecule as it initiates the digestion of dietary proteins but can also play a role on the etiology of peptic ulcer disease (PUD).

GOALS

Overall, this thesis was aimed at studying the functional impact of dietary nitrite-derived $^{\bullet}NO$ and other RNS in the gastric compartment, with emphasis in the modulation of molecular mechanisms and pathways that could trigger either physiological or pathophysiological processes.

Specifically, the goals were:

- To study the effect of polyphenols from different dietary sources on the production of $^{\bullet}NO$ from nitrite in the human stomach.
- To ascertain the diffusional properties of $^{\bullet}NO$ upon nitrite reduction in the simulated gastric compartment.
- To reveal the presence of nitrite-derived nitrating species that could induce modification of biomolecules in the stomach.
- To identify potential post-translational modifications of proteins, in particular nitration, triggered by nitrite within the gastric compartment (lumen and mucosa).
- To assess the functional and physiological impact of nitrated proteins, with emphasis on the development of PUD.

CHAPTER 2

MATERIALS AND METHODS

2.1. Chemicals and reagents

All reagents used to perform the experiments reported herein were of analytical grade unless otherwise stated.

- **Chemicals:** All reagents were purchased from Sigma-Aldrich, except the following: sodium nitrate and [1,2,4] oxadiazolo [4,3]quinoxalin-1-one (ODQ) were from Tocris Cookson. Oleuropein was obtained from Extrasynthèse and both chlorogenic and ascorbic acids from Fluka Biochemika. Procyanidin dimmers B2, B5, B8 and epicatechin-3-O-gallate were kindly provided by Dr. Victor Freitas from the department of Chemistry, Faculty of Sciences University of Porto. ProtoGel was obtained from National Diagnostics and ECF from GE Healthcare. Sodium hydroxide was purchased from Merck, the Bradford reagent from Bio Rad and the A/G Ultralink resin from Thermo Scientific. Voltaren®, Novartis (diclofenac) was obtained at a local pharmacy. The antibodies used are indicated throughout the methods section.

- **Gases:** all gases were from AirLiquide, France

- **Foodstuffs:** The red wine was *Touriga nacional* “Quinta de Cabriz” 2003 (14% v/v), Dão, Portugal and the other foodstuffs were purchased from local shops.

- **Reagents:**

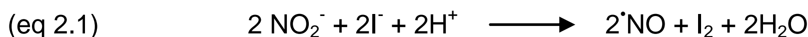
1) *Simulated Gastric Juice* (SGJ) was prepared at pH 2 as described in United States Pharmacopeia (volume 25). Briefly, HCl and NaCl were dissolved in distilled water in order to obtain 7 mM and 50 mM, respectively. Final pH was adjusted to 2 by adding HCl.
2) *Modified Krebs buffer* contained 120 mM NaCl, 4.7 mM KCl, 1.2 mM KH_2PO_4, 25 mM $NaHCO_3$, 1.2 mM $MgSO_4.7H_2O$, 2.5 mM $CaCl_2$ and 11.1 mM Glucose, pH 7.4.
3) *Mitochondrial respiration medium* (MiR05) was composed by 0.5 mM EGTA, 3 mM $MgCl_2.6H_2O$, 60 mM K-lactobionate, 20 mM taurine, 10 mM KH_2PO_4, 20 mM HEPES, 110 mM sucrose, 1 g/L BSA.
4) *Lysis buffer:* 1 mM $NaVO_4$, 10 mM ß-glycine, 5 mM NaF, 1 mM PMSF and protease inhibitor cocktail in 50 mM HEPES buffer solution: 150 mM NaCl, 2 mM EDTA, 10% glycerol and 1% Triton X-100.

2.2. Methods

2.2.1. Electrochemical determination of nitric oxide

The time course of $^{\bullet}NO$ production under acidic pH was followed electrochemically using the ISO-NO Mark II $^{\bullet}NO$ electrode (World Precision Instruments, LTD, Hertfordshire, UK). The rationale of this methodology is based on a 2 mm diameter platinum electrode with an internal reference coated with a teflon membrane permeable to gases. Thus, $^{\bullet}NO$ in solution diffuses the membrane and will be oxidized at 0.8 V generating an electric current monitored and recorded by Apollo 1000 (World Precision Instruments, LTD, Hertfordshire, UK).

For daily experiments, the electrode was calibrated under aerobic conditions in a solution containing H_2SO_4 and KI 1:1 (0.1 M) with increasing sodium nitrite concentrations, according to the manufacturer's instructions. Since the concentration of both H_2SO_4 and KI are in great excess in relation to nitrite, nitrite is completely reduced to $^{\bullet}NO$ and therefore, knowing the concentration of nitrite, one determines $^{\bullet}NO$ (equation 2.1 occurs in a 1:1 ratio).



(eq 2.1) $$2\,NO_2^- + 2I^- + 2H^+ \longrightarrow 2\,^{\bullet}NO + I_2 + 2H_2O$$

To study the nitrite reductase ability of the different polyphenols *in vitro*, a reaction mixture of 10 ml SGJ (pH 2) containing the polyphenol, ascorbic acid or red wine (the

concentrations are indicated in the respective figures) was prepared. The mixture was maintained under stirring and aerobic conditions at room temperature. Once a stable baseline was achieved, the reaction was started by means of nitrite addition.

All stock solutions were prepared in argon-degassed ultra pure water to avoid oxidation. Proper controls with either vehicle or sodium nitrate were performed.

2.2.2. Rate of nitrite decay during polyphenol-induced reduction to nitric oxide

To follow nitrite decay during its reaction with polyphenols we have used the Griess method. In this method nitrite reacts with sulfanilamide (SUFA) at acidic pH to form a diazonium salt. Then, this salt reacts with N-1-naphthylethylenediamine (NNED), producing a complex with a purple color that can be monitored spectrophotometrically. This experiment was performed for three different concentrations of oleuropein and (+)-catechin: 100, 200 and 500 μM. Aliquots (in duplicate) were collected along the incubation time at 0 (before nitrite was added), 1, 10, 20, 30, 45 and 60 minutes after nitrite addition followed by addition of SUFA. After 5 minutes, NNED was added and 5 additional minutes later, absorbance was measured at 540 nm in a Lambda 3, UV/VIS Spectrophotometer (Perkin-Elmer Corporation, Connecticut).

2.2.3. Nitrite reduction to nitric oxide in the gastric lumen and diffusion: the effect of red wine polyphenols

All the procedures used in this thesis are in accordance with Portuguese and European guidelines for animal handling in scientific research. Male Wistar rats weighing between 250 - 350 g (Charles River, Barcelona, Spain) were used in all experiments. Rats were kept under standard conditions of temperature and illumination and 20 hours before the experiments were deprived of food but had free access to tap water.

In order to study $^{\bullet}NO$ production from nitrite at acidic pH (resembling the gastric lumen) and further diffusion within the gastric wall, an Ussing chamber was used. These chambers, named after the physiologist who invented them, Hans Ussing, are widely used to study the transport of ions across gut mucosa. They consist of two halves that are clamped by the tissue or cell culture of interest. In this case, the rat stomach wall was mounted, dividing the chamber into a simulated gastric lumen and serosa.

After euthanasia, the rat was laid supine, an abdominal midline incision was made and the stomach was excised. Then, it was placed in ice-cold Krebs buffer and opened along the lesser curvature. The organ was gently stretched and mounted in a CHM7 Ussing chamber (World Precision Instruments, Sarasota, USA) using a pair of small tweezers. The stomach wall (comprising all the histological layers: serosa, *muscularis externa*, sub-mucosa, *muscularis* mucosa, mucosa and mucus layer) divided the chamber into two distinct compartments: the mucosal side (filled with SGJ, in contact with the gastric epithelium and

mucus) and the serosal compartment (filled with Krebs buffer, in contact with gastric serosa). The $^{\bullet}$NO electrode was placed in the serosal compartment in contact with gastric serosa in a 45º angle and the baseline was let to stabilize. All the reagents (ascorbate, nitrite and red wine) were added to the mucosal compartment at the concentrations indicated in the respective figures.

2.2.4. Functional studies to assess gastric tissue viability

- **Muscle relaxation assays**

In order to evaluate the viability of the gastric tissue after the diffusion experiments, the stomachs were removed from the chamber and four longitudinal *fundal* strips were obtained and submitted to contraction and relaxation experiments. Briefly, the stomach was placed in ice-cold modified Krebs solution and the *fundus* region was carefully separated from the other parts of the organ. Four longitudinal *fundal* strips (3 x 15 mm) were obtained by cutting parallely to the greater curvature using the method first described by Vane [170]. The strips were then mounted separately in a 10 ml organ bath chambers (ADInstruments Australia), with the lower part tied to a steel hook and the upper part to a MLT050/D isometric force transducer coupled to a bridge amplifier ML224 and PowerLab 4/30 data acquisition system (ADInstruments, Australia). The chambers were filled with modified Krebs solution at a constant temperature of 37ºC. An initial resting tension of 2g

was applied to the strips and the preparations were allowed to equilibrate for 2 hours. Afterwards, carbachol was added to each bath in increasing doses: 0.1, 0.5, 1 and 5 µM. Stomachs of rats not exposed to $^{\bullet}$NO in the diffusion experiments were used as controls. In another set of experiments, the strips of both groups (control and $^{\bullet}$NO-exposed stomachs) were challenged with 200 µL of SGJ containing 500 µM ascorbate and 1 mM nitrite and also to an identical volume of SGJ containing only 1 mM nitrite, after pre-contraction with 5 µM of carbachol. These solutions were prepared in closed vials devoided of headspace and added with a Hamilton syringe to the bath. All experiments were performed in triplicate.

- **Cellular respiration assays**

We have used oxygen consumption as a further functional approach to investigate the viability of the stomachs exposed to $^{\bullet}$NO in the diffusion experiments. For this purpose, gastric biopsies from the *corpus* were collected with a small scissor after exposure to 1 mM nitrite and 500 µM ascorbate at pH 2. The biopsies were placed into the chambers (2.2 mL) of high resolution oxygraph (O2K, Oroboros Instruments, Austria) filled with MiR05 at 37ºC.

Before the experiments both O2K chambers were kept with ethanol 70%, washed with water and calibrated at 37ºC in culture medium. Air calibration of the polarographic oxygen sensors was done daily and the oxygraph was let to stabilize at least one hour before each

experiment. Gastric biopsies were added to the chambers and basal respiration was monitored. After a stabilization period of typically 15 minutes, 8.5 mM of succinate was added to each chamber to stimulate mitochondrial respiration; afterwards, 0.45 μM antimycin was added to inhibit respiration.

2.2.5. *In vivo* and *post mortem* nitric oxide diffusion studies

Rats were anesthetized with urethane (1.25 $g.Kg^{-1}$) and laid supine under a heating pad at 37ºC. A small (2 cm) abdominal midline incision was made. The stomach was exposed very carefully in order to avoid damaging the liver. The necessary hepatogastric ligaments needed to cannulate the esophagus were cut. An incision was made in the esophagus and a teflon *cannula* (2 mm diameter) was inserted into the stomach. Care was taken to control hemorrhages and saline at 37ºC was used to obtain a hemoglobin-free preparation and to proportionate an aqueous environment for the electrode positioning. Then, 1ml of 500 μM ascorbate prepared in SGJ was added to the gastric lumen. The electrode (ISO-NO Mark II) was placed in the *fundic* region in contact with the serosal layer and, once a stable base line was obtained, 1 mM nitrite was added to the stomach and the response was monitored for 30 minutes to one hour. The same experiment was repeated *post-mortem* immediately after rat sacrifice by cardiac arrest.

2.2.6. Impact of nitrite-dependent nitric oxide production in the stomach: gastric smooth muscle relaxation studies

The rationale that polyphenols potentiate nitrite reduction to $^{\bullet}NO$ in the gastric lumen and that $^{\bullet}NO$ diffuses the gastric mucosa, prompted us to study the impact on luminal diffusing-$^{\bullet}NO$ in gastric smooth muscle relaxation. For that we used the same organ bath system mentioned above and again, longitudinal fundal strips (3 x 15 mm) from rat stomachs were obtained and processed as aforementioned. After an initial resting tension of 2g was applied, the preparations were allowed to equilibrate for 2 hours. Carbachol (5 μM) was added to each chamber to induce smooth muscle contraction. Once a plateau was achieved, 100 μL of the polyphenol solution was added to each chamber to study muscle relaxation. The concentrations used are indicated in the respective figures.

2.2.7. Production of nitric oxide in the human stomach upon consumption of foodstuffs containing polyphenols

The $^{\bullet}NO$ content in the air expelled from the stomach of six healthy volunteers was measured under overnight fasting conditions, following consumption of 75 g of lettuce (as a source of nitrate. Typically 1 Kg of lettuce contains 1.3 g of nitrate [171]) and, again, after consumption of several dietary products and beverages known to be abundant in distinct classes of polyphenols, namely red wine, green tea, onions, black tea, apples, myrtilles and red cherries. At the times indicated in the legend of the respective figure the air

expelled from the stomach was collected into gas-tight bags by voluntary regurgitation and $^{\bullet}NO$ concentrations were determine by chemiluminescence (CLD88 Exhalyzer, EcoMedics), as described before [84, 159]. The chemical basis of this technique is the reaction of $^{\bullet}NO$ with O_3 generated by the apparatus. From this reaction, NO_2 in a partially excited state is produced (NO_2*). When returning to the ground state, NO_2* emits light in the near infra-red region which is detected by a photosensitive surface. The signal is further amplified by a photomultiplier tube. The detection limit of the instrument was 1 ppb and the light emitted is proportional to $^{\bullet}NO$ concentrations between 1 and 5000 ppb.

2.2.8. Peroxynitrite synthesis

Peroxynitrite was prepared by a quenched flow reaction based on the acid-catalyzed reaction of HNO_2 with H_2O_2. Briefly, 600 mM H_2O_2 prepared in 600 mM HCl was cooled to 4ºC and under constant stirring, 600 mM nitrite were added; immediately after, a yellow color developed (due to the formation of ONOOH) and the reaction was quenched by the addition of 3.6 M NaOH. This solution was frozen at -20ºC and an intense yellow top layer developed afterwards. This layer was the one used in our studies and contained typically 100 to 200 mM of peroxynitrite. The concentration of peroxynitrite was determined daily before each experiment by measuring its absorbance at 302 nm (ε_{302}= 1670 $M^{-1}.cm^{-1}$) as described by Hughes and Nicklin [172].

2.2.9. Detection of nitrated pepsinogen and pepsin *in vitro*

Detection by UV/VIS Spectroscopy. A stock solution of pepsinogen 10 μM (in 50 mM K-Pi pH 7.4) and pepsin 20 μM (in SGJ) were prepared daily and challenged with increasing concentrations of $ONOO^-$: 50, 100, 250 and 500 μM (bolus addition). Peroxynitrite was added under vortex agitation and afterwards the pH was alkalinized to 10.5-11.0 by adding NaOH 5 M. Each test solution was evaluated by spectroscopy in the UV/VIS range (SpectraMax Plus384, Molecular Devices) between 250 and 500 nm; nitrotyrosine confers a light yellow color to the solution that can be measured at 420 nm and converted to concentration attending that ε_{420}= 4400 $M^{-1}.cm^{-1}$ [173].

Pepsin 10 μM was also incubated with nitrite 10 mM for 8 hours in SGJ (pH 2) at 37ºC to assess the nitrating ability of nitrite at acidic pH. Aliquots were collected each hour (for 8 hours) and the media was alkalinized with NaOH 5 M. Pepsin nitration was determined by spectrophotometry as described.

Detection by Western Blot. Pepsinogen (10 μM) and pepsin (20 μM) were nitrated with the aforementioned $ONOO^-$ concentrations (proteins not exposed to $ONOO^-$ were used as control). Samples of pepsin (10 μM) nitrated with acidified nitrite (10 mM) were also prepared.

Proteins (3 μg) were loaded on SDS- 12% polyacrylamide gels and separated by electrophoresis. They were further electroblotted onto PVDF (polyvinylidene fluoride) membranes (2 hours). Nonspecific binding sites were blocked with 5% defatted dry milk in

TBS-T (10% TBS, 0.1% Tween 20), for one hour at room temperature. Membranes were then incubated with rabbit polyclonal antibody against nitrotyrosine (1:2000 dilution, prepared in washing solution, gently provided by Dr Rafael Radi [174]) overnight at 4ºC. After three 10-minutes washes, the immunoblots were probed with a goat anti-rabbit antibody (Santa Cruz Biotechnology) at room temperature for 1 hour. Labeling was detected by incubation with ECF for 5 minutes and analyzed using a fluorescent image analysis system (Thyphoon FLA 9000).

2.2.10. Impact of nitration on pepsinogen function

Absence of collagen. Pepsinogen 10 µM was prepared in 50 mM K-Pi buffer at pH 7.4 and nitrated by adding $ONOO^-$ 500 µM. Another solution of 10 µM pepsinogen was also prepared but was not exposed to $ONOO^-$. Both forms of pepsinogen (nitrated and non nitrated) were transferred to simulated gastric juice (pH 2) for one hour at 37ºC, yielding a final concentration of 2 µM. At this pH, pepsinogen is converted to pepsin in 1-2 minutes [175]. Thus, during this period the cleavage of the inactive pro-segment occurs and an increase in free amino (NH_2) groups is expected. Therefore we have measured this parameter in samples of nitrated and non nitrated pepsinogen at pH 2, as described by Fields [176]. Briefly, each sample was allowed to react with an equal volume of borate buffer (stock solution of 0.1M $Na_2B_4O_7$ in 0.1M NaOH) followed by 22 mM TNBS (final concentration) addition and incubation at room temperature for 5 minutes. The reaction

was stopped by adding a mixture of 98.5 mM $Na_2H_2PO_4$ and 1.5 mM Na_2SO_3. The absorbance was measured at 420 nm (SpectraMax Plus384, Molecular Devices) and free NH_2 expressed in nmol (1 OD equals to 52 nmol free NH_2 groups).

Presence of collagen. Pepsinogen 10 µM was nitrated with 500 µM $ONOO^-$ in order to generate nitrated pepsinogen at pH 7.4 as already mentioned. Nitrated (or non nitrated) pepsinogen was then added to 10 µM collagen in simulated gastric juice (pH 2) for one hour at 37ºC under gentle stirring. Collagen is known to be a substrate for pepsin at acidic pH and therefore its proteolysis may be used to evaluate the enzymatic efficiency of this protease. After one hour, the proteolytic activity was evaluated by measuring the free NH_2 groups using the TNBS method mentioned above.

2.2.11. Impact of nitration on pepsin function

To evaluate the proteolytic activity of nitrated and non-nitrated pepsin, we have selected four different substrates: casein, albumin, collagen and hemoglobin. In the case of the first three proteins, the activity of nitrated pepsin was evaluated by determination of free amino groups using TNBS as described for pepsinogen. On the other hand, the activity of nitrated pepsin on hemoglobin was evaluated by determination the TCA (trichloroacetic acid)-soluble peptides (resulting from proteolysis).

A stock solution of 100 µM pepsin (in SGJ) was prepared daily. Pepsin 20 µM was prepared from the stock solution and was nitrated by adding $ONOO^-$ 500 µM. Afterwards,

nitrated pepsin was added to casein or albumin 10 μM in SGJ at pH2; the final concentration of nitrated pepsin was 1 μM and the mixture was allowed to react for an hour at 37ºC under stirring. Samples of casein or albumin 10 μM alone (in SGJ) or with pepsin 1 μM were allowed to react in the same conditions. In the case of collagen, the conditions were the same but the final concentration of both pepsin and nitrated pepsin was 2.5 μM; additionally, since collagen is an insoluble protein, at the end of one-hour of reaction, the sample was filtrated using a 0.2 μm filter. Free NH_2 groups were then determined as described.

Regarding hemoglobin, the method described by Anson was used [177]. Briefly, hemoglobin 1.5% (w/v) was prepared in HCl 60 mM and warmed to 37ºC. Afterwards, 200 μL of pepsin stock solutions (nitrated and non nitrated) were added yielding a final concentration of 1.25 μM and the reaction was allowed to occur for 10 minutes, under stirring at 37ºC. TCA was added (2 mL) to solubilize the hemoglobin-derived peptides that resulted from the peptic digestion. Blanks were performed similarly, but pepsin solutions were added after TCA and the reaction again occurred under stirring at 37ºC. Then, all the solutions were filtered using a 0.2 μm filter and the absorbance was measured at 280 nm, using a spectrophotometer (SpectraMax Plus384, Molecular Devices).

2.2.12. Detection of nitrated pepsinogen *in vivo* under physiological conditions and acute gastric ulceration

- **Animals**

Rats were anesthetized with urethane (1.25 $g.Kg^{-1}$, intraperitoneal injection) and gastric ulcers were induced by the administration of 30 $mg.Kg^{-1}$ of diclofenac (Voltaren®, Novartis) by oral gavage. The rats were left in their cages lying in a heating pad for 4 hours [110]. Afterward, nitrite 1.3 $mg.Kg^{-1}$ was given also by oral gavage. After one hour the stomach was isolated and processed for further studies. The experimental groups and the protocol used are elucidated in the figure 2.1.

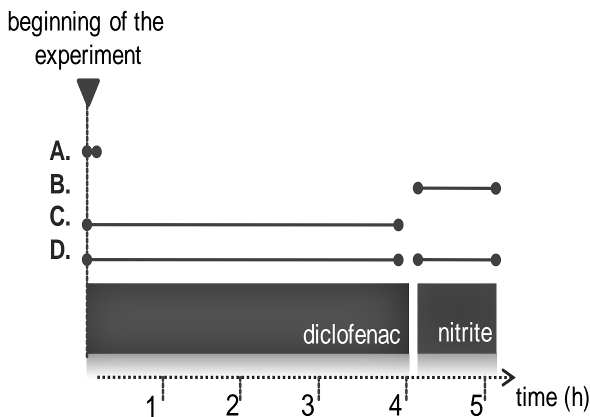


Figure 2.1 – Experimental procedure used to study the effect of dietary nitrite as a nitrating agent in the acidic rat stomach *in vivo*. Wistar rats were divided in 4 groups depicted in the figure as A, B, C and D and the following treatment was performed: (A) no drugs were given to the rats, (B) rats received nitrite 1.3 $mg.Kg^{-1}$ and were sacrificed 1 hour later, (C) rats received diclofenac 30 $mg.Kg^{-1}$ and were sacrificed 4 hours later (D) rats were exposed to diclofenac as in C) followed by nitrite. All drugs were administered by oral gavage. Each group contained 5-6 animals.

2.2.13. Detection of overall protein nitration in the stomach by immunohistochemistry

The presence of nitrated proteins in the gastric mucosa was investigated by immunohistochemistry. The body region of the stomach was fixed in 4% buffered paraformaldehyde, cut using a cryostat and permeabilized with Triton 0.5% (in PBS) for 15 minutes. Non-specific binding was blocked for 2 hours with BSA 5% and 0.6% Tween (in TBS). Then, the sections were incubated with rabbit polyclonal antibody against nitrotyrosine, overnight at 4ºC in a humidity chamber. After two washes with PBS, the sections were incubated with a secondary antibody against rabbit (Alexa fluor 488, Santa Cruz Biotechnology) for 1 hour. Finally the sections were washed again with PBS and the nuclei were stained with Hoechst for 5 minutes. The slides were then observed under a fluorescent microscope (Zeiss Axiovert 200, Carl Zeiss MicroImaging, Germany). Nitrotyrosine expression score was expressed by the judgment of two blind researchers using a four-level scale from 0 to 3 (0, no stain; 1, few stain; 2, moderate stain; 3, intense stain). None of them received any information on diagnose of the other observer.

Two negative controls were performed: firstly, the sections were incubated for one hour with dithionite (1 $mg.mL^{-1}$) which reduces nitrotyrosine to aminotyrosine; secondly, the sections were also treated with the same procedure as for regular immunohistochemistry except for the absence of the primary antibody. The tissue morphology was evaluated by Hematoxylin and Eosin staining under a light microscope.

2.2.14. Morphological analysis: Hematoxylin & Eosin staining

Slices from the region used for immunohistochemistry studies (10 μm) were cut using a cryostat washed with running water and incubated with Mayer hematoxylin for 5 minutes, followed by 10 minutes with running water. Afterward, the slides were washed with distilled water and exposed to Eosin Y for 30 seconds. The tissue was then washed with increasing concentrations of ethanol (80, 90 and 100%) and immersed in xylol until permanent mounting with Permount. The preparations were then observed under a light microscope (Zeiss Axiovert 200, Carl Zeiss MicroImaging, Germany).

2.2.15. Detection of nitrated pepsinogen by immunoprecipitation

The stomachs (from the groups described in figure 2.1) were isolated and immediately immersed in ice-cold lysis buffer. The tissue was roughly minced and then homogenized using a *polytron*. All crude homogenates showed minimal viscosity and were mixed for 30 minutes at 4ºC. The samples were then centrifuged for 10 minutes, 15000 g at 4ºC. The supernatant was then collected and frozen at -80ºC until further analysis. Protein concentration was determined using the Bradford method.

Solubilized proteins (800 μg) were incubated with 2 μg of a polyclonal pepsinogen antibody (Santa Cruz Biotechnology) for 3 hours at 4ºC. The immune complexes were then precipitated (1 hour at 4ºC) with 10 μL of protein A/G Ultralink resin previously washed with

lysis buffer through 5 consecutive centrifugations at 2000 g for 1 minute. The complexes were then washed with PBS (2000 g x 1 minute, 5 times) and nitrated pepsinogen was eluted from the beads by adding loading buffer and further heating at 95ºC for 5 minutes. The samples were then applied in SDS-12% polyacrylamide gels. A western blot against nitrotyrosine was performed as aforementioned. Subsequently, the primary and secondary antibodies were stripped off and the membranes were reprobed with an antibody against pepsinogen (Santa Cruz Biotechnology).

The strong reductant sodium dithionite was used as negative control. Stomach homogenates were incubated with dithionite (1 $mg.mL^{-1}$) for one hour at 4ºC before the immunoprecipitation procedure.

2.2.16. Detection of nitrated pepsin *in vivo*

- **Animals**

Adult male Sprague Dawley rats (320g – 450g) were kept under standardized conditions of temperature and illumination. Before the experiments, the rats were fasted for 20 hours but had access to water *ad libitum.* These experiments were allowed by the ethics committee at Karolinska Institute, Stockholm, Sweden.

Rats were anesthetized by the intraperitoneal (ip) administration of 120 $mg.kg^{-1}$ pentobarbital sodium and laid supine under a heating pad. Pentagastrin 20 $\mu g.Kg^{-1}$ was

administered intraperitoneally to induce gastric secretion 15 minutes before the experiment began. A laparotomy was then performed and the hepatogastric ligaments were cut to facilitate handling the stomach. External clamps were applied in the lower esophagus and pylorus to avoid the passage of air and gastric juice to the adjacent compartments. Luminal levels of $^{\bullet}NO$ were measured by chemiluminescence (CLD88 Exhalyzer, EcoMedics) as previously reported [111, 178]. Briefly, 4 mL of $^{\bullet}NO$-free air (typically less than 4 ppb) were injected into the stomach and incubated for 15 seconds. Then, the same volume was aspirated and injected in the CLD.

Sodium nitrite (1.3 $mg.Kg^{-1}$) was then directly instilled into the stomach lumen through a thin needle and 5 minutes later, intragastric $^{\bullet}NO$ levels were again measured. The rats were sacrificed by cardiac arrest 30 minutes after nitrite instillation. The stomach was dissected out and gastric lesions were evaluated. The tissue was then snap frozen until further analysis.

The same procedure was performed but nitrite was replaced by its vehicle (distilled water) or human saliva collected after the ingestion of lettuce. Nitrite instillation was also performed after urate was injected into the rat stomach. Untreated animals were also used to assess basal nitration.

2.2.17. Macroscopic assessment of gastric lesions

The stomach was opened along the greater curvature, gently washed with phosphate buffer saline and photographed. The lesions were macroscopically graded on a 0 to 6 scale by an observer blinded to the treatment. Each grade corresponded to the following state. 0- no damage; 1- edema; 2- reddening of mucosa; 3- petechial hemorrhages; 4- superficial erosions; 5- ulceration; 6- perforation [179].

2.2.18. Microscopic assessment of gastric lesions

At the end of the experiments, part of the tissue was used for hematoxylin and eosin (H&E) staining as described above.

2.2.19. Detection of nitrated pepsin

Proteins present in the margin of the gastric ulcers were separated by electrophoresis on SDS- 12% polyacrylamide gels and transferred electrophoretically to PVDF membranes. The same procedure as for detection of nitrated pepsinogen by western blot and immunoprecipitation was performed.

2.2.20. Studies of intragastric nitration in germ-free and conventional mice

- **Animals**

All experiments involving germ free animals were approved by the Local Ethics Committee for Animal Experiments at Karolinska Institute. Germ free (GF, n=5) and conventional NMRI mice (conv, n=5) were used in these experiments. The animals were maintained on autoclaved R36 Lactamin chow (Lactamin, Sweden) and kept in 12 h light cycles. Fecal samples were cultured weekly (for up to 4 weeks) to check the GF status.

Both conv and GF mice were divided into 3 groups and their drinking water was supplemented for 7 days as follows: sodium nitrate (10 mM), sodium nitrite (1 mM) or sodium chloride (placebo).

2.2.21. Preparation of stomach homogenates of germ-free and conventional mice

The stomach glandular region of both GF and conv mice was minced in ice-cold lysis buffer (typically 100 mg of tissue were homogenized in 1 mL of buffer) with a pair of small scissors. The suspension was further triturated with a bullet blender (Labmark) and centrifuged at 12 000 g for 10 minutes (4ºC). The supernatant was collected and total protein was quantified by the Bradford method (Bio-rad). Care was taken to avoid artifactual nitration due to media acidification.

2.2.22. Immunoblotting for nitrotyrosine and iNOS

Equal amounts (65 µg for nitrotyrosine and 50 µg for iNOS) of the total protein were blotted to PVDF membranes. The same procedure described for the detection of nitrated pepsin and pepsinogen was also performed for germ-free mice samples using a nitrotyrosine or iNOS (Santa Cruz Biotechnology) antibody, except that labeling was detected by soaking with SuperSignal West Pico Chemiluminescent Substrate (Thermo Scientific) for 5 minutes and analyzed using a luminescence image analysis system (LAS-1000 Plus, Fujifilm).

In all experiments, the antibodies were stripped off and membranes reprobed with a β-actin antibody (1:10000, Santa Cruz, Biotechnology) to guarantee that the same amount of total protein was load in the different samples.

2.2.23. Collection of human saliva

A sample (≈ 10 mL) of saliva was collected from a human volunteer who underwent an overnight fasting. Then, 90 g of iceberg lettuce were ingested and saliva was again collected one hour later. After centrifugation (12000 g x 10 minutes), nitrite content was determined by chemiluminescence (CLD88 Exhalyzer, EcoMedics) and the samples were stored at -80ºC until use.

2.2.24. Assessment of nitrating properties of human saliva

In order to provide further insights on the probable mechanism of nitration, *in vitro* studies using inorganic nitrite, pepsin and human saliva (obtained before and after the intake of 90g of lettuce) were performed. Pepsin 20 μM was allowed to react with human saliva and with inorganic nitrite (100 μM, 1 and 5 mM). The same experiment was also done in the presence of urate 200 μM. The reactions occurred in a closed vial at 37ºC for 1 hour. Nitration was assessed by spectrophotometry and nitrotyrosine concentration was obtained using ε_{420}= 4400 $M^{-1}.cm^{-1}$ [173].

2.3. Statistical analysis

Unpaired and two-tailed t-test was used in two-sample comparison. One-way Anova variance followed by Bonferroni multiple comparison test was used to compare more than 2 groups. A probability value (p value) of less than 0.05 was considered significant and unless otherwise stated, all values are presented as mean±SEM.

CHAPTER 3

INTRAGASTRIC INTERACTION BETWEEN NITRITE AND POLYPHENOLS: NITRIC OXIDE PRODUCTION AND DIFFUSION THROUGH THE GASTRIC WALL

3.1. Introduction

As already highlighted in chapter 1, although the health benefits of polyphenols are now recognized, other mechanisms beyond their acute antioxidant activity have been forwarded to explain such effects [155-156]. In addition to the modulation of signaling pathways, they have also been described to modulate $^{\bullet}$NO production at acidic pH [159-162]. We have explored the possible modulation of $^{\bullet}$NO metabolism in the stomach, the compartment where, due to the likely high concentrations of polyphenols present in an unmodified form, the phenol:nitrite interaction is favored. For that, we selected a group of different compounds, distributed among diverse dietary products in order to demonstrate that $^{\bullet}$NO production is a general phenomena following consumption of polyphenols. In addition to being kinetically facilitated in the stomach due to the high concentrations achieved locally, the observation that the redox potential of polyphenols (mostly ranging from 250 to 700 mV) are generally lower than that of nitrite (900 mV) affords thermodynamic feasibility to nitrite reduction, although the precise mechanism remains unclear. Moreover, and in order to impart biological significance to phenolic-dependent nitrite reduction we evaluated the impact of phenol:nitrite mixtures in an *in vitro* model of stomach muscle relaxation.

Nevertheless, $^{\bullet}$NO is a *Dr Jekyll and Mr Hyde* kind of molecule as in addition to its recognized gastroprotective actions, it has also been shown to induce harmful effects, being implicated in the development of some types of cancer, namely gastro-esophageal [180-181]. Furthermore, nitrite-derived $^{\bullet}$NO was also observed to cause molecular changes

in the stomach wall [140], increasing its permeability and thereby predisposing to gastric lesions. Yet, since a wide number of both oxygen and nitrogen species are formed in the stomach when nitrite is protonated in the presence of a reducing agent (*e.g.* ascorbate), it is possible that species other than •NO are responsible for these feats. In an attempt to clarify the direct effects of •NO arising from the interaction between nitrite and dietary polyphenols in the stomach, we assessed its diffusional properties. For that, we used an Ussing chamber with a hemi chamber simulating the gastric lumen (at acidic pH) and the other the serosal environment. In both cases the hemi chambers were separated by a rat stomach wall. Nitrite, ascorbate and red wine (in different combinations) were added to the simulated gastric lumen and •NO was detected electrochemically in the serosal side.

Here we demonstrate that •NO generated from the chemical reduction of nitrite by ascorbate and wine polyphenols is able to diffuse the entire stomach wall at the µM range without causing meaningful tissue loss of functionality (as evaluated in terms of muscle relaxation and mitochondrial respiration) and thus, may be directly responsible for physiological actions such as the modulation of local blood flow. Moreover, we show that •NO generated from the interaction between nitrite and polyphenols at acidic pH induces smooth muscle relaxation likely modulating physiological phenomena such as gastric emptying. Finally, and most importantly, we established the *proof of concept* that the ingestion of dietary products rich in polyphenols (e.g., onions, berries, red wine, apples) enhance the production of •NO in the human stomach upon consumption of foodstuffs with high nitrate content (e.g., lettuce).

3.2. Results

3.2.1. Electrochemical determination of nitric oxide in phenol: nitrite mixtures

Figure 3.1A shows the production of $^{\bullet}NO$ in a reaction mixture containing the polyphenols and nitrite in simulated gastric juice (pH 2) at concentrations easily attainable in the gastric compartment after a meal. Results are expressed as the maximal $^{\bullet}NO$ produced for similar conditions. Among the polyphenols, epicatechin-3-O-gallate was the most efficient in producing $^{\bullet}NO$ from nitrite, yielding a peak of 4.6 ±0.6 μM of $^{\bullet}NO$ for 20 μM of phenol and 50 μM of nitrite. That is, even for such a low concentration of the phenol, c.a. 10% of the nitrite added is converted to $^{\bullet}NO$. Overall, the efficiency of the compounds can be ordered as follows: B5 (1.0±0.1 μM) < catechin (1.4±0.0 μM) < epicatechin (1.8±0.0 μM) < chlorogenic acid (2.6±0.2 μM) < B2 (2.8±0.1 μM) < oleuropein (2.9±0.1 μM) < B8 (3.4±0.1 μM) < quercetin (3.8±0.1 μM) < Epicatechin-3-O- gallate (4.6±0.6 μM).

Typically, the initial rates of $^{\bullet}NO$ production under these experimental conditions fall roughly in a range between 1.0 and 5.0 $\mu M.s^{-1}$. Nitric oxide concentration increased with a T80 of about 2 minutes, decreasing afterwards but at rates that vary as a function of the phenolic structure and concentration. For the lower concentrations used (20 μM) in the case of flavanols (catechin and epicatechin) a plateau is apparently reached, and a slow decay was afterwards observed during one hour of recording, whereas for the other compounds (oleuropein, chlorogenic acid and quercetin) the decay is faster and follows

distinct rates, suggesting the occurrence of several competing reactions for the decay of $^{•}$NO. Figure 3.1B illustrates this phenomena for some of the compounds (for the sake of clarity not all the compounds are shown). From these experiments, and taking into consideration the distinct profiles of $^{•}$NO production and also the distinct chemical structures, as well as their relative abundance in quite distinct dietary products, we have selected catechin (abundant in tea) and oleuropein (present in olive oil) for further studying the interaction with nitrite and the possible physiological consequences in terms of stomach muscle relaxation.

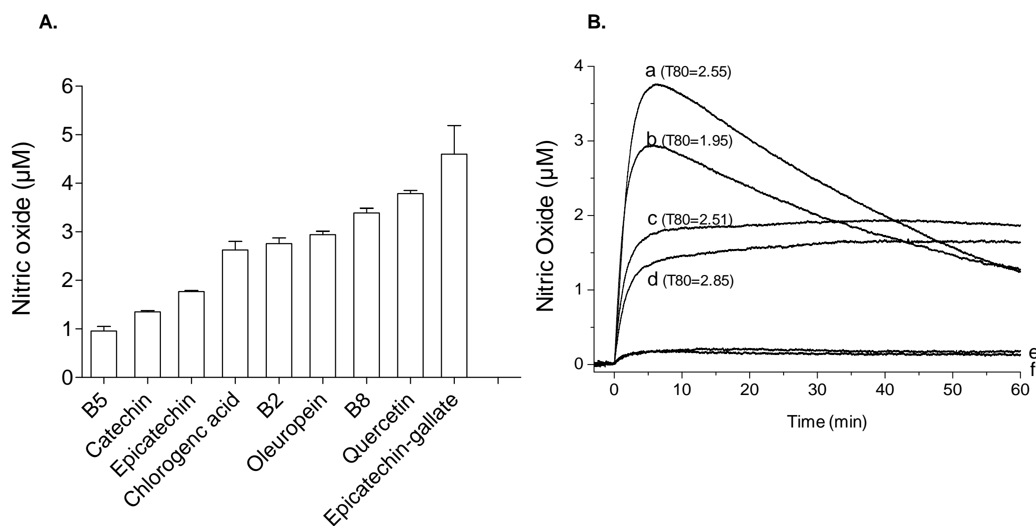


Figure 3.1 - Production of $^{•}$NO measured electrochemically in phenol:nitrite mixtures at pH 2. For each experiment, the selective electrode was inserted into the reaction mixture in simulated gastric juice equilibrated with normal atmosphere. A- peak of $^{•}$NO obtained from the kinetic recordings for 20 μM of polyphenol and 50 μM of nitrite (values are presented as mean±SEM, n=3). B- Typical time courses of $^{•}$NO production for the selected compounds shown: a, quercetin; b, oleuropein; c,

epicatechin; d, catechin; e, control nitrite (without polyphenols); f, DMSO control. Nitrite was added at time zero.

3.2.2. Nitrite decay upon reaction with phenolic compounds

The increase of $^{\bullet}$NO peak value was concomitant with a decrease in nitrite concentration along time, as shown in figure 3.2, for several concentrations of catechin and oleuropein. It is apparent that, for each compound, the higher the $^{\bullet}$NO produced, the faster the decay of nitrite concentration (table 1). However, it is also apparent that the kinetics of the decay of $^{\bullet}$NO and nitrite is distinct for both compounds. For instance, although oleuropein produces a higher peak of $^{\bullet}$NO as compared with catechin, in the latter the decay of nitrite is faster than in the former, indicating competing reactions governing $^{\bullet}$NO decay.

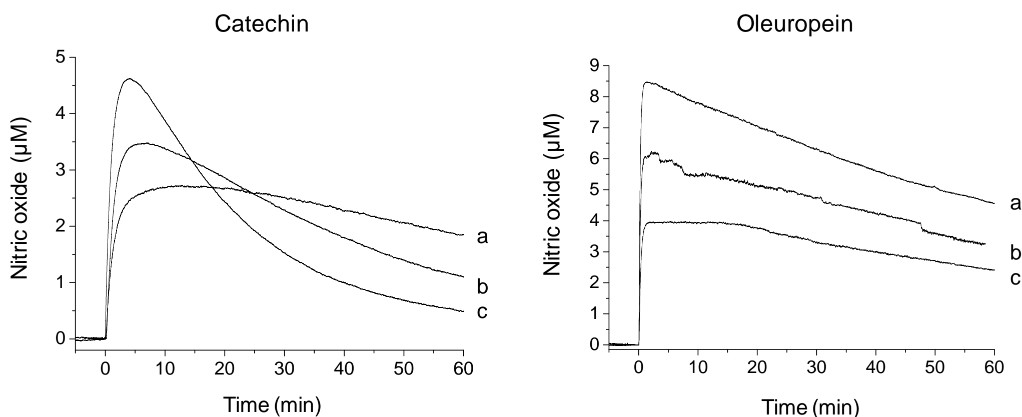


Figure 3.2 - Time courses of $^{\bullet}NO$ production in catechin and oleuropein mixtures with nitrite in SGJ pH 2. The initial concentration of nitrite in the mixture was 50 µM. The phenols were added at 100 (lines a), 200 (lines b) and 500 µM (lines c).

Phenol assayed (µM)		**Nitrite (µM) along the reaction time**		
		1 min	**30 min**	**60 min**
Catechin	100	50.5 ± 4.9	18.8 ± 1.5	4.4 ± 1.5
	200	46.7 ± 4.3	8.7 ± 1.1	1.8 ± 1.3
	500	36.0 ± 2.3	0.7 ± 0.5	0.1 ± 0.0
Oleuropein	100	53.6 ± 2.8	42.3 ± 1.9	31.8 ± 2.7
	200	45.6 ± 0.2	33.9 ± 1.5	23.8 ± 2.7
	500	44.0 ± 2.9	21.4 ± 0.8	8.1 ± 1.7

Table 1 - Time course of nitrite concentration upon reaction with polyphenols. Nitrite concentrations at 1, 30 and 60 min after mixing each of the indicated compounds with 50 µM nitrite (in SGJ). All values are presented as mean±SEM, n=3.

3.2.3. Gastric smooth muscle relaxation by mixtures of catechin and oleuropein with nitrite at pH 2

The mixtures of the selected dietary phenolic compounds, oleuropein and catechin, with nitrite at pH 2 induced gastric smooth muscle relaxation. The extent of the relaxation was dependent on the phenolic structure. Oleuropein 20 µM induced a much powerful relaxation than catechin for a range of nitrite concentrations (figure 3.3). Of note, oleuropein also induced a higher production of $^{\bullet}NO$ as compared with catechin for the same nitrite concentration range (figure 3.4). For 1 µM nitrite, oleuropein provoked

35.7±7.9 % of relaxation whereas catechin induced only 5.4±4.8 % (p=0.032). Increasing nitrite concentrations induced higher relaxations for both catechin and oleuropein but in the case of the latter a plateau was achieved (figure 3.3). For the highest concentration of nitrite (50 μM), 20 μM catechin induced 46.0±9.9 % of relaxation but oleuropein induced 59.5±9.4 % of relaxation (p=0.377). Typical relaxation recordings are shown for mixtures of 20 μM of catechin and oleuropein with 1 and 50 μM of nitrite (figure 3.3B). Controls were also performed with oleuropein and catechin 20 μM (final concentrations) prepared in SGJ in the absence of nitrite. Under these conditions no relaxation was observed (data not shown).

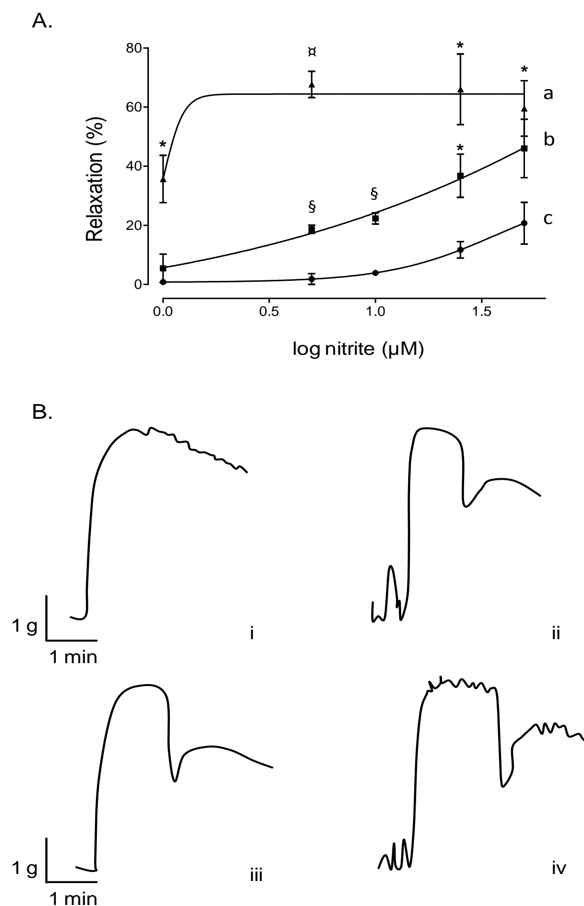


Figure 3.3 - Dose-response curve to oleuropein and catechin induced gastric smooth muscle relaxation in rat fundal strips. A- percentage of relaxation for (a) 20

μM oleuropein, (b) 20 μM catechin and (c) control SGJ without phenol. *p<0.05, §p<0.01, ¤p<0.001 (n=3). Values are presented as % of relaxation (mean±SEM, n=3). B- Typical recordings of relaxation induced by mixtures of the phenols with nitrite during 1min and afterwards added (arrow) to pre-contracted strips. 20 μM catechin and 1μM (i) or 50 μM (ii) nitrite; 20 μM oleuropein and 1 μM (iii) or 50 μM (iv) nitrite.

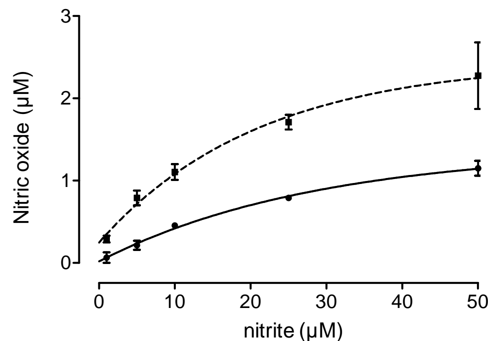


Figure 3.4 - Nitric oxide production in catechin and oleuropein mixtures as a function of nitrite concentration in simulated gastric juice at pH 2. The phenols (oleuropein, dashed line and catechin, full line) were used at 20 μM and data is shown as mean±SEM, n=3.

3.2.4. Production of nitric oxide in the stomach of human volunteers upon consumption of diet-containing polyphenols

Figure 3.5 shows the content of $^{\bullet}NO$ in the air expelled from the stomach of healthy volunteers following consumption of lettuce (as a source of nitrate) and different polyphenol-containing dietary compounds. Following consumption of lettuce (which, in addition to nitrate, also contains polyphenols) a slight increase of $^{\bullet}NO$ is observed in the

stomach (time zero). However, a sharp increase occurs after consumption of the dietary phenolic products at c.a. 5th min that tends to decrease for later times (roughly between 20-30 min), as it is particularly evident in the case of onion, apple, tea and wine.

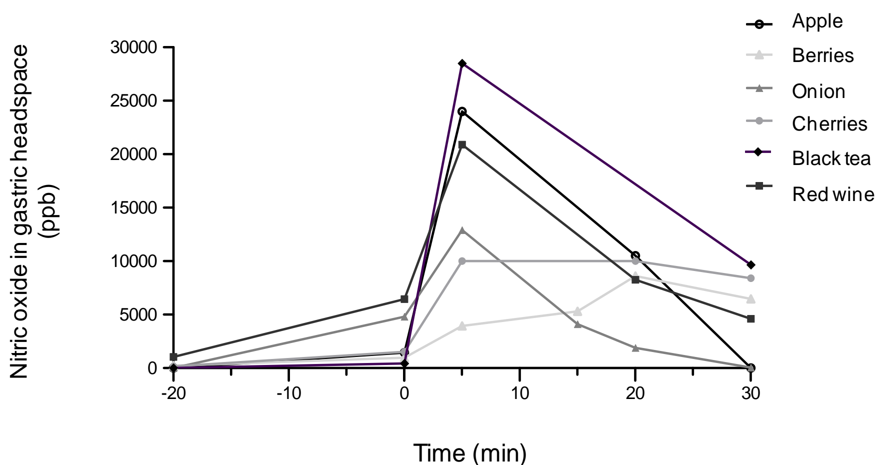


Figure 3.5 - *In vivo* $^{\bullet}$NO production in the stomach of healthy volunteers following consumption of lettuce and the dietary products and beverages indicated. Air was collected under fasting conditions (-20 min). Five minutes later (-15 min) 75 g of lettuce were ingested and $^{\bullet}$NO was measured 15 minutes later (0 min). The volunteers ate the food at time 0 min and gastric samples were collected at 5, 15, 20 and 30 minutes later). $^{\bullet}$NO was “collected” to gas-tight bags and measured by chemiluminescence.

3.2.5. Nitric oxide production from nitrite in simulated gastric juice in the presence of ascorbate and red wine

The production of $^{\bullet}$NO in the mixtures of nitrite, ascorbate and red wine was investigated under different conditions at 37ºC using a highly selective electrochemical approach. When

10 mL SGJ were supplemented with 500 μM ascorbate and 1 mM nitrite 113±10.8 μM of •NO (maximal •NO peak) were measured. When the same concentration of ascorbate and nitrite was added to a 10% red wine dilution in SGJ, 297±39.9 μM of •NO was detected ($p<0.01$). For the same dilution of red wine in the absence of ascorbate it was detected 95±23.1 μM of •NO. 17±11.3 μM of •NO was detected when 1 mM nitrite was added to 10 mL of SGJ (figure 3.6).

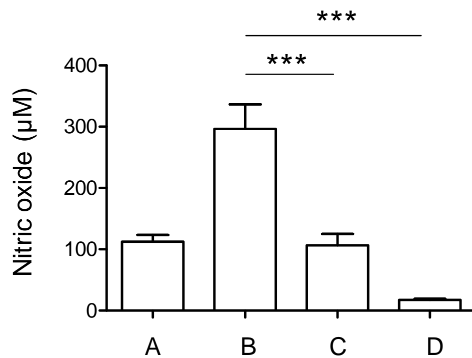


Figure 3.6 – Nitric oxide production at pH 2 from: 1 mM nitrite and 500 μM ascorbate (A), 1 mM nitrite, 500 μM ascorbate and 10% red wine (B), 1 mM nitrite and red wine (C) and 1 mM nitrite (D). All experiments were performed under stirring in open vessels at 37ºC. Values are presented as mean±SEM, *** $p<0.001$, n=3.

3.2.6. Nitric oxide diffusion through the gastric wall

The use of the diffusion chamber mounted with the rat stomach separating two compartments enabled to simulate the gastric lumen environment and to measure the diffusion of •NO generated in the luminal compartment from physiologic concentrations of nitrite and ascorbate. Upon production in the mucosal side of the diffusion chamber, •NO was detected in the other compartment (the serosal side) in a time dependent way (figure

3.7). Typically a lag-phase of c.a. 5 min was noticed between the initial ˙NO production in the mucosal side, as elicited by nitrite addition, and the onset of ˙NO detection in the serosal side (figure 3.7).

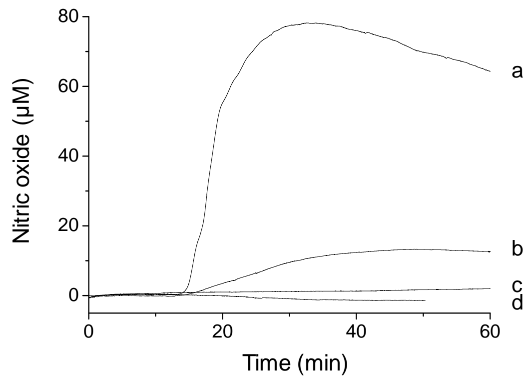


Figure 3.7 - Typical ˙NO recording in the serosal compartment of the diffusion chamber. 1 mM nitrite was added at t=2 min to 500 μM ascorbate and 10% red wine in SGJ (a); 1 mM nitrite was added at to 500 μM ascorbate (b); water (20 μL) added to 500 μM ascorbate (c) and 1 mM nitrate added to the same mixture (d). Nitrate was used as an osmolarity control. These recordings are representative for an n=3.

In the case of the mixture containing 500 μM ascorbate and 1 mM nitrite, about 8 % (in terms of maximum amplitude of the signal) of ˙NO produced in the initial compartment (mucosal) was detected in the serosal compartment. Expectedly, red wine, that strongly

potentiated the production of ${}^{\bullet}NO$ (figure 3.6), increased the percentage of ${}^{\bullet}NO$ found across the stomach wall (20 %) (figure 3.8).

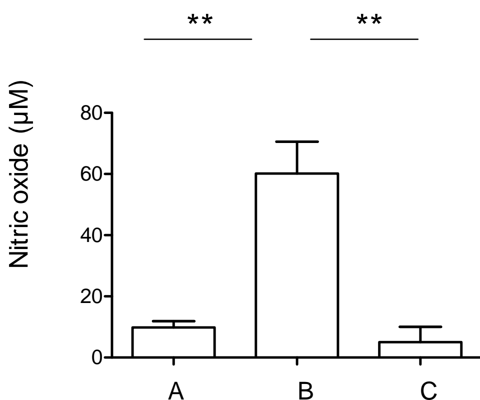


Figure 3.8 – Nitric oxide detected in serosal compartment after the following reaction in mucosal side: 1 mM nitrite and 500 μM ascorbate at pH 2 (A), 1 mM nitrite, 500 μM ascorbate and 10% red wine (B) and nitrite 1 mM and 10% red wine (C). No ${}^{\bullet}NO$ was detected when 1 mM nitrite was added alone to the mucosal hemi chamber. ** $p<0.01$, n=3.

In the absence of ascorbate but in the presence of red wine diluted to 10%, 1mM nitrite generated 95 μM ${}^{\bullet}NO$ (figure 3.6) of which 5% was detected in the serosal side. However, ${}^{\bullet}NO$ was not detected in the serosal hemi chamber when 1 mM nitrite was added to SGJ in the mucosal hemi chamber, which suggests the occurrence of a concentration threshold for ${}^{\bullet}NO$ to cross the stomach wall. Table 2 summarizes the peaks of ${}^{\bullet}NO$ achieved and the diffusion fraction under the several conditions studied.

	•NO produced at pH 2 (μM)	•NO detected at pH 7.4 (μM)	% diffused
500 μM asc + NO_2^- 1 mM	113 (n=3)	9 (n=3)	8
500 μM asc + NO_2^- 1 mM + 10% RW	297 (n=3)	60 (n=3)	20
NO_2^- 1 mM + 10% W	95 (n=3)	5 (n=3)	5

Table 2 - •NO produced in SGJ from nitrite, ascorbate (asc) and red wine (RW) under different conditions in the mucosal hemi chamber and percentage of diffused and detected in the serosal hemi chamber.

3.2.7. Carbachol-dependent contraction of gastric tissue exposed to nitric oxide

In order to assign biological relevance to •NO diffusion through the stomach wall and to exclude cellular dysfunction associated with such levels of this free radical, the exposed stomach was submitted to a functional assessment *via* carbachol-dependent contraction. The stomach was exposed to 0.1, 0.5, 1 and 5 μM of carbachol and dose-dependent contractions were measured (figure 3.9). In both conditions (control and strips exposed to •NO-generating mixture) 5 μM of carbachol induced a maximum contraction (the plateau for 5 μM did not change if higher concentrations were used). A difference between the curves is however noticed for 1 μM carbachol, as shown in figure 3.9.

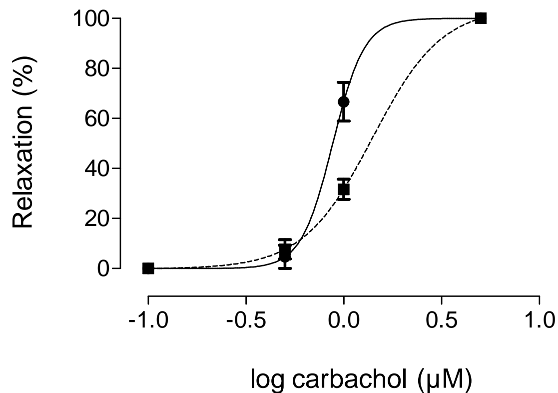


Figure 3.9 - Gastric *fundal* strips response to carbachol. Longitudinal *fundic* strips were mounted in an organ chamber filled with Krebs buffer at 37ºC. Gastric tissue exposed in the diffusion chamber to nitrite-derived $^{\bullet}$NO (dashed line) and control (solid line) were challenged with increasing doses of carbachol: 0.1, 0.5, 1 and 5 μM. Each experimental group was composed by 3 rats.

This observation may be due to the variability inherent to the use of different animals and, to some extent, to slight cellular dysfunction as when both tissues were exposed to 1mM nitrite:500 μM ascorbate mixture, similar effects were observed in terms of relaxation, namely 86.5±5.5 % and 75.0±4.0% ($p>0.05$) in control and $^{\bullet}$NO-exposed stomachs, respectively (figure 3.10).

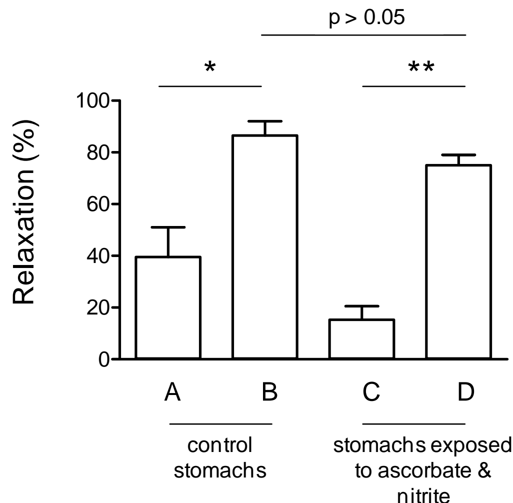


Figure 3.10 - *Fundal* strips relaxation induced by 1 mM nitrite (A) 1 mM nitrite and 500 μM ascorbate (B), 1 mM nitrite (C) and 1 mM nitrite and 500 μM ascorbate (D). (A),(B) correspond to control stomachs and (C), (D) to nitrite-derived $^{\bullet}$NO-exposed stomachs in the diffusion chamber. * $p<0.05$ and ** $p<0.01$.

3.2.8. Cellular respiration assays

Oxygen consumption was used as an additional approach to evaluate gastric tissue functionality following the exposure to nitrite:ascorbate mixtures. When gastric biopsies were added to the oxygraph chamber an initial rate of oxygen consumption of 5.3±0.64 pmol/(sec.mg) was observed. In order to exclude a major contribution of other pathways rather than mitochondrial respiration for oxygen consumption (*e.g.* lipid peroxidation), electron transfer was stimulated by adding succinate, a substrate of complex II, succinate dehydrogenase. Succinate 8.5 mM induced an increase in the rate of oxygen consumption: 20.7±5.2 pmol/(sec.mg) (figure 3.11A). Antimycin 0.45 μM clearly inhibited oxygen

consumption 6.0±1.0 pmol/(sec.mg) (figure 3.11B). An increase in the rate of oxygen consumption in the presence of succinate in respect to basal respiration (p=0.0418) and a dramatic decrease after antimycin addition (p=0.0497) demonstrate that gastric mitochondria are still functional after exposure to nitrite and ascorbate at pH 2.

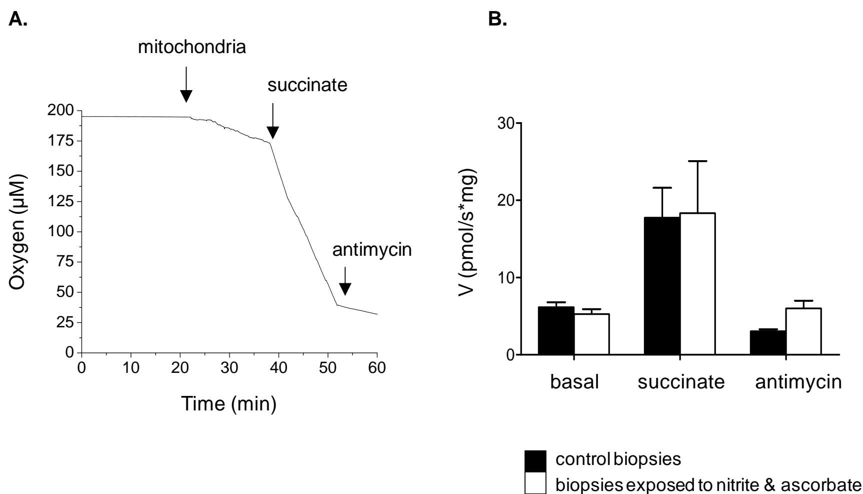


Figure 3.11 A - Real time recording of oxygen consumption by a gastric biopsy after exposure to 1 mM nitrite and 500 µM ascorbate at pH2. All experiments were performed in triplicate in closed chambers devoided of headspace. B - Rates of oxygen consumption after the addition of gastric biopsies (basal), succinate 8.5 mM and antimycin 0.45 µM. Values are presented as mean±SEM, n=3.

3.2.9. Nitric oxide diffusion studies in the stomach *in vivo* and *post mortem*

The studies with the diffusion chamber were complemented with diffusion experiments *in vivo*. When 1 mM nitrite was administered to the stomach of an anesthetized rat, previously filled with 1 ml 500 µM ascorbate at pH 2, $^{\bullet}$NO was not detected outside the stomach. Care was taken to have a hemoglobin-free environment as otherwise gastric-derived $^{\bullet}$NO could be easily scavenged by the hemeprotein, avoiding its electrochemical detection. However, when the same experiment was repeated *post-mortem*, $^{\bullet}$NO was detected across the stomach wall, outside the organ, peaking at 31 µM (figure 3.12).

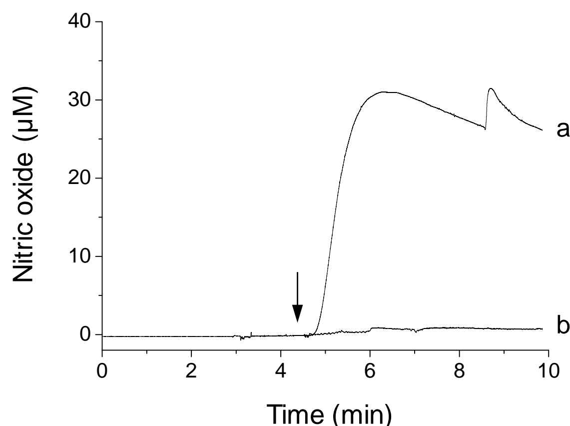
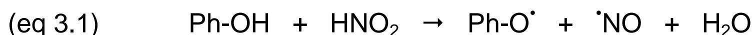


Figure 3.12 - *In vivo* and *post mortem* detection of $^{\bullet}$NO in the serosal side of the stomach. The arrow represents 1 mM nitrite addition inside the stomach filled with 500 µM ascorbate prepared in SGJ (b); (a) represents the same experiment*, post mortem* in the same animal.

3.3. Discussion

Despite the extensive intestinal metabolism, it is apparent that polyphenols may act as reductants before absorption which affords them an antioxidant activity during digestion. This notion has been forwarded years ago by Jovanovich et al [182]. However, the ability of dietary polyphenols to reduce nitrite in the gastric compartment and the search for ensued biological effects resulting from such interaction are issues that have remained largely unappreciated [159-161, 183]. We have addressed this question for a collection of polyphenols, from diverse dietary sources and distinct chemical structures to infer, among other reasons, whether this can be considered a general phenomenon after a meal. On basis of the low E^o range for polyphenols (for flavonoids is broadly 350 - 700 mV), it is expected that nitrite (E^o= 900 mV) is univalently reduced to $^{\bullet}NO$. Thus, thermodynamically, the production of $^{\bullet}NO$ in the phenolic:nitrite mixtures is feasible [184]. Accordingly, the results here obtained support the notion that polyphenols (Ph-OH) from different dietary sources and endowed with distinct chemical structures interact with nitrite at pH 2 in SGJ and in the stomach *in vivo*, producing $^{\bullet}NO$, with the concomitant intermediary formation of phenol phenoxyl radical ($Ph\text{-}O^{\bullet}$), as proposed before [159-160]:



(eq 3.1) $$Ph\text{-}OH + HNO_2 \rightarrow Ph\text{-}O^{\bullet} + {}^{\bullet}NO + H_2O$$

The kinetics of $^{\bullet}NO$ formation and particularly its decay is dependent on the polyphenolic structure, suggesting that competition reactions for $^{\bullet}NO$ are likely to occur. In fact, although

the reaction of $^{\bullet}NO$ with oxygen is not a major contributor for $^{\bullet}NO$ decay in tissues (because of the low rate constant), in the oxygenated environment of the stomach $^{\bullet}NO$ auto-oxidation is kinetically pushed and nitrosating (N_2O_3) as well as nitrating species ($^{\bullet}NO_2$) may be formed. These, may compete for polyphenols and phenoxyl radicals, thus affecting $^{\bullet}NO$ decay. Considering that the formation of the phenoxyl radical is a required step after the univalent reduction of nitrite and that, in contrast to flavonols (e.g. quercetin), the radicals of flavanols (e.g., catechins) decay *via* phenolic coupling reactions forming stable compounds such as dimmers and oligomers [185-186], it is likely that in the case of quercetin the faster decay of $^{\bullet}NO$ signal, may be contributed by reactions of the phenoxyl radical of quercetin with $^{\bullet}NO$ and derived species (*e.g.* $^{\bullet}NO_2$).

The production of $^{\bullet}NO$ from the interaction of a variety of polyphenols with nitrite *in vitro* was corroborated *in vivo*, in healthy volunteers consuming distinct dietary products and beverages known to be abundant sources of several classes of polyphenols. *This experiment establishes the proof of concept that, in the presence of nitrite, phenol-containing dietary products induce a strong increase of* $^{\bullet}NO$ *in the human stomach.*

Catechin and oleuropein, due to their prevalence in Mediterranean diet, their different distribution among dietary products and the discrepancies as nitrite reductants, were selected to study the potential effect of phenolic:nitrite mixture in gastric smooth muscle relaxation. As expected, oleuropein being a more effective nitrite reductant induced a more evident relaxant effect, as compared with catechin. However, as nitrite concentration increased, differences in smooth muscle relaxation became tinier, being non significant for

50 μM nitrite ($p=0.377$), albeit the increase of $^{\bullet}NO$ production observed for both compounds as the concentration of nitrite increased from 1 to 50 μM. Probably, a maximum response in terms of relaxation has been achieved (*e.g.,* due to sGC saturation). It can also be presumed that attending to the faster decay of $^{\bullet}NO$ produced in the reaction of catechin with nitrite, as compared with oleuropein, $^{\bullet}NO$ produced by the former reaction is involved in secondary reactions being consumed much faster than the one produced by the latter and thus less amount is available to diffuse to muscle cell layer.

At acidic pH the biochemistry of nitrite and $^{\bullet}NO$ can be quite complex for, under these conditions, and through the intermediacy of HNO_2, different RNOS can be produced. Due to this complexity, and to previous reports on the diffusion of $^{\bullet}NO$ in the stomach [187], we assessed the ability of nitrite-derived $^{\bullet}NO$ to diffuse the gastric mucosa. We observed that $^{\bullet}NO$ generated from physiologic concentrations of nitrite and ascorbate (regular components of stomach content) diffuses across the entire gastric wall *in vitro* and in the rat stomach after cardiac arrest. The fraction of $^{\bullet}NO$ diffusing across the stomach wall is a function of time and of the concentration of $^{\bullet}NO$ generated in the lumen as ascertained by modulating $^{\bullet}NO$ production *via* reduction of nitrite by red wine. Conversely, in the living animal, with operative vascular blood flow, $^{\bullet}NO$ generated in the stomach lumen was not detected outside the organ. Given the abundance of hemoglobin in the vascular system and the high rate constant of $^{\bullet}NO$ reaction with hemoglobin ($k \approx 10^7\ M^{-1}s^{-1}$), red blood cells act as a sink for $^{\bullet}NO$ preventing its diffusion across the entire stomach. *Thus, if $^{\bullet}NO$ produced in the lumen is not seen in the serosa in the living animal but can be seen*

immediately after cardiac arrest that implies that •NO diffuses from the lumen reaching the blood vessels of the stomach wall.

Given this scenario, distinct reaction pathways for •NO may be envisaged, namely a) decomposition to RNOS that can further induce nitrosation of gastric biomolecules but also nitration of specific tyrosine residues which, in turn, may be responsible for important functional changes; b) •NO may be scavenged by hemeproteins namely muscular myoglobin and residual hemoglobin that may still be present in gastric blood vessels and c) may modulate mitochondrial respiration. Overall these notions provide a rationale for the consumption of •NO within the gastric cellular and extracellular levels and thus, only the fraction that is able to escape from these pathways is detected in the serosa. However the key finding of the present study is the observation that in the absence of normal blood flow (*in vitro* and *post mortem* experiments), •NO is able to reach gastric *muscularis externa* which is a strong evidence that, due to the absence of the typical •NO signal *in vivo*, in the living animal •NO reaches mucosal blood vessels being, at least partially, responsible for the increase of mucosal blood flow measured by other groups under these conditions [102, 104].

The discussion of the results in terms of physiological relevance requires that the gastric tissue is viable and thus •NO diffusion is a feasible event and is not due to irreversible damages of gastric wall induced by RNOS produced in the lumen.

Accordingly, the physiological status of the gastric tissue, following exposure to •NO, was evaluated in terms of: (a) the functionality of the signaling pathway leading to contraction

and relaxation of the gastric smooth muscle and (b) mitochondrial respiration. Both experiments showed that the exposure to such $^{\bullet}NO$ concentrations did not compromise the tissue functionality.

Ascorbic acid, secreted locally in the gastric environment, reduces nitrite and thus, has been proposed to protect against the formation of carcinogenic N-nitrosamines by competing with secondary amines for the reaction with nitrosating agents [188-189]. Likewise, polyphenols, by reducing nitrite may be engaged in the potential prevention of nitrosamine formation [190-191], competing with the amines for nitrosating species. These effects of polyphenols would add to those arising from $^{\bullet}NO$ production and diffusion through the gastric mucosa, where it has been shown to exert protective effects [102, 192].

3.4. Conclusions

In this chapter we may resume the findings in two key aspects:

- Polyphenols, commonly found in our every-day diet, reduce nitrite to •NO not only at acidic gastric conditions *in vitro* but also in the human stomach. Therefore, we established the *proof of concept* that the ingestion of foodstuffs rich in nitrate/nitrite and polyphenols enhance intragastric generation of •NO in humans.
- Nitric oxide generated from the univalent reduction of nitrite by dietary polyphenols in the simulated gastric lumen is able to diffuse the gastric mucosa, inducing smooth muscle relaxation and likely directly modulating mucosal blood flow.

Since •NO may interfere with several gastric pathophysiological functions, polyphenols can be regarded as modulators of these biological pathways. Such a concept adds to the new role of polyphenols beyond their acute antioxidant activities and helps to explain their notorious health benefits.

CHAPTER 4

PEPSIN NITRATION AND INACTIVATION BY DIETARY NITRITE IN THE STOMACH: A NOVEL ANTI-ULCEROGENIC AGENT

4.1. Introduction

The modulation of physiological pathways by dietary nitrite in connection to $^{\bullet}$NO biology is now largely recognized [79, 93, 193]. However, few studies have reported the ability of nitrite to act as a signaling molecule through the induction of post-translational modifications, namely protein nitration. The ability of nitrite to nitrate proteins at acidic pH is recognized *in vitro*, but has been ignored from a physiological point of view. The need of a low pH and relatively high levels of nitrite justify this disregard. Though, on the light of the new findings on the nitrate-nitrite-nitric oxide dynamics *in vivo*, the hypothesis of nitrite-dependent nitration in the stomach must be revised. Moreover, since the stomach is continuously exposed to nitrite from swallowed saliva, especially after consumption of nitrate/nitrite-containing foodstuffs (but even under fasting conditions, human saliva contains 50-250 µM likely due to NOS's activity), there may be a compartmentalized, continuous and high output generation of nitrating species in the gastrointestinal tract originating from nitrite in saliva. Both, the gastric lumen and mucosa contain putative targets for nitration, not only proteins and lipids from ingested aliments but also endogenous proteins secreted by the oxyntic glands.

Following this rationale we hypothesized the nitrite-induced nitration of endogenous proteins on a diet dependent fashion.

Pepsin, the major gastric protease, is an enzyme enrolled in the digestion of not only dietary but also endogenous proteins. For this reason, it has a physiological role on

digestion but a pathological effect on peptic ulcer disease (PUD). Apart from these physiological aspects, pepsin is an endopeptidase secreted abundantly from the chief cells of the gastric mucosa into the lumen after a meal. Thus, it fulfills one *criteria* that favors nitration: exists in a high amount in a compartmentalized region. Moreover, it will act precisely on the site of generation of nitrating agents: the gastric lumen (where nitrite first encounters the acid). In addition, the amount of Tyr residues (17 tyr residues per mol [194]), made of pepsin (at acidic pH) and its zymogen, pepsinogen (at physiological pH) possible targets for nitration (in fact, nitrated pepsinogen has already been detected in the human plasma [195]). The rationale is that, after a meal, the rate of pepsinogen release increases and pepsin becomes available in the gastric lumen. Here, a dynamic interaction between pepsin and the ingested nutrients would occur. That is to say, not only pepsin will have an effect on dietary proteins by cleaving peptide bonds, but it could be simultaneously targeted for biochemical modifications induced by dietary products. Ultimately, what one eats may also modify pepsin structure and possibly its function.

Here we propose that dietary nitrite, through the intermediacy of $^{\bullet}$NO, yields nitrating agents in the stomach and that pepsin is nitrated in vivo. Whereas pepsin nitration takes place at the luminal acidic pH, $^{\bullet}$NO produced herein is able to diffuse large distances within the gastric mucosa (see previous chapter) and might trigger nitration reactions in the deep regions of the gastric mucosa. Therefore, pepsinogen contained in the cytoplasmic vesicles of the chief cells may also be nitrated. We further address the impact of nitration on protein function and demonstrate that nitration of both pepsin and pepsinogen is

translated into a decreased peptic activity *in vitro*. Accordingly, *in vivo*, nitrated pepsin prevents the development of gastric ulcers, demonstrating not only a functional but also a physiological consequence of intragastric nitration.

Our results support the notion that the ingestion of nitrate- and nitrite- rich foodstuffs induces pepsin nitration in the stomach, decreasing its ability to erode the gastric mucosa. Nitrated pepsin can therefore be regarded as an anti-ulcerogenic molecule with the peculiarities of being endogenously produced and dietary driven.

4.2. Results

4.2.1. Detection of nitrated pepsinogen: *in vitro* studies

Peroxynitrite 50 µM yielded 12 ± 4.5 µM of nitrotyrosine whereas 500 µM produced 26.7 ± 2.3 µM (figure 4.1 A). To exclude any oxidative effect of nitrate, nitrite or H_2O_2 on pepsinogen nitration, $ONOO^-$ was added to the K-Pi buffer and allowed to decompose for 5 minutes before pepsinogen was added. In this situation when the protein is added to the solution, $ONOO^-$ has already decayed and therefore no nitration is observed (data not shown). A representative UV visible spectrum is depicted in figure 4.1 B where it is demonstrated the dose-dependent increase of pepsinogen (tyrosine) nitration yields by means of the increase of absorbance at 420 nm.

Western blot was also used as an approach to confirm pepsinogen nitration by $ONOO^-$ and indeed nitration was again observed to occur and depend on the concentration of $ONOO^-$ (figure 4.1C).

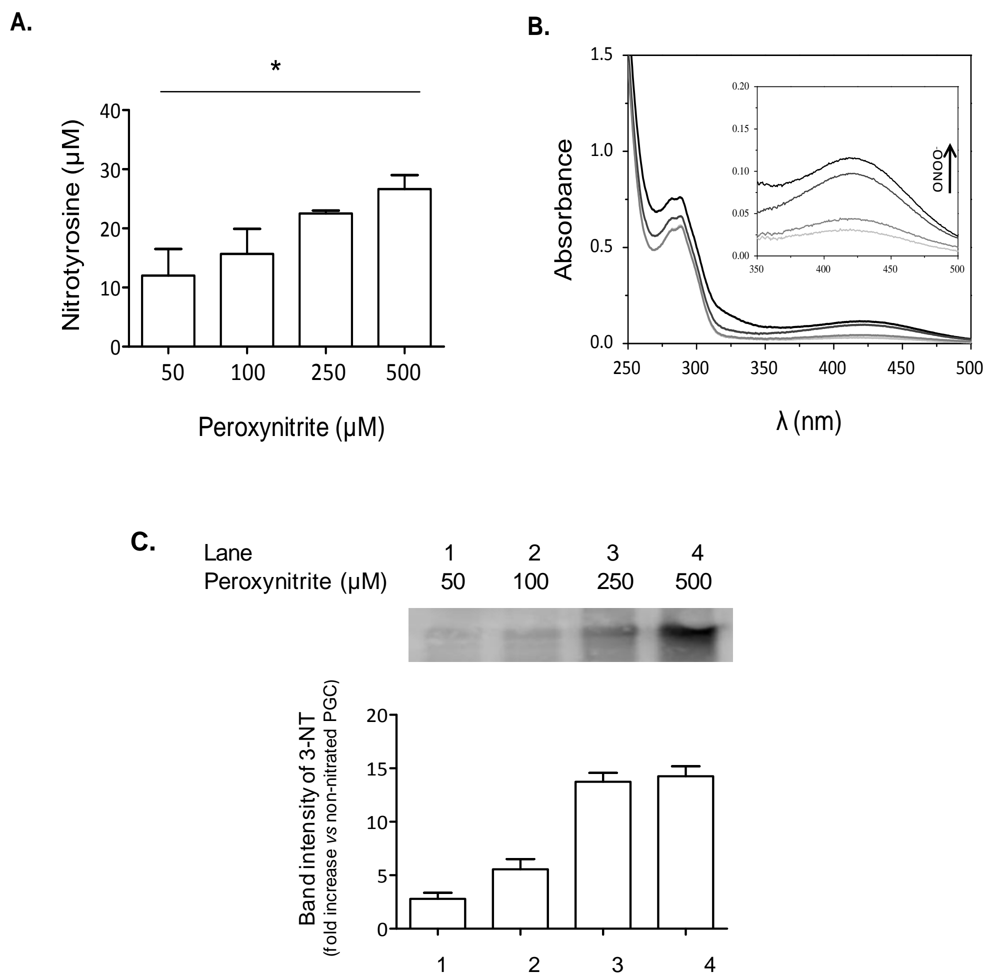


Figure 4.1 – Pepsinogen nitration by $ONOO^-$ *in vitro*. Pepsinogen 10 μM was nitrated by bolus addition of increasing concentrations of $ONOO^-$ (50, 100, 250 and 500 μM) under vortex agitation. (A), nitrotyrosine yields were determined by spectrophotometry attending to the value of absorbance at 420 nm and ε_{420}= 4400 $M^{-1}.cm^{-1}$. Values are mean±SEM (n=3 in each group, *p<0.05). (B), representative spectrum of pepsinogen nitrated with the same $ONOO^-$ concentrations mentioned

above. In the inset an increase at 420 nm (referring to nitrated tyrosine residues) is observed. (C), detection of nitrated pepsinogen by western blot. 3-NT, 3-nitrotyrosine; PG, pepsinogen.

The percentage of nitrated tyrosine residues *per* molecule was determined attending to the studies of Ryle [196] and Arnon [194] which showed that 1 mol of pepsinogen contains 17 to 20 mol of tyrosine residues (table 3).

Peroxynitrite (µM)	**Nitrotyrosine (µM)**	**Nitrated tyrosine residues (%)**
50	12.0 ± 4.5	7.1
100	15.7 ± 4.3	9.2
250	22.5 ± 0.5	13.2
500	26.7 ± 2.3	15.7

Table 3 – Percentage of nitrated tyr residues *per* mol of pepsinogen.

4.2.2. Detection of overall protein nitration in the stomach by immunohistochemistry

Protein tyrosine nitration is a physiological phenomenon in the stomach since nitrated proteins were detected in the deep mucosa of untreated rats (Figure 4.2). However, nitration yields typically decreased after nitrite exposure. On the contrary, administration of diclofenac increased the levels of nitration both in the deep and intermediate mucosa (figures 4.2 and 4.3). Nitration was predominant in the extracellular matrix rather than in

the gastric glands. This observation is in accordance with previous studies showing an increase of nitrated proteins in extracellular components of *Helicobacter pylori*-infected gastric biopsies [197]. In addition, the immunoreactivity to nitrated proteins has further increased when, after diclofenac, nitrite was administered to the rats (Figure 4.2).

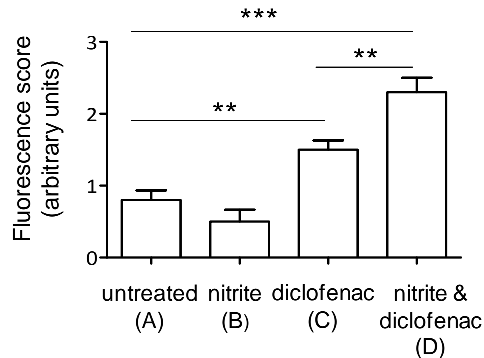


Figure 4.2 - Effect of dietary nitrite on the overall nitration in healthy and ulcerated stomachs *in vivo*. Each plotted data represents mean±SEM of 5-6 rats (***p<0.001, **p<0.01).

Once again, the staining was predominant in the deep mucosa, but in the intermediate mucosa it was also observed abundant protein nitration that was more intense than in the stomachs exposed to diclofenac alone (Figure 4.3).

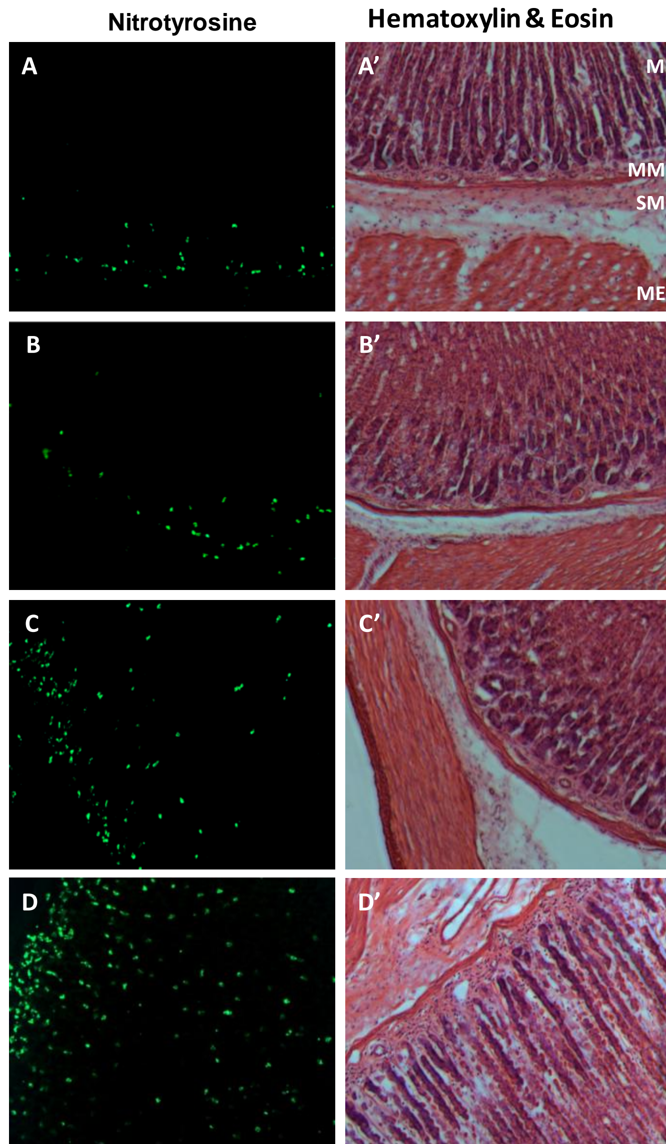


Figure 4.3 - Representative photomicrographs of the immunohistochemical labeling for nitrotyrosine in the rat stomach. Basal nitrotyrosine positive staining (green) is observed for both untreated (A) and nitrite-treated rats (B). The immunoreactivity increased in the rats that received diclofenac (C) but was further enhanced when, after diclofenac, rats received nitrite (D). The right panels stand for the H&E findings of representative regions of the correspondent panel on the left. M - mucosa, MM – *muscularis mucosa,* SM – sub-mucosa, ME – *muscularis externa* (original magnification X200).

In rats exposed to diclofenac and nitrite, it was also possible to observe nitration of proteins within the gastric glands of the deep mucosa, indicating selective nitration of specific gastric secretions (Figure 4.4). Furthermore, nitrotyrosine staining was also evident in submucosal arterioles and venules and the cytoplasmatic pattern indicated that the immunoreactivity observed in the lamina propria was most likely polymorphonuclear cells (PMN cells) (Figure 4.4 B). No staining was observed when the incubation with the primary antibody was replaced by overnight incubation with blocking solution or when the tissue was incubated with dithionite (data not shown).

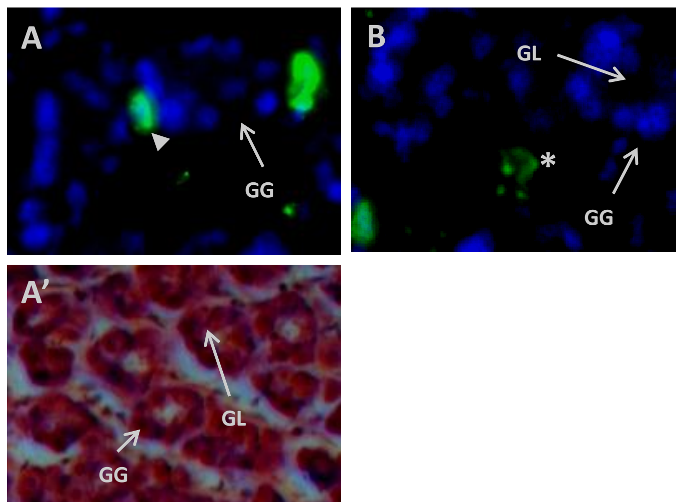


Figure 4.4 - Spatial distribution of nitrotyrosine staining in the rat gastric mucosa.
(A) Immunoreactivity (green labeling) was detected within glandular cells (arrowhead) and (B) scattered through the *lamina propria* (asterisk). Nuclei were stained with Hoechst (blue). Transversely orientated gastric glands (GG) and the respective lumen (GL) are represented in figure (A') by H&E staining (original magnification X200).

4.2.3. Detection of nitrated pepsinogen by immunoprecipitation

Immunoprecipitation was used to concentrate and purify pepsinogen from stomach homogenates. Western blot analysis of the immunoprecipitates was performed using a nitrotyrosine antibody and a clear band at 44 KDa (pepsinogen molecular weight) was detected. This band was clearly resolved from the ones of the heavy and light chains of immunoglobulins and coincided with the one of pepsinogen nitrated *in vitro* with $ONOO^-$ 500 µM (Figure 4.5 A and C). In accordance with the results of the immunohistochemistry, low levels of nitrated proteins were detected in control conditions (untreated rats), indicating that tyrosine nitration exists under physiological conditions in the stomach. Previous studies have already reported protein nitration under basal conditions in different organs. Inflammatory conditions increased the yields of pepsinogen nitration *in vivo*, but subsequent exposure to nitrite further enhanced nitration (p=0,0289, Figure 4.5 B). The administration of nitrite (alone) decreased the efficiency of pepsinogen nitration in respect to untreated rats. Treatment of total protein extracts with $ONOO^-$ 500 µM confirmed pepsinogen nitration. In contrast, incubation of the total extracts with dithionite 1 $mg.mL^{-1}$ abolished the signal (Figure 4.5 C).

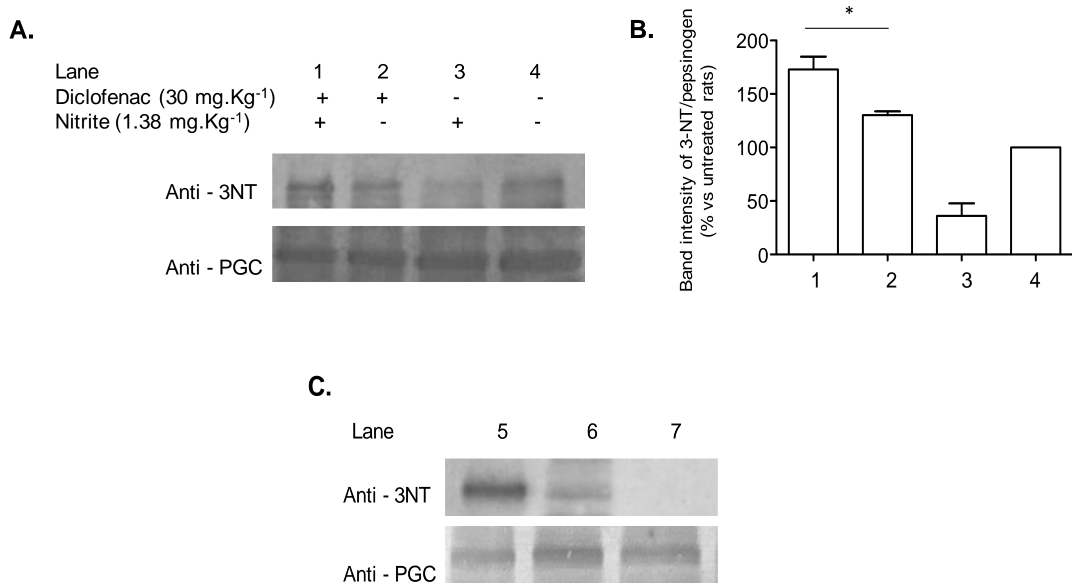


Figure 4.5 - Impact of dietary nitrite in the nitrative status of pepsinogen in healthy and ulcerated stomachs *in vivo*. (A) Pepsinogen is shown to be nitrated under physiological conditions (lane 4), but nitration yields increase under inflammatory states (lane 2). The exposure of stomachs with active gastric ulcers to physiological concentrations of nitrite further increases pepsinogen nitration (lane 1). Dietary nitrite decreases the levels of nitrated pepsinogen in healthy stomachs (lane 3). (B), densitometric analysis of the data is depicted in figure (B). (C) The bands of nitrated pepsinogen coincided with the one of pepsinogen (10 μM) nitrated *in vitro* by peroxynitrite (500 μM) (lane 5). Similarly, the addition of 500 μM of peroxynitrite to a stomach homogenate confirmed overall nitration (lane 6). The incubation of the stomach for 1 hour with dithionite (1 $mg.mL^{-1}$) abolished the signal (lane 7). Values are mean±SEM of 4 rats *per* condition (*p<0.05).

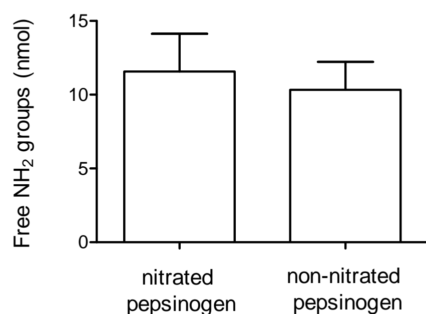
4.2.4. Impact of nitration on pepsinogen function: *in vitro* studies

Once at acidic (pH 2), the 44-aminoacidic pro-segment of pepsinogen is cleaved and the active pepsin is released. We evaluated how nitration could influence the separation of

pro-segment from the rest of the molecule, taking into consideration that when this occurs there is an increase in free NH_2 groups. We observed that there were no differences in free NH_2 groups when both nitrated and non-nitrated pepsinogen were placed at pH 2 (SGJ). In the latter there were 10.3±1.9 nmol of NH_2 and in the former 11.6±2.6 nmol ($p=0.719$) (Figure 4.6 A), indicating that the release of the pro-segment was not affected by nitration.

When nitrated and non nitrated pepsinogen were allowed to react with collagen in SGJ what one was measuring was indeed the proteolytic activity of pepsin derived from both pepsinogen forms. In this case we observed that the proteolytic activity of pepsin derived from nitrated pepsinogen was severely (c.a. 5 times) decreased when compared with the activity of the protease obtained from activation of the non nitrated zymogen. In the latter, it were detected 87.6 ± 6.5 nmol of NH_2 whereas in the former there were 12.3 ± 2.8 nmol ($p=0.0004$) (figure 4.6 B).

A.



B.

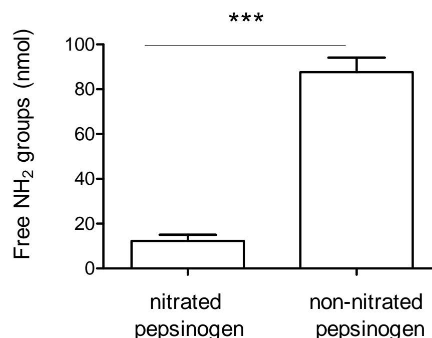


Figure 4.6 - Impact of pepsinogen nitration in its activation into pepsin at acidic pH. (A) Both native and nitrated pepsinogen (peroxynitrite 500 μM at pH 7.4) were acidified to a final pH of 2 and free

NH_2 groups were determined to evaluate the efficiency of the pro-segment cleavage. (B) Nitrated and non-nitrated pepsinogen were allowed to react with collagen at pH 2 for 1 hour under gentle stirring and free NH_2 groups determined as a predictor of the proteolytic activity of derived pepsin. The values are expressed as mean±SEM (n=3, ***p<0.001).

4.2.5. Detection of nitrated pepsin: *in vitro* studies

Peroxynitrite induced pepsin nitration dose dependently. Concerning pepsin 20 µM, 50 and 100 µM $ONOO^-$ induced the production of 1.6±0.58 µM and 4.0±0.74 µM of NT, respectively. On the other hand, the yields of tyrosine nitration greatly increased for 500 µM of $ONOO^-$, being detected 11.0±0.85 µM (figure 4.7 A). It is known that pepsin has about 20.1 mol of tyr *per* 1 mol of protein [196] thus, 0.8±0.29 % of nitration was observed for $ONOO^-$ 50 µM, but for 500 µM the percentage increased to 5.5±0.43 %. Figure 4.7 B depicts a representative spectrum showing an increase at 420 nm corresponding to an increase of nitrotyrosine yields. An $ONOO^-$ dose-dependent increase in the levels of nitrated pepsin was also observed by western blot (figure 4.7 B).

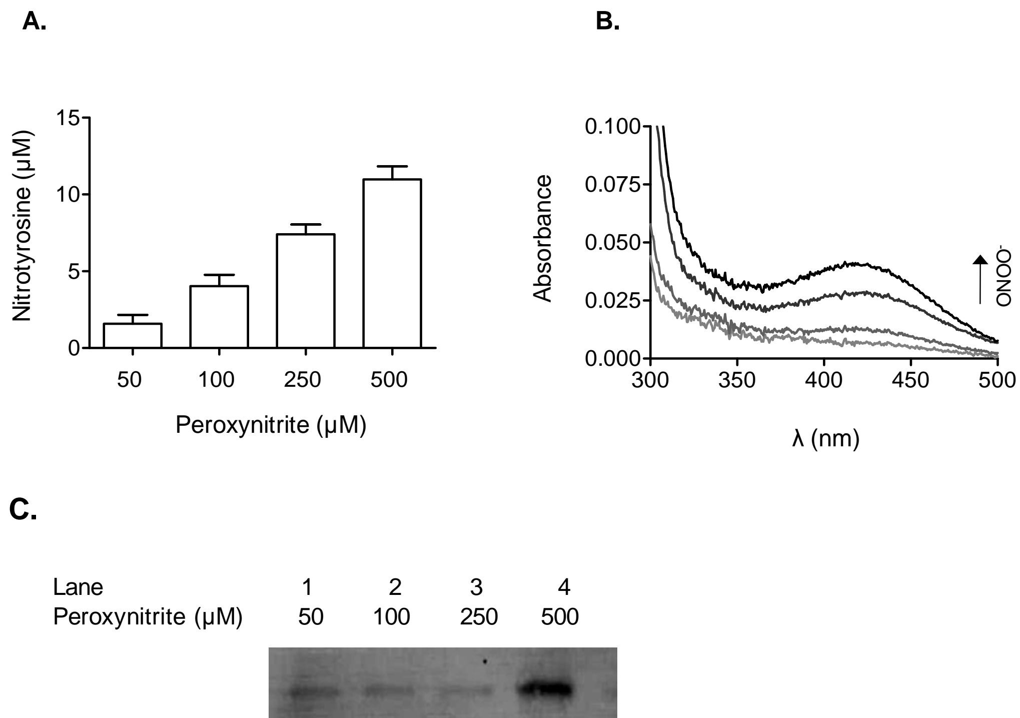


Figure 4.7 - Pepsin nitration by $ONOO^-$ *in vitro*. (A), pepsin 20 µM was nitrated by bolus addition of increasing concentrations of $ONOO^-$ (50, 100, 250 and 500 µM) under vortex agitation. (B), representative spectrum of pepsin nitrated with $ONOO^-$. (C), representative membrane of nitrated pepsin detected by western blot. Values are mean±SEM.

Similarly, acidified nitrite also induced pepsin nitration *in vitro* but at slower rates than $ONOO^-$. Nitration was detected both by spectrophotometry and western blot (figure 4.8)

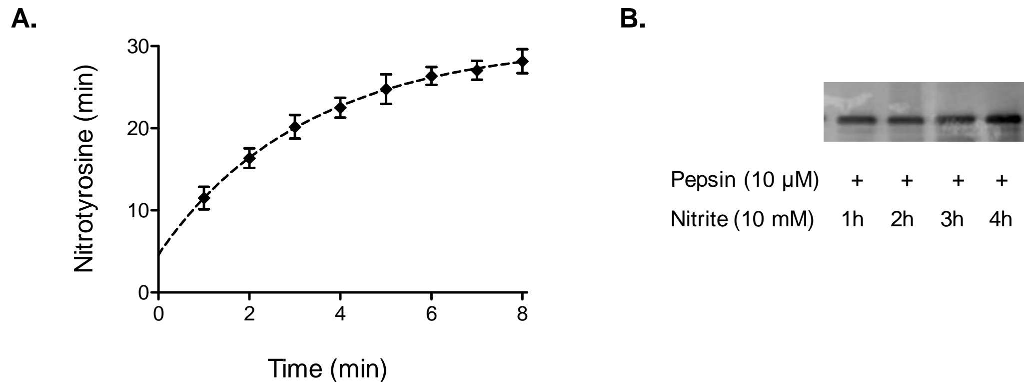


Figure 4.8 – Pepsin nitration by nitrite *in vitro.* (A), determination by spectrophotometry and (B), western blot.

4.2.6. Detection of nitrated pepsin in the stomach

Instillation of physiological concentrations of nitrite in the stomach of rats actively secreting gastric juice (both acid and pepsinogen) induces pepsin nitration. Although traceable amounts of nitrated proteins were also detected in the gastric juice (see chapter 5), pepsin nitration was obvious in the mucosa surrounding the lesions (Figure 4.9).

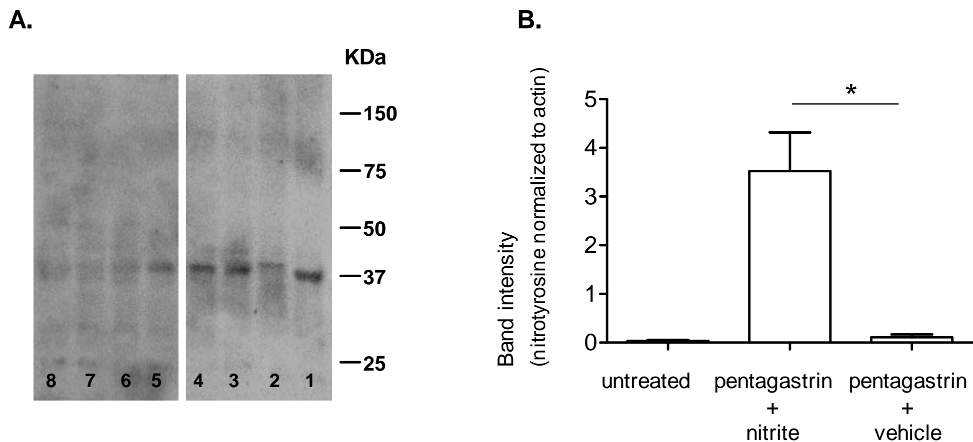


Figure 4.9 – Pepsin nitration in the stomach through a nitrite-dependent pathway. (A) Nitrated pepsin (molecular weight 37 KDa) was detected at the margin of the lesions obtained when gastric secretion was stimulated by pentagastrin followed by nitrite instillation (lanes 1-4). When nitrite vehicle was administered instead, only residual amount of nitration were detected (lanes 6-5). Untreated animals were used to assess basal nitration (lanes 7-8). Each lane corresponds to one animal. (B) Quantification of band intensities in relation to actin. Values are presented as mean±SEM (*p<0.05).

Since both acid and pepsinogen are secreted into the oxyntic gland canaliculi, it is expectable that pepsinogen is converted into pepsin still inside the mucosa. Furthermore, under these experimental conditions, the gastric pH was 1.5 – 2.0 and nitrite was readily reduced to $^{\bullet}NO$ as the levels in the headspace increased more than tenfold in comparison to basal values ($p < 0.001$) (Figure 4.10). On the contrary, when nitrite vehicle was instilled, no changes were observed in intragastric $^{\bullet}NO$ levels (Figure 4.10). Still under these conditions, only residual levels of nitrated pepsin were detected (Figure 4.9 A).

Taken together these results suggest that pepsin nitration is directly dependent on the ability of nitrite to generate •NO and possibly other nitrogen oxides in the gastric lumen.

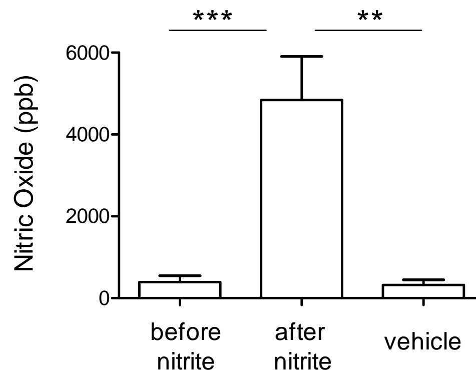


Figure 4.10 – Peak •NO values (ppb) in gastric headspace before and after nitrite instillation. Each values represents mean±SEM of 6 (for nitrite) and 4 rats (for the vehicle) (***$p<0.001$, **$p<0.01$).

4.2.7. Impact of nitration on pepsin function: *in vitro* studies

The proteolytic activity of nitrated pepsin is decreased in respect to non nitrated pepsin and is dependent on the substrate (Figure 4.11). Pepsin is most efficient in cleaving peptide bonds between aromatic amino acids, and therefore its ability to decompose proteins is dependent on the substrate structure. This specificity is also noticeable in the nitrated form of pepsin. Nitrated pepsin exhibits lower proteolytic activity not only towards dietary (e.g. casein, albumin) but also endogenous (e.g. collagen) proteins.

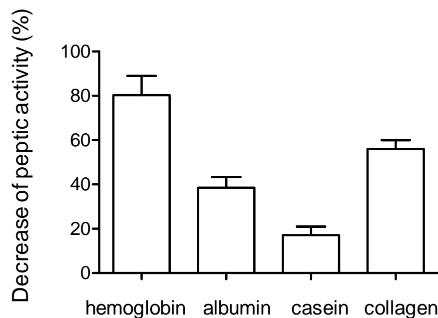


Figure 4.11 – Decrease of pepsin's proteolytic activity by nitration. The proteolytic activity was evaluated by measuring the free amino groups and compared to the peptic activity of non-nitrated pepsin (results are expressed as the % of the decrease in relation to the native pepsin).

4.2.8. Impact of nitration on pepsin function *in vivo*: anti-ulcerogenic properties of nitrated pepsin

Pentagastrin induced gastric injuries in all the animals included in this study. In control experiments (when nitrite was substituted by its vehicle) the lesions ranged typically from ulcers to gastric perforations with intense bleeding. Yet, animals receiving nitrite showed a reduced injury index reflecting a reduction in both number of lesions and degree of damage (Figure 4.12). This effect coincided with pepsin nitration.

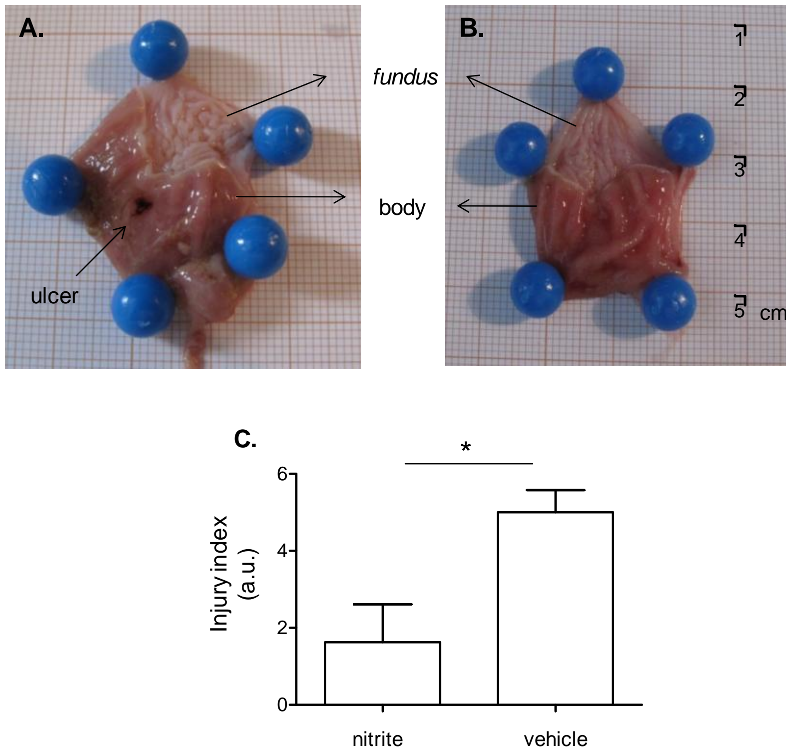


Figure 4.12 - Effect of pepsin nitration on the development of gastric ulcers. (A) Pentagastrin-induced ulcerogenesis was evident throughout the study but (B) when nitrite was instilled into the stomach the development of the ulcers was prevented. (C) The injury index was evaluated by a researcher blinded to the treatment and scored in a 0 to 6 scale. Values are presented as mean±SEM of 4 rats (*$p<0.05$).

4.3. Discussion

In this chapter we demonstrate that nitrite induces pepsin and pepsinogen nitration in the stomach and that nitration has not only a functional but also a therapeutic impact which is translated into a decreased peptic activity.

The GI mucosa is permanently under a “controlled inflammatory state” characterized by the recruitment of PMN cells, such as neutrophils [198]. In agreement we observed that in the stomach of rats under acute inflammation the overall yields of nitration are enhanced in respect to healthy animals. In this situation, extensive nitrotyrosine labeling was located within the *lamina propria* in the deep regions of the gastric mucosa, an observation that is in accordance with infiltrating PMN cells and activation of MPO which, in turn increases the nitration levels. In fact, increased protein nitration has already been reported under inflammatory conditions [197, 199], but here we demonstrate that subsequent exposure to dietary nitrite further increases the nitration yields (Figures 4.2 and 4.3). This result can be explained by the existence of two operative nitrating pathways within the gastric mucosa: 1) a biochemical pathway: nitrite-derived $^{\bullet}NO$ produced in the lumen is able to diffuse towards the glandular mucosa [187, 200], likely inhibiting cytochrome c oxidase and thereby promoting electron leakage of the mitochondrial respiratory chain with consequent generation of $O_2^{\bullet-}$ [201-202]. Nitric oxide and $O_2^{\bullet-}$ react at a diffusion-limited rate to generate $ONOO^-$, which nitrates tyrosine [173, 203]. 2) MPO-dependent pathway: luminally-diffusing $^{\bullet}NO$ can be oxidized to nitrite which is converted to nitryl chloride

(NO_2Cl) and $^{\bullet}NO_2$ by MPO and thus induce protein nitration [204]. Therefore, activation and migration of PMN cells in addition to the biochemical production of $ONOO^-$ may account for the increased immunoreactivity to nitrotyrosine in the rats with gastric ulcers exposed to physiological concentrations of nitrite. The spatial distribution of nitrotyrosine labeling follows a reproducible and specific pattern, being more abundant in the deep regions of the gastric mucosa. Furthermore, the immunoreactivity, albeit being more intense within the *lamina propria*, occurs also inside glandular cells (Figure 4.4), indicating that specific gastric secretions are being nitrated. This staining is characterized by an intense reactivity within the cytoplasm suggesting that nitrated proteins are stored in considerable amounts in this compartment, probably in vesicles. Given these facts (intense staining in cells located in deep regions of the gastric glands and with the cytoplasm filled with vesicles containing proteins able to be nitrated), chief cells and their major secretion, pepsinogen, became logical targets.

Pepsinogen nitration *in vivo* was clearly demonstrated by immunoprecipitation. Pepsinogen was one of the proteins nitrated under physiological conditions (Figure 4.5); still, nitration yields increased in ulcerated stomachs and were further enhanced after exposure to nitrite, as occurred with overall nitration. The mechanisms stated above to explain the nitration dynamics in healthy and ulcerated stomachs, exposed or not to nitrite, also fit in the outcome of pepsinogen nitration. Surprisingly, both immunohistochemistry and immunoprecipitation results show that pepsinogen and overall nitration are decreased when rats are given nitrite (alone) by oral gavage (Figures 4.3 and 4.6). Indeed, nitrite has

been shown to exert a protective role in different contexts, ranging from cardiovascular, urinary and GI systems [102, 104, 205-208]. Regarding the latter, Björne et al have shown that salivary nitrite increases mucosal blood flow and mucus thickness and thus, has a gastroprotective effect.[102]. An increase of mucosal blood flow would more efficiently wash out PMN cells infiltrated within the mucosa and thereby inhibit nitration reactions. On the contrary, in an already injured tissue (as in the case of acute inflammation), the mucosa would be more vulnerable and the defense mechanisms would be overwhelmed, increasing the nitration yields. Indeed, NSAIDs have been shown to induce hypoxia in the gastric mucosa [209] and consequently cells expressing enzymes involved in nitrating mechanisms would accumulate in the tissue. This rationale might explain why pepsinogen nitration is decreased in the rats exposed to nitrite as compared to untreated animals but the nitration yields increase when nitrite is given to animals with gastric ulcers.

Moreover, simulating the composition of gastric juice after a meal, with increasing acid and pepsinogen (due to pentagastrin administration) and exogenous nitrite, we identified pepsin as a major target for nitration in the stomach. It is conceivable that other proteins can also be nitrated, however pepsin is not only nitrated but also its proteolytic function is decreased. One obvious consequence would be the impairment of dietary proteins digestion but considering that only 15% of the exogenous proteins are breakdown by pepsin [210], the clinical significance of this finding is diverted to the role of nitrated pepsin as a preventive agent on PUD. Interestingly, the main sources of nitrate (and thus, nitrite) are green leaf vegetables that have been suggested to be beneficial for patients with

peptic ulcer [211-212]. Nitration reactions between nitrite from the diet, and endogenous proteins in the gastric lumen, may be envisaged as a probable mechanism underlying such advantageous effects.

The heme-peroxidase-dependent nitrating pathway is a likely source for nitrating agents in the gastric mucosa, and may explain the presence of low levels of nitrated pepsin when the animals received pentagastrin but no nitrite. However, the fast reduction of nitrite to $^{\bullet}NO$ and other RNOS endowed with nitrating ability, increases the yields of pepsin nitration and seems to be the predominant nitrating pathway. In untreated animals nitrated pepsin failed to be detected. This observation suggests that only when pepsin is available in an amount resembling the one obtained after a meal, it is possible to detect its post-translational modification. Furthermore, pepsin nitration is prone to occur during the postprandial phase, precisely when the gastric mucosa is being challenged with foreign agents (microorganisms, spices, NSAIDs) that may contribute to the disruption of the protective mechanisms orchestrated by the mucosa. If these mechanisms are overwhelmed and the gastric epithelium starts to be eroded, nitrated pepsin would prevent the progression of the lesion. In accordance, the injury index in the rat mucosa where pepsin was nitrated was lower than the one where pepsin was in its native form (Figure 4.12).

These results indicate that dietary-dependent nitration in the stomach occurs and produces physiologically-active compounds. Moreover, we demonstrate that pepsin is nitrated *in vivo* and that this modification confers a preventive feature in the development of PUD. Clearly,

gastric homeostasis can be actively modulated by the diet and the dynamics between nitrate, nitrite and nitric oxide are a key player.

4.4. Conclusions

In this chapter we reveal that nitrite, from dietary products, triggers nitrating pathways *in vivo* in the stomach. Nitrite, at acidic pH, is known to be a nitrating agent but has been ignored from a physiological point of view. Here, we show that not only it induces nitration of gastric mediators with a pivotal role on digestion and PUD (pepsin and pepsinogen), but also impacts on their biological function.

Pepsin is able to digest not only dietary but also endogenous proteins and, therefore, has been implicated in the progression of gastric ulcers by eroding the mucosa. In this regard we show that nitration decreases the efficiency of pepsin proteolytic activity, preventing the progression of gastric ulcers. Thus, when nitrated, pepsin acquires an anti-ulcerogenic activity, effectively preventing PUD.

CHAPTER 5

THE ROLE OF $^{\bullet}NO_2$, MYELOPEROXIDASE AND GUT MICROBIOTA ON THE MECHANISMS OF NITRITE-DEPENDENT NITRATION IN THE STOMACH: IMPLICATIONS FOR PEPTIC ULCER DISEASE

5.1. Introduction

This last chapter aims to provide further insights on the mechanisms of nitrite-dependent nitration at the acidic gastric pH. Although we have previously shown that pepsin is nitrated and inactivated by nitrite in the rat stomach, diverse nitrating agents can be produced in a biological setting at the same or different time points depending on the stimulus itself and its location [49, 213]. In this regard, $ONOO^-$, $^{\bullet}NO_2$ and acidified nitrite figure as the most notable nitrating agents [213]. Moreover, the stomach has the peculiarity of displaying a wide range of pH, comprising the acidic lumen, the physiological pH in the mucosa and the gradient along the unstirred mucus layer. As a result, different nitrating agents can be formed in these microenvironments. As an example, $ONOO^-$ exists mostly in the protonated form (ONOOH) in the gastric lumen (pKa = 6.8), whereas in the mucosa the anionic form will be the most abundant. This is quite important for nitration reactions because ONOOH, but not $ONOO^-$, isomerizes into $^{\bullet}NO_2$ and $^{\bullet}OH$. Moreover, is ONOOH and not $ONOO^-$, that reacts with HCO_3^- to produce nitrosoperoxycarbonate that spontaneously decays in $CO_3^{\bullet -}$ and $^{\bullet}NO_2$. Carbonate radical and $^{\bullet}NO_2$ are critical oxidizing and nitrating radicals, respectively. Therefore, different nitrating agents or nitrating routes may be operative in the stomach. The goal of the work described herein is to unravel nitrite-derived nitrating agents in the gastric lumen and mucosa. Using a model of germ-free mice we show that salivary nitrite is a key modulator of nitration reactions in the stomach through a mechanism dependent on gut microbiota. In another set of

experiments, after inducing gastric secretion (release of HCl and pepsin into the rat gastric lumen), nitrated pepsin was identified by immunoprecipitation. Besides, pepsin nitration was coincident with the amelioration of pentagastrin-induced gastric ulcers. Both, pepsin nitration and gastric ulcer relieve were prevented when urate was instilled intragastrically in the stomach, suggesting that $^{\bullet}NO_2$ is most likely the nitrite-derived nitrating agent at acidic pH.

5.2. Results

5.2.1. Salivary nitrite is a key modulator of nitration reactions in the stomach and depends on gut microbiota

The gut is colonized with more than 1000 species of bacteria and some of these are now thought to exist in a truly symbiotic relationship with the host [130]. Some oral bacteria use nitrogen instead of oxygen as final electron acceptor in the mitochondrial respiration, thereby reducing nitrate to nitrite and playing a crucial role on the salutary effects attributed to nitrite and nitrite-derived $^{\bullet}NO$ [110, 214]. For this reason, we investigated the role of oral bacteria in the generation of nitrating agents in the stomach. Germ-free animals fed with placebo or nitrate are not able to produce nitrite in the buccal cavity due to the lack of bacterial nitrate reducing enzymes and, consequently do not produce $^{\bullet}NO$ in the stomach (no nitrite available). However, when fed with nitrite, the step of bacterial nitrate reduction is surpassed and $^{\bullet}NO$ is normally produced in the gastric lumen. On the contrary, conventional mice generate intragastric $^{\bullet}NO$ when fed with nitrate or nitrite due to the well-functioning bacterial system. Using this experimental design we observed that nitration occurs physiologically in the gastric mucosa of conventional and GF mice, but is less intense in the latter (43.7 ± 7.7% of the conv, figure 5.1A,B). The western blots appear quite clear except for a band at around 25 kDa, which is the one we used to make comparison between groups. Curiously, $^{\bullet}NO$ production in the stomach seems to decrease the yields of nitration in the gastric mucosa: GF mice fed with nitrate exhibit higher levels of

nitrated proteins than the GF fed with nitrite (**p=0.0028) whereas no differences are observed in the same groups of conventional mice (p=0.941). When both GF and conventional mice are fed with nitrite, the yields of nitration are lower than the placebo but a slight difference is observed between them (*p=0.0454). We also evaluated the expression of iNOS in the gastric tissue and, as expected, conventional mice express higher levels of the inducible form of $^{\bullet}$NO synthase than the GF mice (figure 5.1C), probably due to the longstanding exposure to microorganisms. No differences were observed when both groups were fed with nitrate or nitrite (data not shown).

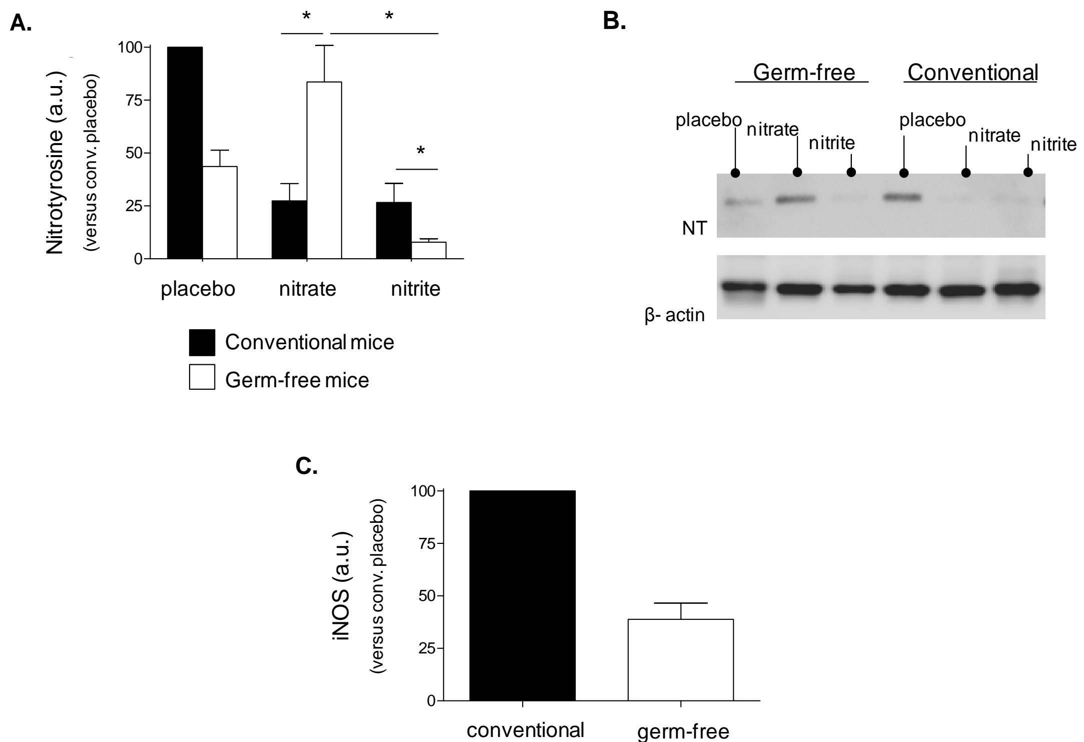


Figure 5.1 - Impact of gut microbiota on overall nitrite-dependent nitration in the stomach. A) Intragastric generation of ˙NO from dietary sources (GF supplemented with nitrite or conv mice fed with nitrate or nitrite) decreases the yields of mucosal nitration. B) Representative western blot of the data presented on A). C) iNOS expression in the gastric mucosa of GF and conv mice which had access to water supplemented with placebo (NaCl). No differences were observed when they were supplemented either with nitrate or nitrite. All values are presented as mean±SEM and were normalized to conv placebo (100%); n=5, *p < 0.05.

5.2.2. Intragastric ˙NO production from salivary nitrite *in vivo*

The univalent reduction of nitrite to ˙NO in the stomach has already been reported both in rats and humans [83-84, 178]. In this study we show that inorganic urate, naturally present in human saliva [215], impairs ˙NO production (*p=0.0174) in the rat stomach *in vivo* (figure 5.2). Though, when human saliva (obtained after the ingestion of 90 g of lettuce, with 945 µM of nitrite) was instilled into the stomach, the levels obtained for inorganic nitrite were recovered (p=0.598). Despite no statistical differences were seen between the instillation of saliva and urate plus nitrite, there is a clear trend for the inhibition of ˙NO production in the presence of urate (p=0.0536).

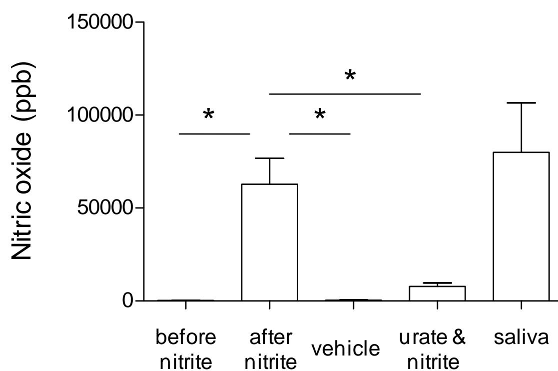


Figure 5.2 - Intragastric generation of ˙NO (ppb) from inorganic sodium nitrite, nitrite and urate and human saliva obtained after the consumption of lettuce (nitrate load). Presented values (mean±SEM) were obtained for at least three animals, *p < 0.05.

5.2.3. Salivary nitrite induces pepsin nitration in the stomach and this reaction is inhibited by urate

Pentagastrin 20 µg.Kg^{-1} induced gastric lesions in all animals included in this study. The lesions ranged from erosions to ulcers with profuse bleedings. Samples of gastric mucosa were collected from the ulcer margin and analyzed for nitrated pepsin. Since both pepsinogen (the inactive precursor of pepsin) and HCl are secreted to the gland lumen, the pH in this microenvironment may be low enough to activate pepsinogen into pepsin. Pentagastrin favors the release of both compounds and therefore facilitates pepsin activation still in the mucosa. It was based in this rationale that pepsin was immunoprecipitated from the ulcer margin. A western blot against nitrotyrosine was then performed to detect nitrated pepsin. As we have previously reported, intragastric nitrite induces pepsin nitration (*p=0.0474 in respect to control) but here we add to that information that inorganic urate inhibits nitrite-dependent nitration at the acidic gastric pH (figure 5.3). On the contrary, when nitrite-enriched human saliva (nitrite 945 µM), which contains other compounds that may influence nitration reactions (promoting – such as peroxidases – or inhibiting – such as urate) was injected into the stomach, nitrated pepsin was again detected. This result suggests that pepsin nitration is a feasible process in the stomach after a nitrate-containing meal.

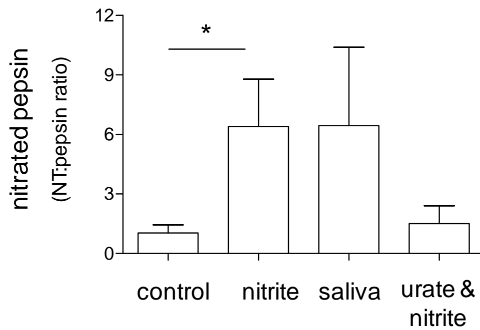


Figure 5.3 - Detection of nitrated pepsin in the margin of pentagastrin-induced gastric ulcers. Pepsin was immunoprecipitated and resolved by electrophoresis. Nitrotyrosine was then detected by western blot. Values are presented as mean±SEM, n=3, *p < 0.05.

Although pepsinogen can be activated within the mucosa, it is at the highly acidic pH of the stomach lumen (pH ≈2) that pepsin will preferably be formed. Therefore, we investigated the presence of nitrated pepsin in the gastric juice of rats which received nitrite or urate plus nitrite directly in the stomach. Since pepsin is the most abundant gastric enzyme and that pentagastrin increases its luminal levels, we used western blot to detect nitrated proteins and then re-probed the membranes with an antibody specific for pepsin. In all conditions, a single band at 37 kDa was detected. This band was coincident with pepsin molecular weight and was also present after the reprobing with the pepsin antibody (figure 5.4).

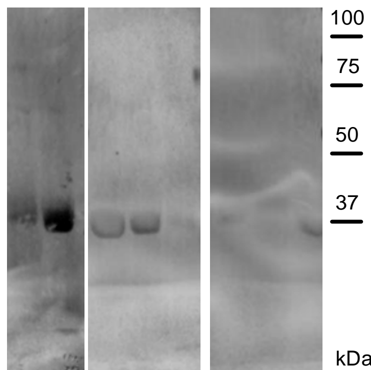


Figure 5.4 - Identification of nitrated pepsin in the gastric juice of rats which received inorganic nitrite, urate and nitrite or distilled water (vehicle). Each lane represents one animal.

It is evident that nitrite induces pepsin nitration in comparison to control (vehicle) and that urate inhibits nitrite-dependent nitration. This result establishes the *proof of concept* that nitrite nitrates pepsin at acidic pH, a reaction that is inhibited by urate.

5.2.4. Salivary nitrite decreases the levels of mucosal MPO, an effect that is prevented by urate

Myeloperoxidase is highly expressed in PMN leukocytes and has been shown to mediate nitration reactions *in vivo*, due to the production of $^{\bullet}NO_2$ and NO_2Cl [204, 216-217]. Since nitrite-derived $^{\bullet}NO$ has been shown to increase mucosal blood flow and PMN cells are constantly being carried and entering the tissues *via* blood vessels, we assessed the impact of nitrite-derived $^{\bullet}NO$ on the levels of intragastric MPO. We observed that inorganic nitrite decreases the levels of MPO by about twofold (*p=0.0481) in respect to control. Salivary nitrite also decreased mucosal MPO, even though there are no statistical differences (p=0.066). In the presence of urate, MPO levels remained similar to control (p=0.585) (figure 5.5).

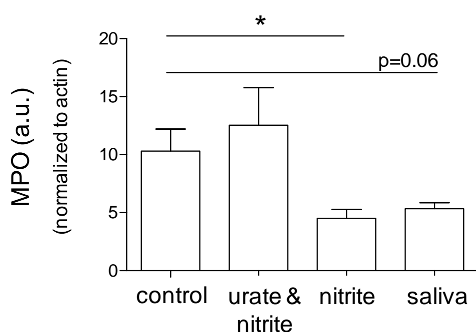


Figure 5.5 - Myeloperoxidase immunoreactivity in the rat gastric mucosa after instillation of nitrite, nitrite plus urate or nitrite-enriched saliva. Values are presented as mean±SEM, n=3, *p < 0.05.

5.2.5. Assessment of gastric injury: the anti-ulcerogenic effect of nitrated pepsin

The administration of pentagastrin 20 µg.Kg^{-1} followed by the instillation of nitrite vehicle induced gastric lesions in all animals. The lesions were macroscopically visible and ranged from serious erosions to ulcers with bleedings. When nitrite 1.3 mg.Kg^{-1} was instilled into the stomach, an obvious amelioration was observed; in this case, in two animals the gastric damage was resumed to small abrasions (figure 5.6). This observation was paralleled by nitrite-dependent pepsin nitration (figure 5.6 and 5.3).

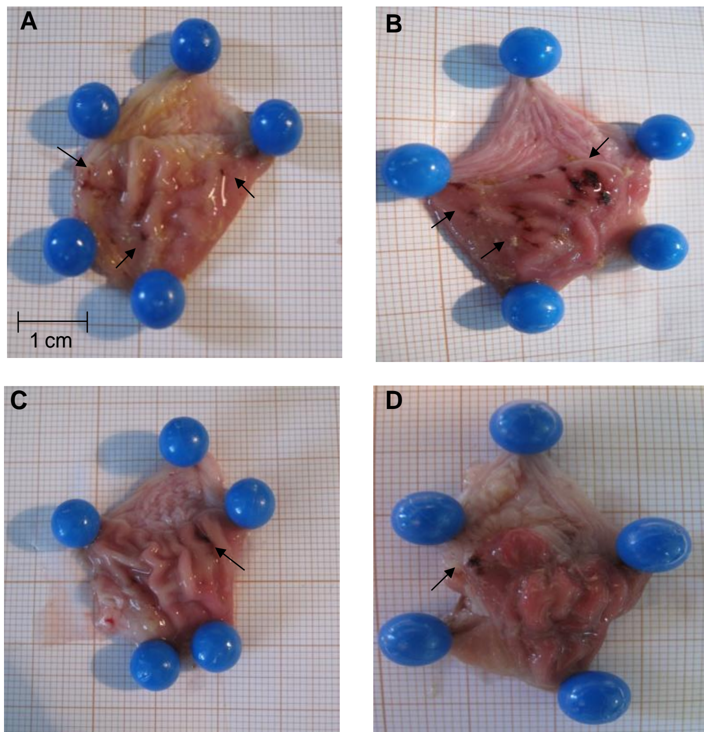


Figure 5.6 - Effect of nitrite on secretagogue ulcers.
A) Pentagastrin induced gastric ulcers in all animals included in this study.
B) Macroscopic lesions developed upon gastric instillation of urate and sodium nitrite.
C) Intragastric instillation of nitrite ameliorated the prognostics of gastric damage.
D) nitrite-enriched human saliva resembled the effect of sodium nitrite and inhibited the development of gastric ulcers. These are representative photographs of at least three animals per condition.

To study the physiological relevance of this effect, that is to say, the ability of salivary nitrite to participate in ulcer prevention, human saliva was instilled in the stomach, since it was impossible for the rat to swallow. The preventive effects of inorganic nitrite were mimicked by saliva (containing nitrite 945 µM) and again were accompanied by pepsin nitration. Opposingly, when urate was injected into the stomach, both the macroscopic appearance and microscopic morphology of the lesions worsened considerably and all were complicated by profuse bleedings (figure 5.6 and 5.7). Interestingly, in the presence of urate, nitrite fails to induce pepsin nitration (figure 5.3). Urate alone was instilled into the stomach to confirm that at acidic pH, it wouldn't damage *per se* the gastric mucosa and indeed no macroscopic lesions were observed (not shown).

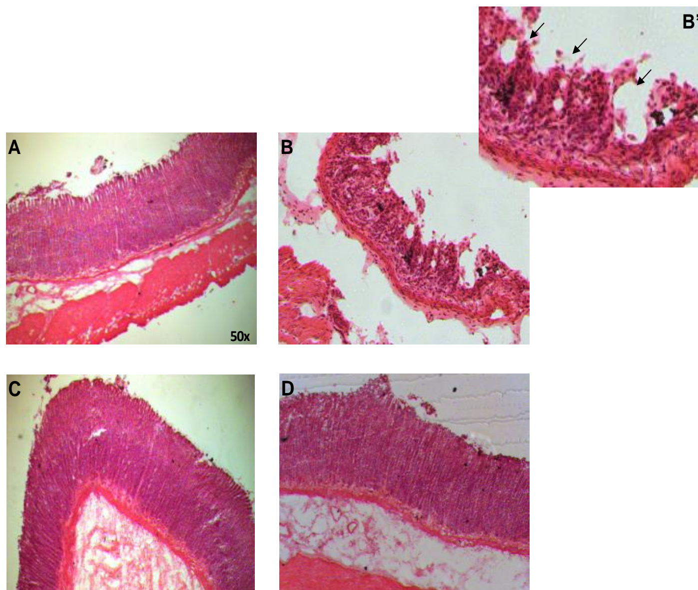
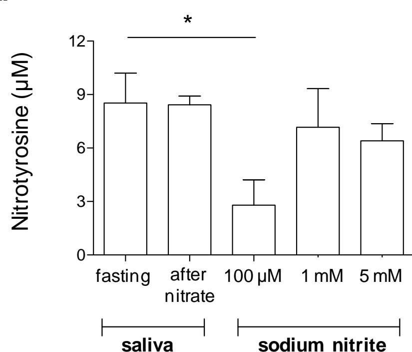


Figure 5.7 - Evaluation of gastric ulcers by H&E. A) Control rats. B) Gastric instillation of urate and nitrite. B') Mucosal derangement. C) Instillation of nitrite. D) Instillation of saliva. Representative photographs of at least three animals per condition. Amplification 50x.

5.2.6. Other salivary components rather than nitrite contribute to pepsin nitration at acidic pH

Human saliva is a slightly acidic mucoserous mix of fluids coming from different salivary glands [218]. It is composed of several electrolytes (e.g. sodium, potassium), immunoglobulins, enzymes (including MPO and lactoperoxidase), mucins and nitrogenous products (such as urate). Some of these may influence nitration reactions, for instance MPO may function as a promoter and urate as an inhibitor of nitration. Our results fit in this apparently complex chemical setting: fasting saliva (nitrite 93 μM) induced pepsin nitration much more efficiently than sodium nitrite 100 μM (*p=0.0404) (figure 5.8A). An apparent plateau is achieved for higher nitrite concentrations, both from salivary origin and inorganic form. Urate prevented pepsin nitration by salivary components not only under fasting but also after the ingestion of 90 g of lettuce, reaching significant differences in the latter (**p=0.036, figure 5.8B).

A.



B.

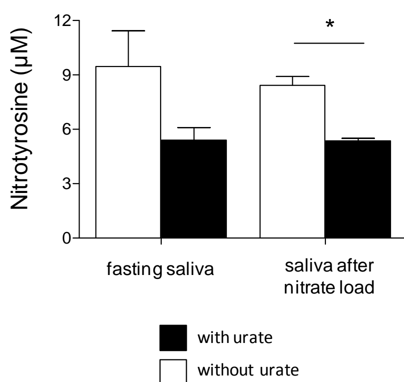


Figure 5.8 - *In vitro* pepsin nitration by inorganic or salivary nitrite in the absence (A) or presence (B) of

urate. A) Pepsin 20 μM was allowed to react with fasting and after-lettuce saliva, nitrite 100 μM, 1 and 5 mM in simulated gastric juice (pH 2) for 1 hour at 37ºC in a closed vial. B) The same experiment with pepsin and saliva was repeated in the presence of urate 200 μM. Values are presented as mean±SEM, n=4, *$p < 0.05$.

5.3. Discussion

In the past few years the paradigm of $^{\bullet}NO$ synthesis *in vivo* has shifted considerably and the main reason is that nitrite, a supposedly inert intermediate of its oxidation, was shown to be recycled back to $^{\bullet}NO$ either physiologically (e.g., in the stomach) or under hypoxic distresses [93, 219]. Despite the promising therapeutic opportunities based on the physiological effects of nitrite, few studies deal with the biochemical interactions of nitrite with both endogenous and exogenous biomolecules in connection to post-translational modifications (particularly nitration) at acidic pH. We have previously shown that dietary nitrite induces pepsin nitration thereby decreasing its proteolytic function. In this chapter we pursued the nitrite-derived nitrating agents involved in intragastric nitration. Using a model of GF mice we show that nitrite modulates nitration reactions in the stomach. Nitrite-derived $^{\bullet}NO$, produced by GF mice fed with nitrite and conventional mice fed with nitrate and nitrite, decreases the yields of overall nitration in gastric tissue. Of note, GF mice fed with nitrate are not able to generate $^{\bullet}NO$ in the stomach because they lack oral microbiota which is essential for the reduction of nitrate to nitrite. These mice will therefore possess a nitrate- but not nitrite-enriched saliva and whereas nitrite is reactive at acidic pH (yielding HNO_2 and higher nitrogen oxides), nitrate is fairly stable leaving the stomach with the chyme [99, 120]. On the contrary, when GF mice are fed with nitrite, this deficiency is overwhelmed and $^{\bullet}NO$ is produced. Recent evidences show that nitrite-derived $^{\bullet}NO$ increases gastric mucosal blood flow [102, 104], likely increasing the number of circulating

PMN cells which express MPO, a peroxidase able to produce nitrating agents [204, 216]. However the increased vascular tone could also contribute to a more efficient wash out of these cells from the gastric mucosa thereby decreasing the availability of nitrating species. In this situation, a decrease of nitrated proteins would only be observed when •NO is produced in the stomach, which is in accordance with the data presented herein (figure 5.1). Moreover, we also demonstrate that gut microbiota is a critical modulator of nitration reactions since oral commensal bacteria are necessary for nitrate reduction to nitrite and, consequently •NO production. As expected, conventional mice express higher levels of iNOS since they are exposed not only to foreign (and potential harmful) agents but also to endogenous bacteria. Some of these bacteria live in a close intimacy with the host and have been proposed to contribute to important physiological processes [130]. The co-habitation of this microflora with the host and between themselves may contribute to the permanent, but yet physiological, inflammatory sate of the stomach [220]. In this context, iNOS overexpression is thereby expected due to the involvement of •NO in inflammatory conditions.

Pepsin nitration by nitrite was confirmed using a model of pentagastrin-induced gastric ulcers in the rat stomach. Intragastric instillation of nitrite induced an increase of •NO levels by about two hundredfold and pepsin nitration (figures 5.2 and 5.3). At the first glance this observation seems to contradict the results obtained with the GF mice in which intragastric •NO apparently inhibits nitration. However, in this case nitration was studied at physiological pH (in the unstimulated gastric mucosa), whereas in the case of

pentagastrin-induced ulcers the pH was fairly acidic (ranged between 1 and 3). As already mentioned, pH differences may be responsible for the production of different nitrating agents. Jansson et al have shown that dietary nitrate prevents the development of NSAIDs –induced ulcers and attributed this effect to the gastroprotective feats of $^{\bullet}NO$ (in particular by increasing the mucus thickness) [111], but here we suggest that nitration reactions may be the molecular mechanism underling the anti-ulcerogenic properties of nitrite-derived $^{\bullet}NO$. Besides, we demonstrate that urate, a salivary component and a $^{\bullet}NO_2$ scavenger [221], inhibits pepsin nitration suggesting that $^{\bullet}NO_2$ may be the ultimate nitrating agent arising from nitrite. Another observation that implicates $^{\bullet}NO_2$ as the nitrite-derived nitrating agent relies on the inhibition of $^{\bullet}NO$ production in the stomach (figure 5.2). Urate is not an efficient scavenger of $ONOO^-$ but instead of $^{\bullet}NO_2$ and $^{\bullet}NO$ [222]. Indeed, uric acid (the predominant form at acidic pH, pKa= 3.3) reacts with $^{\bullet}NO_2$ with $k= 1.8x10^7\ M^{-1}.s^{-1}$ and with $^{\bullet}NO$ with a $k= 1.0x10^9\ M^{-1}.s^{-1}$, meaning that $^{\bullet}NO_2$ and $^{\bullet}NO$ compete for uric acid [222]. In agreement, urate decreases intragastric $^{\bullet}NO$ c.a. eightfold in comparison with nitrite alone (figure 5.2). Interestingly, pentagastrin-induced lesions were aggravated when nitrite was instilled after urate. Ulcers complicated by extensive bleedings were observed in all animals suggesting that $^{\bullet}NO_2$ scavenging by urate, thus inhibiting pepsin nitration, prevented the repairing effects of nitrite. These results are in accordance with the profile of nitrated proteins in the gastric juice.

Saliva is a key player in nitrite biology since dietary nitrate is reduced to nitrite in the oral cavity and mixed with saliva which will carry it further to the stomach (reviewed in [79]).

Regarding nitration reactions, is even more tricky because on one hand it contains compounds that may inhibit protein nitration (e.g., urate, ascorbate) and on the other, others that may favor this post-translational modification (e.g. MPO and lactoperoxidase) [223]. Myeloperoxidase is the major peroxidase in the oral cavity and comes mostly from PMN leukocytes migrating at the gingival sulcus [224]. Thus, the dynamics of the agents inhibiting or enhancing nitration reactions led us to study the impact of nitrite in a more physiological context by using human saliva. When 1 mL of saliva obtained after the consumption of lettuce (nitrite-enriched) was instilled into the stomach, an increase of intragastric $^{\bullet}NO$ levels and pepsin nitration were observed. The impact on the prognostic of gastric ulcers was similar to that obtained with inorganic nitrite, being characterized by small abrasions or erosions instead of the pronounced lesions obtained in the presence of urate. This observation confers biological significance to our results as nitration reactions occur with salivary concentrations of nitrite and urate. Moreover, the functional impact on acute peptic ulcers is also observed in this physiological setting. These evidences support the notion that nitrite-dependent nitration with impact on local physiological mechanisms is prone to occur in the stomach. We also addressed the nitrating ability of saliva *in vitro*. These studies confirmed that other salivary components rather than nitrite can induce pepsin nitration under simulated gastric conditions. Fasting saliva (nitrite 95 μM) increased pepsin nitration by about 3 times compared to inorganic nitrite 100 μM. Interestingly no differences were observed between fasting and post-lettuce saliva, nitrite 1 and 5 mM

suggesting that probably a maximum of nitrated tyrosine residues was achieved. Similarly, urate decreased pepsin nitration yields both by fasting and non-fasting saliva.

As already mentioned, MPO induces protein nitration through the generation of different nitrating species [204, 217]. Therefore we also assessed if MPO could have any influence on mucosal nitration reactions. We observed that nitrite instillation decreases the immunoreactivity of this peroxidase, an effect that is mimicked by nitrite-enriched saliva (figure 5.5). We hypothesized that nitrite-dependent $^{\bullet}NO$, by enhancing mucosal blood flow, would improve the wash out of PMN cells expressing MPO.

In the study presented herein, we demonstrate that protein nitration is facilitated in the acidic gastric lumen and is modulated by salivary nitrite, gut microbiota and intragastric generation of nitrogen oxides. Furthermore, we show that urate impairs gastric ulcer relieve, an observation that underpins $^{\bullet}NO_2$ as the most likely nitrating agent deriving from salivary nitrite. The biological significance of gastric nitration products locally in the gastrointestinal tract, and systemically after absorption, should be further investigated.

5.4. Conclusions

Here we add a new concept to the field of nitrite biology by showing that dietary nitrite induces nitration of endogenous proteins with impact on the prognostics of gastric ulcers and that $^{\bullet}NO_2$ is most likely the nitrating agent involved. Moreover the inhibition of the anti-ulcerogenic effect of nitrated pepsin in the presence of a $^{\bullet}NO_2$ scavenger, suggests that nitrite-dependent pepsin nitration may be at least partially responsible for the gastroprotective effects of $^{\bullet}NO$.

CHAPTER 6

FINAL DISCUSSION AND CONCLUSIONS

Following its initial identification with the EDRF, $^{\bullet}NO$ has been one of the most studied endogenous molecules and is currently regarded as an ubiquitous cell messenger, mediating both physiological and pathophysiological processes. Until recently, the conceptual framework for $^{\bullet}NO$ actions relied on its regulated synthesis by a family of nitric oxide synthases and its decay to stable end products, notably nitrite and nitrate. Nevertheless, the appreciation that $^{\bullet}NO$ and nitrite could embark in a reversible redox cycle revealed that $^{\bullet}NO$ production out of control of its synthases could be operative *in vivo* and, notable in certain cases, could result from a pure chemical reaction from nitrite univalent reduction. This has open the new field of “Nitrite Biology”, in which $^{\bullet}NO$ has a center role.
Dietary nitrite has been shown to exert several effects, ranging from the decrease of blood pressure, protection against myocardial infarction and attenuation of ischemia-reperfusion injury. In the particular case of the gut, nitrite activates gastroprotective mechanisms, ameliorates the prognosis of ulcerative colitis in mice and kills invading pathogens. A number of these effects can ultimately be mediated by $^{\bullet}NO$, either directly or indirectly, but a direct role of nitrite cannot currently be ruled out. Although the health benefits of nitrite are now being extensively studied, the biochemical interactions of this anion with endogenous molecules has remained largely elusive. In the third chapter of this thesis we report that in the acidic stomach, nitrite reacts with dietary compounds namely polyphenols, to produce $^{\bullet}NO$. This reaction is thermodynamically facilitated and the yields of $^{\bullet}NO$ are increased up to thirtyfold. Moreover, we established the *proof of concept* that in a typical meal containing vegetables (as source of nitrite) and foods rich in polyphenols,

$^{\bullet}NO$ levels in the gastric headspace strongly increase. Noteworthy and considering the potential benefits of dietary polyphenols, these results support novel redox properties of polyphenols beyond their antioxidant activity that, as it is now generally accepted, cannot support their actions *in vivo*. Following the rationale of nitrite reduction to $^{\bullet}NO$ in the human stomach, we later demonstrated that $^{\bullet}NO$ generated in the gastric lumen diffuses towards the deep layers of the gastric mucosa, directly implicating this free radical in physiological pathways, such as smooth muscle relaxation. It may be envisage that $^{\bullet}NO$ may trigger different signaling pathways within the mucosa, namely a) may decompose to RNS that can further induce nitrosation or nitration of gastric biomolecules likely inducing loss, gain or change of function; b) may be scavenged by hemeproteins such as muscular myoglobin and circulating hemoglobin and c) may compete with molecular oxygen for mitochondrial cytochrome c oxidase.

In this regard and taking into account that nitrite has been already identified as a nitrating agent *in vitro*, we investigated whether this anion could also induce such post-translational modification *in vivo* by targeting endogenous proteins. Until now, nitrite-dependent nitration has been overlooked from a physiological point of view since both low pH and high nitrite concentrations are necessary for the reaction to proceed. However, on the light of the nitrate-nitrite-nitric oxide pathway (in which diet fuels high amounts of nitrite into the gastric lumen), nitrite-dependent nitration of gastric proteins became an obvious scenario. In the final chapters of this thesis we provide experimental evidences for this hypothesis and demonstrate that dietary compounds endowed with adequate redox properties can target

endogenous biomolecules and induce post-translational modifications with functional consequences. Accordingly, it is shown that pepsinogen and pepsin, at physiological and acidic pH, respectively, are targeted for nitration by dietary nitrite. The results presented herein strongly suggest that nitrite-dependent nitration involves a rather complex network of chemical reactions and is highly dependent both on the physiological status of the tissue and on the pH. Most importantly, it is demonstrated that nitration of pepsinogen and pepsin decreases the proteolytic activity of the later. This finding has obvious functional but most importantly, physiological consequences, such as the inhibition of gastric ulceration. Overall it is provided data supporting an axis involving dietary products, biochemical modifications and physiological outcomes in which $^{\bullet}NO$ is the key *ingredient.* Conceptually, the demonstration that dietary nitrite induces nitration of gastric proteins such as pepsinogen and pepsin, opens a new and wide stage in which the nitrate-nitrite-nitric oxide pathway plays a central role. For instance, other proteins may be targeted for nitration. Proteins from the tight junctions (e.g., occludin), due to the close proximity to the site of generation of nitrating agents and to the high content of Tyr residues in extracellular loops, are a feasible target *in vivo*. Both functional and physiological consequences may be also expected since these proteins are responsible for the fence function of the gastric epithelium and the derangement of these structures may have consequences for mucosal cytoarchitecture. Furthermore, dietary compounds other than proteins may also be selectively nitrated by nitrite in the stomach. Obvious examples are dietary lipids, such as oleic acid (important component of olive oil) that could be nitrated in the acidic gastric

lumen. Nitrated lipids have been subject of intense research in the past few years due to their anti-inflammatory and signaling properties *in vivo*. Upon production, they could act locally in the stomach or be absorbed and modulate signaling pathways systemically. These are some examples illustrating the impact of the novel nitrating pathway that is reported in this thesis and have been discussed in an “hypothesis paper” recently published by us. It is now challenging to understand how our daily diet can serve the biochemical interactions in the stomach and how our organism can benefit from such dynamics.

As a framework for the conclusions that can be drawn from this thesis, three key aspects can be highlighted: $^{\bullet}NO$ production by the interaction of nitrite and polyphenols in the human stomach, $^{\bullet}NO$ diffusion through the gastric mucosa and $^{\bullet}NO$-dependent post-translational modifications with physiological relevance. In detail, the following findings should be emphasized:

- Foodstuffs rich in different classes of polyphenols efficiently reduce dietary nitrite to $^{\bullet}NO$ in the human stomach.
- Once produced in the gastric lumen, $^{\bullet}NO$ diffuses towards the gastric mucosa, inducing smooth muscle relaxation.
- Nitrite triggers nitrating pathways in the acidic gastric lumen and mucosa, being pepsin and pepsinogen targets for nitration.

- The nitration of pepsin decreases its proteolytic function, a process that taking place *in vivo* is accompanied by the inhibition of gastric ulcers progression.
- Myeloperoxidase and $^{\bullet}NO_2$ are the likely nitrating agents arising from nitrite.

Overall, the experimental evidences provided by this thesis contribute to the gain of significance that the new field of “The Biology of Nitrite” is acquiring in the scientific community, supporting that dietary nitrite may perturb biochemical processes with both physiological and pathophysiological implications: firstly, nitrite yields the ubiquitous messenger $^{\bullet}NO$ in the stomach that, in turn, may embark into the modulation of several biochemical pathways, notably smooth muscle relaxation. Secondly, nitrite triggers nitrating pathways *in vivo* inducing critical biochemical modifications in proteins with pathophysiological implications that can be tuned by the diet.

Schematic summary

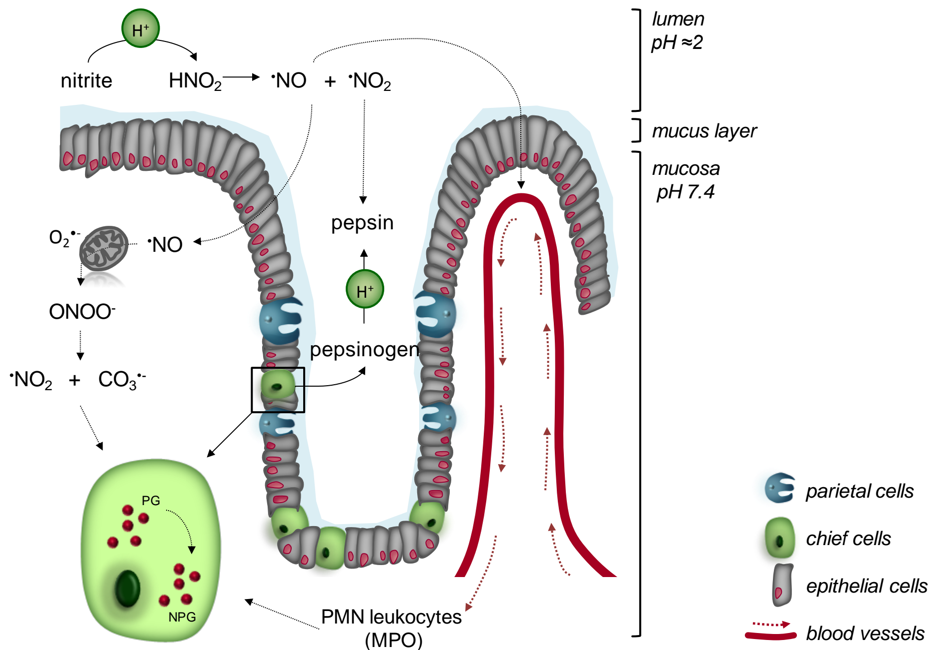


Figure 6.1 – Schematic representation of some of the physiological and biochemical effects of $^{\bullet}NO$ in the gastric mucosa. Dietary nitrite is protonated and reduced to $^{\bullet}NO$ in the acidic gastric lumen. Nitric oxide diffuses towards the gastric mucosa modulating different physiological pathways, such as blood flow. In addition to $^{\bullet}NO$, acidified nitrite may also yield other RNOS, such as $^{\bullet}NO_2$ which may react and nitrate pepsin released from the chief cells. Alternatively, $^{\bullet}NO$ may also inhibit mitochondrial respiration in the mucosa, leading to the generation of $O_2^{\bullet-}$ that reacts with $^{\bullet}NO$ producing $ONOO^-$. Peroxynitrite produces $^{\bullet}NO_2$ and, in the presence of CO_2, $CO_3^{\bullet-}$. Both these radicals induce protein tyrosine nitration at physiological pH and may explain pepsinogen nitration in the chief cells cytoplasm. Additionally, circulating polymorphonuclear cells (PMN, eg leukocytes) express MPO which is also described to induce protein nitration and may be an alternative nitrating pathway operating in the stomach. Curiously, this pathway is also dependent on nitrite-derived $^{\bullet}NO$ since an increase of mucosal blood flow shapes the availability of PMN by increasing their wash out.

CHAPTER 7

ABBREVIATIONS AND REFERENCES

Abbreviations

Cl^-	Chloride anion
CO_2	Carbon dioxide
$CO_3^{\bullet-}$	Carbonate radical
cGMP	cyclic Guanosine monophosphate
DNA	Deoxyribonucleic acid
EDRF	Endothelium Derived Relaxing Factor
EtNO	Ethyl nitrite
FAD	Flavin adenine dinucleotide
FMN	Flavin mononucleotide
GI tract	Gastrointestinal tract
GTP	Guanosine triphosphate
H_2O_2	Hydrogen peroxide
HOCl	Hypochlorous acid
HNO	Nitroxyl
HNO_2	Nitrous acid
MPO	Myeloperoxidase
NMDAR	N-methyl-D-aspartate receptors
NO^+	Nitrosonium ion
N_2O_4	Dinitrogen tetraoxide
N_2O_3	Dinitrogen trioxide
$^{\bullet}NO$	Nitric Oxide
NOS	Nitric Oxide Synthase
NO_3^-	Nitrate
NO_2^-	Nitrite
$^{\bullet}NO_2$	Nitrogen dioxide radical
NO_2^+	Nitronium ion
NO_2Cl	NO^+
NSAID	Non steroidal Anti-Inflammatory Drugs
NT	Nitrotyrosine

$^{\bullet}OH$	Hydroxyl radical
$ONOO^-$	Peroxynitrite anion
$ONOOCOO^-$	Nitrosoperoxycarbonate
ONOOH	Peroxynitrous acid
$O_2^{\bullet-}$	Superoxide radical
pO_2	Oxygen tension
PUD	Peptic Ulcer Disease
RNOS	Reactive Nitrogen and Oxygen Species
SOD	Superoxide dismutase
sGC	soluble Guanylate Clyclase
Tyr	Tyrosine
$Tyr–O^{\bullet}$	Tyrosil radical
XO	Xanthine oxidase

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