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The role of nitrite-derived nitric oxide in gastric physiology: biochemical mechanisms, molecular targets and the modulatory effect of red wine.

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Abbreviations

CaM	Calmodulin
CcOx	Cytochrome c oxidase
cGMP	cyclic Guanosine Monophosphate
CGRP	Calcitonin Gene-Related Peptide
COX	Cyclooxygenase
EDRF	Endothelium Derived Relaxing Factor
ETC	Electron Transfer Chain
FAD	Flavin Adenine Dinucleotide
FMN	Flavin Mononucleotide
GI tract	Gastrointestinal tract
GKN2	Gastrokine 2
GTP	Guanylate Triphosphate
HIF	Hypoxia-inducible Factor
NADPH	Nicotinamide Adenine Nucleotide Phosphate
3-NT	3-Nitrotyrosine
NMDAR	N-methyl-D-aspartate receptors
NOS	Nitric Oxide Synthase
NSAID	Non-Steroidal Anti-Inflammatory Drug
OG	Oral gavage
PG	Prostaglandin
pO_2	Oxygen tension
RNOS	Reactive Nitrogen Oxide Species
ROS	Reactive Oxygen Species
$-RS^{\bullet}$	Thyil radical
RW	Red Wine

sGC	soluble Guanylate Cyclase
SNO	S-Nitrosothiols
SOD	Superoxide dismutase
TFF	Trefoil Factor peptide
VEGF	Vascular Endothelial Growth Factor

Resumo

É hoje globalmente aceite que diferentes aspetos do estilo de vida, nomeadamente a dieta, podem ter um impacto significativo na qualidade de vida e no surgimento de determinadas doenças. Neste contexto, o nitrato e o nitrito, constituintes de vegetais consumidos regularmente na dieta e presentes no sangue humano e de outras espécies animais, foram identificados como sendo compostos bioativos envolvidos em processos biológicos que contribuem para uma melhoria da saúde humana.

O óxido nítrico (•NO) é um mediador celular ubíquo com papel relevante nos sistemas cardiovascular, imunitário e nervoso. As propriedades físico-químicas do •NO, nomeadamente o facto de ser uma molécula diatómica, difusível e hidrofóbica tornam-no distinto de outros mensageiros celulares, uma vez que permeia membranas biológicas facilmente, não sendo, portanto, armazenado em vesículas. A dinâmica da concentração do •NO, ou seja, o perfil de variação da concentração no tempo e no espaço, em grande parte determinado pelo equilíbrio entre a sua síntese (via óxido nítrico sintases, NOS ou por redução química do nitrito) e a sua inativação (por reação com heme proteínas ou via oxidação a nitrito e nitrato), determina a sua bioatividade.

O nitrito tem sido considerado quer como um produto do metabolismo do •NO cujo destino é a excreção, quer como um tóxico existente em determinados alimentos causador de cancro gástrico pela formação de N-nitrosaminas e envolvido em casos de meta-hemoglobinemia infantil. A biologia do nitrito chamou a atenção da comunidade científica quando foi descoberta a produção de •NO a partir de nitrito inorgânico no estômago. O consumo de alimentos como alface, beterraba, espinafres, brócolos e outros vegetais de folhas verdes ricos em nitrato (e algum nitrito) levam a um aumento de nitrato e nitrito no plasma. Na cavidade oral, bactérias comensais reduzem nitrato a nitrito, que misturado com a saliva, chega ao estômago onde o pH ácido promove a redução do nitrito a •NO. Esta sequência de eventos designada por *Nitrate-Nitrite-•NO pathway*

origina no estômago a maior concentração de $^{\bullet}$NO formada *in vivo*. Por outro lado, têm sido propostos diversos mecanismos para a redução de nitrito a $^{\bullet}$NO *in vivo*, nomeadamente os que envolvem a catálise por heme-proteínas (e.g. hemoglobina) que mostram atividade de nitrito reductases em condições de baixa tensão de oxigénio e de baixo pH, implicando o envolvimento do nitrito na sinalização em hipoxia. O nitrito representa, portanto, um vasto reservatório de $^{\bullet}$NO no organismo e tem também sido implicado na modulação de funções celulares de uma forma independente do $^{\bullet}$NO.

No trato gastrointestinal, o $^{\bullet}$NO derivado do nitrito demonstra propriedades antimicrobianas e modula o fluxo sanguíneo, a produção de muco, a motilidade gástrica e está envolvido na prevenção ulcerogénica. No meio acídico do estômago, o nitrito da dieta leva à formação de diversos óxidos de nitrogénio (RNOS) além do $^{\bullet}$NO, que podem induzir modificações pós-tradução como a nitrosação e a nitração em proteínas com impacto biológico. A ingestão concomitante de alimentos contendo compostos redox ativos, tais como os polifenóis do vinho tinto, potencia a formação de $^{\bullet}$NO por redução univalente do nitrito e consequente oxidação do polifenol ao seu radical semiquinónico.

Em termos gerais, o trabalho apresentado aborda a bioquímica redox de nitrito no estômago, nomeadamente a sua conversão a $^{\bullet}$NO por compostos fenólicos do vinho tinto e consequente impacto funcional em termos de expressão e modificação de proteínas, e respiração mitocondrial. Assim, nesta tese mostra-se que o nitrito derivado da dieta tem a capacidade de induzir nitrosação, especialmente S-nitrosação, em proteínas constituintes da camada de muco (mucinas) que cobre a mucosa gástrica. Além disso, foi observado que proteínas do epitélio gástrico são alvos para nitrosação por nitrito acidificado. Estes resultados apontam para o muco como filtro ativo ao estresse nitrosativo e para potenciais efeitos celulares mediados pelo $^{\bullet}$NO. O perfil de nitrosação é modulado pela presença de vinho tinto o que sugere uma nova atividade para os polifenóis do vinho tinto relativamente à formação de compostos S- e N-nitrosados no compartimento gástrico. A quantificação de nitrosação na mucosa gástrica foi conseguida recorrendo a uma

metodologia de quimiluminescência de elevada sensibilidade e seletividade. Os S-nitrosotióis são compostos relativamente estáveis e que podem funcionar como transportadores e dadores de •NO, com efeitos locais e sistémicos. De facto, foi observado que o muco de estômago de rato nitrosado com nitrito acidificado liberta •NO a pH fisiológico. Em condições inflamatórias *in vivo* a extensão de nitrosação por nitrito é aumentada, particularmente na fração correspondente aos S-nitrosotióis.

Além dos conhecidos efeitos do nitrito derivado da dieta relativamente à produção de muco gástrico, neste trabalho foi observado que o nitrito estimula a produção de TFF1 (trefoil factor 1), um importante peptídeo para proteção e regeneração da mucosa e com propriedades anti-tumorogénicas, contribuindo para a manutenção da integridade mucosal. Também aqui a modulação redox pelo vinho tinto tem impacto, aumentando a expressão de TFF1.

As elevadas concentrações de nitrito e •NO atingidas no estômago derivadas em função da dieta, levantam questões sobre o seu impacto na função mitocondrial da mucosa. A mitocôndria é um conhecido alvo para o •NO e mais recentemente foi também reconhecida como alvo para o nitrito. Nos resultados apresentados, pode observar-se que além da capacidade para lidar com elevadas concentrações de •NO e nitrito, a função mitocondrial da mucosa gástrica surge melhorada por efeito do nitrito *in vivo*. A análise da função mitocondrial foi efetuada utilizando a respirometria de alta-resolução. Considerando que a respiração mitocondrial é essencial no funcionamento celular e em vias de sinalização, a modulação da função mitocondrial por constituintes da dieta como nitrito inorgânico pode ter implicação na fisiologia e patologia gástrica.

No seu conjunto, estes resultados destacam a atividade biológica de nitrato e de nitrito da dieta, da sua interação com outros componentes da dieta como o vinho tinto e o seu impacto coletivo na fisiologia e patologia gástricas.

Abstract

As the endeavour for a healthy life and disease fighting continues to be a global matter of concern, there has been increasing interest in gaining a more comprehensive understanding of how different aspects of life style, in particular diet, may impact on human health. In this regard, nitrate and nitrite, consumed in vegetables as part of a normal diet, are permanent constituents of blood in animal species and have been identified as bioactive compounds capable of influence biological processes, resulting in improvements for human health.

Nitric oxide ($^{\bullet}$NO) is a ubiquitous messenger implicated in several important signalling pathways. Critical physiological functions such as regulation of the vascular tone, immune response and neuromodulation depend on $^{\bullet}$NO dynamics. In between its synthesis (by $^{\bullet}$NO synthases or by chemical reduction of nitrite) and its inactivation (by heme globins or oxidation to nitrite and nitrate), $^{\bullet}$NO diffuses trough biological milieu reaching its molecular targets.

Considered for long as waste product, capable of induce gastric cancer via the formation of carcinogenic nitrosamines, nitrite is now proving that is more than a stable $^{\bullet}$NO metabolite. Nitrite represents a vast $^{\bullet}$NO reservoir in the body and has been implicated in many modulatory pathways itself. The nitrite biology gained attention upon the report of $^{\bullet}$NO production in the stomach from inorganic nitrite. Nitrate from diet is reduced to nitrite in the saliva that reaches the gastric lumen where the acidic pH promotes the univalent reduction to $^{\bullet}$NO, in the so-called *Nitrate-Nitrite-$^{\bullet}$NO pathway*. This pathway originates the highest yield of $^{\bullet}$NO *in vivo*. Moreover, several proteins have been shown to acquire nitrite reductase (e.g., haemoglobin) properties at low oxygen tensions, suggesting a role for nitrite in the hypoxic signalling in the body.

In the gastrointestinal tract, nitrite-derived $^{\bullet}$NO has been shown to modulate host defence, blood flow, mucus production and gastric motility and protection. At the acidic

pH, nitrite generates several nitrogen oxides (RNOS) beside $^{\bullet}NO$ such as nitrogen dioxide ($^{\bullet}NO_2$) and dinitrogen trioxide (N_2O_3) that can induce post-translational modifications of endogenous proteins with consequent physiological impact. Other redox active dietary components, such as red wine polyphenols, are known to be implicated in the nitrite chemistry in the gastric lumen, enhancing $^{\bullet}NO$ production by univalent reduction of nitrite and consequent oxidation of the polyphenols to its o-semiquinone radical.

Overall, the work presented here addresses the redox biochemistry of nitrite that via $^{\bullet}NO$ production and in a process modulated by wine polyphenols impact on gastric physiology in terms of protein expression and modifications and mitochondrial respiration. More specifically, this thesis shows that dietary nitrite is able to induce nitrosation (mostly S-nitrosation) of mucus glycoproteins (mucins) and of gastric mucosa cells, pointing towards $^{\bullet}NO$-mediated actions in the mucosa and to the filter effect of the mucus. This pattern is redox-modulated by red wine, suggesting novel actions for wine polyphenols *in vivo* via the balance of S- an N-nitroso compounds in the gastric wall. A highly sensitive chemiluminescence methodology was used to quantify the formation of nitroso compounds. S-nitrosothiols are fairly stable compounds that may act as $^{\bullet}NO$ carriers, exerting both local and systemic impact. In fact, upon exposure to acidified nitrite, nitrosated mucus of rat stomach is shown to release $^{\bullet}NO$ at physiological pH. Additionally, the alteration of the gastric environment by inflammation *in vivo* increases nitrite-induced nitrosation, particularly the S-nitrosothiols fraction.

Alongside with mucus production and blood flow regulation, dietary nitrite and its derivatives demonstrated to contribute to the maintenance of gastric mucosal integrity via the stimulation of the expression of an important signalling peptide, the trefoil factor 1 (TFF1) involved mucosal protection and anti-tumorigenesis. Again, the redox modulation of the nitrite chemistry by red wine plays an important role, particularly under inflammatory conditions, by increasing TFF1 expression.

The high concentration of nitrite and •NO achieved in the stomach raised the question of how can gastric mitochondria cope with such challenge. Mitochondria are known targets for •NO and more recently were identified as targets for nitrite also. In fact, we observed that not only gastric mitochondria can deal with both •NO and nitrite amounts easily achieved by a vegetables rich diet, but also mitochondrial function is improved with the nitrate-nitrite-•NO pathway. The analysis of mitochondrial function was achieved by means of high-resolution oxygraphy. Mitochondria are key in cell function and signalling, and the modulation of their functionality by dietary derived inorganic molecules such as nitrite and •NO can have major impact in gastric physiology and disease.

Taken together, these results highlight the relevance of bioactive compounds in everyday diet such as nitrate and nitrite, their interaction with other diet components as red wine and their impact in the gastric physiopathology.

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1

General Introduction

1.1 Nitric oxide

1.1.1 Historical context

The scientific community was not aware of the physiological role played by nitric oxide (•NO) until the 1980 decade. Until then, •NO was known as toxic and an atmospheric pollutant. The perspective on the physiological relevance of •NO started to change when Furchgott and Zawadzki published their observations on the vasodilatory effect of acetylcholine being dependent of either an intact epithelium or a factor that was essential for muscular relation named *Endothelium Derived Relaxing Factor* (EDRF) [1]. Other groups have identified soluble guanylate cyclase (sGC) and cyclic guanylate monophosphate (cGMP) as a target and intermediary, respectively, of the EDRF-dependent actions [2, 3]. In the later 80's, •NO was identified as being the EDRF by Moncada and Ignarro's groups [4, 5] and L-arginine was identified as the •NO precursor in endothelial cells by Palmer's group [6]. This observations brought new relevance to the findings published in the 1970's where it was described that glutamate induced increase in cGMP levels [7] and that •NO activated cerebral sGC [8]. In 1989, Garthwaite and co-workers clarified the mechanism whereby glutamate activates N-methyl-D-aspartate receptors (NMDAR) with subsequent production of •NO which, in turn, is able to convert guanylate triphosphate (GTP) to cGMP [9]. In the years that followed, more evidence supported a physiological relevance for •NO in humans, ranging from vasodilation [2, 3] and neuromodulation [9] to the immune response [10, 11].

1.1.2 Chemical and physical properties

Under the apparent simplicity of the •NO molecule hides a complex biochemistry that has been the target of numerous studies during decades. •NO is a small sized diatomic molecule constituted by an atom of oxygen and an atom of nitrogen in the oxidation state +2 bound by a 2.5 order covalent bond. •NO has 11 valence electrons with

an unpaired electron in the antibonding π orbital, meaning it is a free radical. The removal of the unpaired electron leads to the formation of the oxidized specie NO^+ (nitrosonium ion). It can also be reduced, leading to the formation of NO^- (nitrosyl ion) [12].

At atmospheric pressure (101.3 kPa) $^{\bullet}NO$ is a colourless gas with a solubility in water of 1.93 mM at 25 ºC and 1.63 mM at physiological temperature (37 ºC), evidencing temperature dependence for solubility. Increasing ionic strength of the solution also diminishes solubility of $^{\bullet}NO$ and thus the $^{\bullet}NO$ solubility at physiological ionic strength and temperature is 1.55 mM [13]. This characteristics, particularly its small size and hydrophobicity, allow the diffusion of $^{\bullet}NO$ for distances great as many cell sizes, *in vivo*. Moreover, $^{\bullet}NO$ is a signalling messenger but it does not interact with cell membrane receptors in a structural complementary basis, as review in [14]. It diffuses through the lipid membrane and triggers intra and extracellular events seeing no barrier in biological membranes. Although $^{\bullet}NO$ is a radical it is relatively stable and not very reactive [13]. The reactivity of $^{\bullet}NO$ depends on the environment where is produced due to conversion to more reactive species and its half-life can vary from about 2 milliseconds inside a blood vessel and 2 seconds inside a cell [15]. The parameter that most adequately translates $^{\bullet}NO$ reactivity is its concentration dynamics, the profile of change in time and space, as affected by its rate and site of production, its consumption (availability of molecular targets, oxygen tension (pO_2)) and diffusion.

1.1.3 Nitric oxide synthesis

Moncada and colleagues demonstrated in the early 1990's that $^{\bullet}NO$ is produced endogenously by an enzyme named nitric oxide synthase (NOS) in a tightly regulated manner [16]. NOS catalyses the oxidation of L-arginine to L-citrulline and $^{\bullet}NO$. The reaction also requires oxygen (O_2) and nicotinamide adenine nucleotide phosphate (NADPH) as co-substrates [17]. There are three NOS isoforms described, that differ from

each other not only because they are the product of three distinct genes but also because they differ in terms of their localization, regulation, catalytic properties and sensitivity to inhibitors, as reviewed in [18]. Initially the NOS isoforms were classified according to the tissue where they were first localized: neuronal-NOS I (NOS I or nNOS) [19, 20], inducible-NOS (NOS II or iNOS) [21] and endothelial-NOS (NOS III or eNOS) [22]. Despite the differences, NOS isoforms share some structural characteristics. They are only active as homodimers and each monomer consists of two domains: an oxigenase (N-terminal) and a reductase (C-terminal) domain, linked by a polypeptide which is the calmodulin (CaM) binding site [23, 24]. The oxigenase domain contains the binding site for iron protoporphyrin IX [25-27], tetrahydrobiopterin (H_4B) [28, 29] and L-arginine and the reductase domain contains the binding sites for flavin adenine dinucleotide (FAD), flavin mononucleotide (FMN) [30, 31] and NADPH. NOS isoforms I and III are constitutively expressed and activated by the binding of the complex Ca^{2+}-CaM when the intracellular Ca^{2+} concentration is high. The binding of CaM works as a molecular switch that allows electron flow from the reductase towards the heme, which facilitates the oxidation of L-arginine to citrulline and $^{\bullet}NO$, as reviewed in [18]. Constitutive NOS isoforms generate low fluxes of $^{\bullet}NO$ for short periods of time [32]. Isoform NOS II is active for basal concentrations of Ca^{2+}, being its regulation dependent on expression via cytokine or endotoxin activation of immune cells (macrophages, monocytes and neutrophils) [16, 19, 33]. Unlike the constitutive isoforms NOS I and III, NOS II is able to produce higher and longstanding $^{\bullet}NO$ concentrations as long as L-arginine and the co-factors are available [34]. This is a key aspect for the antimicrobial and antitumorigenic properties of iNOS-derived $^{\bullet}NO$ [35, 36].

1.1.4 Biological effects

Nitric oxide shows a unique chemistry in biological systems. Where, when, and how much $^{\bullet}NO$ is present or is being produced under a given circumstance determines the

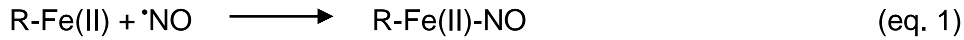
biological response. The biological effects of •NO are normally divided in two categories: direct and indirect effects [32, 37]. The first are those where •NO interacts directly with biological molecules whereas indirect effects are derived from the reaction of •NO with either superoxide ($O_2^{\bullet-}$) or oxygen, which yields reactive nitrogen oxide species (RNOS) [32]. When low concentrations of •NO (< 1 μM is commonly accepted) are generated in cells for a short period of time, direct effects of •NO are the predominant chemistry and indirect effects are limited. On the other hand, higher production of •NO (> 1 μM) allows indirect effects such as nitrosation, nitration and oxidation reactions to occur via production of more reactive nitrogen species. Also the distance from the •NO-generating source to the targets is crucial. Spatial and temporal factors are therefore important when considering the chemistry responsible for the specific biological effects, as reviewed in [32].

1.1.4.1 Direct effects

The relevant direct reactions of •NO in biology are those whose rates are fast enough to be considered physiological relevant. The reaction rate constant and the stability of the products dictate their biological relevance [32]. The most relevant direct reactions of •NO can be divided as following.

- Reaction with metalloproteins

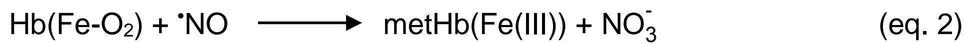
•NO binds to the ferrous (Fe^{2+}) heme of the protein forming a 5-coordinated stable nitrosyl complex (Fe–NO), as represented in equation 1. Good examples of this reaction are: 1) the •NO interaction with sGC [38, 39], the most recognized biological target for •NO and responsible for the regulation of the vascular tone, platelet function and neuromodulation [40]; 2) the interaction with several enzymes of the cytochrome P450 family involved in the metabolism of drugs and the cholesterol biosynthesis; 3) Cytochrome c oxidase (CcOx), a key enzyme in the mitochondrial respiratory chain [41]; 4) and the NOS [42].



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

- Reaction with oxygen metal complexes

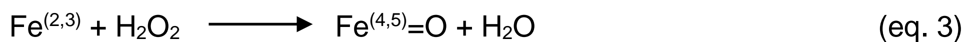
The reaction between ${}^{\bullet}NO$ and an oxygen metal complex such as in oxyhaemoglobin (Hb) is one of the most relevant ${}^{\bullet}NO$ removal pathways in biological systems [43, 44]. From this reaction nitrate (NO_3^-) and methaemoglobin (metHb) are also produced (eq. 2).



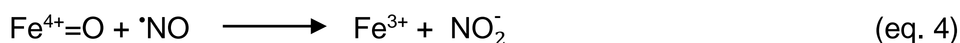
$$Hb(Fe\text{-}O_2) + {}^{\bullet}NO \longrightarrow metHb(Fe(III)) + NO_3^- \qquad \text{(eq. 2)}$$

- Reaction with metallo-oxo complexes

Metallo-oxo complexes are formed during the oxidation of metals or metal-O complexes by hydrogen peroxide (H_2O_2) (eq 3). This complexes are potent oxidants due to their high valence states, that can inflict cellular damage [45]. The reduction of these complexes by ${}^{\bullet}NO$ acting as an antioxidant prevents the occurrence of other reactions potentially harmful [46, 47] (eq. 4). An example of this reaction is the interaction of ${}^{\bullet}NO$ with catalase.



$$Fe^{(2,3)} + H_2O_2 \longrightarrow Fe^{(4,5)}{=}O + H_2O \qquad \text{(eq. 3)}$$



$$Fe^{4+}{=}O + {}^{\bullet}NO \longrightarrow Fe^{3+} + NO_2^- \qquad \text{(eq. 4)}$$

- Reaction with other radicals (radical-radical combination)

${}^{\bullet}NO$ reacts with other free radicals at high rate. An example is the reaction of ${}^{\bullet}NO$ with alcoxyl ($LO^{\bullet}$) and peroxyl ($LOO^{\bullet}$) radicals formed during lipid peroxidation (eq. 5). A further very important reaction is the formation of peroxynitrite upon interaction with superoxide radical (see below). This reaction is controlled by diffusion occurring as soon as ${}^{\bullet}NO$ and the other radicals meet ($k \sim 10^9$ $M^{-1}.s^{-1}$) and, by this way, ${}^{\bullet}NO$ acts as a terminator of the propagation of lipid peroxidation [48-50].

$$LOO^{\bullet} + {}^{\bullet}NO \longrightarrow LOONO \qquad \text{(eq. 5)}$$

1.1.4.2 Indirect effects

As described above, these effects are usually associated with higher concentrations of •NO but not mediated by •NO itself. The reactions of •NO with other biomolecules in order to induce post-translational modifications such as oxidation, nitros(yl)ation and nitration occur at a low rate in biological systems, since an activation step of •NO via interaction with oxygen or superoxide radical to produce RNOS is required. The modifications induced by RNOS cascades can perturb the function of proteins and lipids [32, 51].

- Reaction with molecular oxygen (auto-oxidation)

The reaction between •NO and O_2 can yield RNOS with higher nitrosative and/or oxidative potential than •NO and oxygen individually. The trimolecular reaction occurs both under gas or liquid phases, depends on the square concentration of •NO and the concentration of O_2 and yields nitrogen dioxide radical (•NO_2) (eq. 6), in an overall third order rate constant [52, 53]. •NO_2 could either dimerize to form dinitrogen tetroxide (N_2O_4, that decomposes in nitrite (NO_2^-) and nitrate (NO_3^-)) or react to a third •NO molecule and yield dinitrogen trioxide (N_2O_3) (eq. 7) that is hydrolysed to NO_2^- [32]. In aqueous phase, the formation of free •NO_2 is unlikely due to its instability in water.

$$2\ {}^{\bullet}NO + O_2 \longrightarrow 2\ {}^{\bullet}NO_2 \quad \text{(eq. 6)}$$

$${}^{\bullet}NO_2 + {}^{\bullet}NO \longrightarrow N_2O_3 \quad \text{(eq. 7)}$$

Considering that O_2 is a di-radical (has two unpaired electrons with the same spin in the valence orbitals), the oxidation of other molecules would imply the acceptance of two electrons with parallel spin otherwise one of the electrons would have to change spin. Such transition is not kinetically favourable which explains the low O_2 reactivity [54]. *In vivo*, the reaction of •NO with molecular oxygen is very slow and is probably not relevant unless booth •NO and O_2 fluxes increased beyond typical values. The •NO flux and its

half-life are closely linked: for low levels auto-oxidation is considered less relevant and $^{\bullet}NO$ can diffuse away from the site of production but for higher $^{\bullet}NO$ levels, the formation of RNOS will increase along with the relevance of $^{\bullet}NO$-indirect effects, decreasing its diffusion and half-life [32, 55].

As hydrophobic molecules, $^{\bullet}NO$ and O_2 are more soluble in hydrophobic compartments where they may concentrate and diffuse at similar extent favouring their mutual interaction. Thus, likely, lipid layers are the primary sites for $^{\bullet}NO$ oxidation *in vivo* [56]. Another fact regarding the hydrophobic environment is the inhibition of the N_2O_3 hydrolysis by the absence of water, and consequently stabilization of this molecule which is a mild oxidant but a powerful nitrosating agent [57].

- Reaction with superoxide anion radical

A radical-radical interaction between $^{\bullet}NO$ and $O_2^{\bullet -}$ (product of the univalent reduction of O_2), leads to the formation of peroxynitrite anion ($ONOO^-$) (eq. 8), a molecule of notorious biological impact [58]. This reaction is the fastest non-catalysed reaction described in Biology with an accepted rate constant of $k \approx 10^{10}\ M^{-1}s^{-1}$ ($k = 16\text{-}20 \times 10^9\ M^{-1}s^{-1}$ [59]), meaning that the formation of $ONOO^-$ is controlled by the diffusion rates of $^{\bullet}NO$ and $O_2^{\bullet -}$.

$$^{\bullet}NO + O_2^{\bullet -} \longrightarrow ONOO^- \quad \text{(eq. 8)}$$

Both $^{\bullet}NO$ and $O_2^{\bullet -}$ are fleeting in biological systems which implicates that for $ONOO^-$ formation the two species have to be generated in the same cellular compartment. $^{\bullet}NO$ has an half-life of seconds and easily goes through biological membranes [44, 60] but $O_2^{\bullet -}$ persists merely for milliseconds and needs anionic channels to cross membranes [61], therefore, $ONOO^-$ occur preferentially near a source of $O_2^{\bullet -}$.

Superoxide dismutase (SOD, the enzyme that catalyses the dismutation of $O_2^{\bullet-}$ [62]) can efficiently compete with $^{\bullet}NO$ for $O_2^{\bullet-}$, however with a lower rate constant ($k \approx 2 \times 10^9\ M^{-1}s^{-1}$) [63] which allows $ONOO^-$ to be formed in the presence of SOD, as reviewed in [64]. Furthermore, in physiological conditions, chloride anions can interfere with the electrostatic field that attracts $O_2^{\bullet-}$ to the SOD active site, compromising the superoxide dismutation and making $ONOO^-$ formation even more likely *in vivo* than *in vitro* [65, 66].

$ONOO^-$ is a powerful nitrating and oxidizing agent [58] that is unstable at physiological pH due to the equilibrium with peroxynitrous acid (ONOOH, pKa=6.8) which, in turn, might decompose into an intermediary species with similar reactivity to hydroxyl radical ($^{\bullet}OH$) and $^{\bullet}NO_2$ [67, 68]. At a molecular level, $ONOO^-$ oxidizes a large range of molecules from low molecular weight compounds such as glutathione and α-tocopherol, aminoacid residues as cysteine and tyrosine, proteins like albumin, myeloperoxidase and SOD, polyunsaturated fatty acids and DNA [69]. Furthermore, it is also able to inhibit mitochondrial respiration by the irreversible inactivation of electron transport chain complexes, decreasing ATP synthesis, act as a cytotoxic agent and induce organ damage such as pulmonary emphysema, acute lung injury atherogenesis and neurotoxicity. $ONOO^-$ has been implicated in diabetes, cancer, inflammation, sepsis along with cardiac, vascular and neurodegenerative disorders [32, 64, 68].

- Oxidation, nitration and nitrosation reactions

The indirect effects of $^{\bullet}NO$ in biological systems are associated to a nitroxidative, nitrative, and nitrosative stress. Depending on the predominant RNOS formed and the biological conditions one or more of these reactions can occur [55, 70].

Oxidation reactions implicate electron transfer between substrates. The RNOS resulting from the reaction of $^{\bullet}NO$ and O_2 and/or $O_2^{\bullet-}$ exhibit a wide range of redox potentials, from N_2O_3 which is considered a relatively weak oxidant, to $ONOO^-$ a potent oxidant [32]. In the central nervous system, $ONOO^-$ is considered a primary responsible

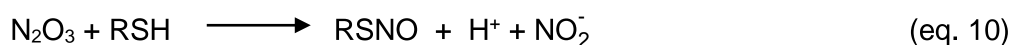
for oxidative stress [71], and the impossibility to be measured in biological systems due to its short half-life raises doubt on its participation in oxidative damage imputed to $^{\bullet}NO$ [72]. In addition to the above described oxidation induced by $ONOO^-$, its decomposition yields other oxidizing species such as *trans*-ONOOH, $^{\bullet}NO_2$ and $^{\bullet}OH$ [69, 73].

Nitration is a protein post-translational modification characterized by the electrophilic addition of a nitronium ion (NO_2^+) in the *ortho* position of a phenolic ring of aminoacids, typically tyrosine (tyr), being 3-nitrotyrosine (3-NT) the primary biological marker of nitration, but also tryptophan. To form this covalent bond a two-step mechanism is required being the first step the oxidation of a tyrosine residue by oxidants such as $^{\bullet}OH$, $^{\bullet}NO_2$ and carbonate radical ($CO_3^{\bullet-}$), yielding tyrosil radical (tyr-O$^{\bullet}$) followed by the insertion of NO_2^+ in the ring or addition of $^{\bullet}NO_2$. $ONOO^-$ has been implicated in protein nitration since it can decompose in several oxidizing and nitrating species, as reviewed in [74], though nitration can be achieved through other pathways [75]. This modification has been identified in several pathologies and diseases such as atherosclerosis, Alzheimer's disease, diabetes and inflammatory conditions, strongly suggesting that it is tightly involved in $^{\bullet}NO$ toxicity [76-80]. The formation of nitrating species will depend on other factors such as the nitrogen oxides produced, kinetics and compartmentalization and the presence of pro-nitration agents (inflammatory cells) or scavengers for nitrating species (e.g. antioxidants) [81].

Nitrosation is the result of the reaction of a NO^+ with a nucleophile like a thiol (cysteine residue), amine, aromatic compound or a hydroxyl group in an aliphatic alcohol, yielding, respectively, S-, N-, C- and O- nitroso compounds [82-85]. On the other hand, heme moieties can bind $^{\bullet}NO$ (nitrosylation), yielding heme-nitrosyls (heme-nitrosylation), as indicated above (eq. 1) [86, 87]. N_2O_3, $^{\bullet}NO_2$ and nitrous acid (HNO_2, that under acidic conditions originates NO^+) are the primary nitrosative agents [84, 88, 89]. The biological significance for nitroso species remains unclear, however S-nitrosation has received

particular attention since the posttranslational modification of a critical cysteine residue in a protein can be relevant on the regulation of protein function [90]. In fact, S-nitrosation has been implicated in the regulation of biological functions such as oxygen delivery to tissues as well as in the function and activity of transcription factors, enzymes, membrane receptors and ion channels [87, 91, 92]. S-nitrosothiols (SNO), are found *in vivo* and have a half-life of about 40 minutes [93]. Physiologically, this is of particular interest since unlikely $^{\bullet}NO$, SNO are not inactivated/removed by haemoglobin and therefore can act as stable carriers for $^{\bullet}NO$ in order to spread its biological effects [93]. Vasodilation, antimicrobial properties and regulation of redox signalling have been associated with SNO involving nitrosohaemoglobin, nitrosoalbumin and nitroglutathione [32, 94-96]. Mechanistically, S-nitrosation may occur either due to the reaction between $^{\bullet}NO$ and a thyil radical ($-RS^{\bullet}$) previously formed in the cysteine residue (eq. 4) [97] or, by the action of RNOS (formally the addition of a NO^{+} equivalent), such as N_2O_3 (formed by $^{\bullet}NO$ autoxidation or from acidified nitrite) (eq. 10) with a thiol group [55, 97]. In addition, two distinct thiols can undergo fast transnitrosation reactions (eq. 11), which may explain in part the liability of SNO [98, 99]. S-nitrosation presents unique features, including the fact that its formation and degradation depend solely on chemical reactions without enzymatic catalysis [100]. Being fairly stable in solution, SNO may decompose by photochemical and thermal reactions or via a metal ion-catalysed route, particularly with copper, yielding the corresponding dissulphide and $^{\bullet}NO$ (as reviewed in [83]).

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



$$N_2O_3 + RSH \longrightarrow RSNO + H^{+} + NO_2^{-} \qquad \text{(eq. 10)}$$

$$RSH + R'SNO \longrightarrow RSNO + R'SH \qquad \text{(eq. 11)}$$

1.2 Nitrite

1.2.1 Historical context

Inorganic nitrite has been used as a food preservative for as long as 5000 years, particularly in cured meat. However, in the 1960s and 1970s a major public health concern was raised when nitrite consumption was associated with endogenous formation of carcinogenic N-nitrosamines [101]. Despite the numerous studies dedicated to associate nitrite consumption and endogenous formation of N-nitrosamines to gastric cancer development in humans, a casual relation between nitrite exposure and cancer is still missing [102, 103]. The negative connotations of nitrite and nitrate consumption towards human health led to a restrict regulation of their levels in food and drinking water. It is of note that the acknowledgement of a biological impact of inorganic nitrite occur nearly a century before the recognition of $^{\bullet}$NO *in vivo* effects. In fact, since 1880 that vasodilatory properties have been imputed to nitrite [104] and only later acidified nitrite was used to relax aortic strips [105] and the involvement of sGC in such an effect suggested [106].

In the late 1970s, early findings by Tannenbaum et al [107] on nitrogen balance in humans indicated that nitrite and nitrate are formed *de novo* in the human intestine. Till then the steady-state of nitrite and nitrate was attributed to diet and nitrogen fixing enteric bacteria. These findings altered the perception of nitrite and nitrate effects *in vivo* [108]. In the mid-90s, both Lundberg and Benjamin's groups brought physiological relevance to nitrite showing that nitrite-rich saliva generated $^{\bullet}$NO in the human stomach at a pH and nitrite concentration-dependent rate [109, 110] and that the nitrite-derived $^{\bullet}$NO exhibited antimicrobial properties. The traditional view that nitrite was only a metastable intermediary of $^{\bullet}$NO oxidation to the more stable metabolite nitrate and that, under biological conditions, this cycle was irreversible was dispelled by several studies that uncover an active nitrite recycling to $^{\bullet}$NO along the oxygen gradient [111]. In addition to the nitrite reduction to $^{\bullet}$NO by acidification, several enzymes have been shown to acquire

a nitrite reductase activity and reduce nitrite to •NO when the oxygen tension is low [112-118]. From then after, nitrite is considered a critical player in the hypoxic signalling as a storage for •NO [111]. The biomedical community has a new look at nitrite as a health promoting molecule, considering it as a •NO oxidation product, [111, 119], and nitrite was been pharmacologically used as vasodilator, bronchodilator, intestinal relaxant and even as an antidote for cyanide poisoning, as reviewed in [108].

1.2.2 Sources of nitrite

In mammalian systems, nitrite originates from: 1) endogenous •NO oxidation; 2) reduction of salivary nitrate by commensal bacteria in the mouth; 3) diet [119, 120].

The major pathway contributing to the nitrite pool *in vivo* is the nutritional source. Green leafy vegetables (such as lettuce, broccoli and spinach) supply up to 86% of the daily ingestion of nitrate and 16% of nitrite and cured meats, baked goods and cereals contribute with 34% of nitrite [121, 122]. The reduction of dietary nitrate to nitrite in the oral cavity by commensal bacteria [123, 124] raises the nitrite levels and contributes up to 90% of the nitrite intake [125]. This pathway named entero-salivary circulation of nitrate will be addressed later, in detail.

Nitrite is a permanent constituent of blood in all animal species at concentrations that vary with the diet. Nitrite concentrations through the whole body are maintained in a strictly regulated steady-state that varies depending on tissue, compartment and NOS activity, being usually more concentrated in tissues than in circulation [126, 127]. The observation that the concentration in tissues (varying between 0.5 and 20 micromolar among different mammalian tissues) is higher than in plasma, indicates the presence of transport mechanisms (e.g., anion transporters) that are still largely unknown, although passive transmembrane transport in the protonated form, HNO_2, has been described [128].

In plasma, the nitrite concentration is conserved across mammals in the range of 50-600 nM [129-133] and nitrite remains stable for several hours, as summarized by Bryan [108]. In whole blood, though, $^{\bullet}$NO and nitrite are rapidly oxidized to nitrate, limiting the half-life of nitrite to 110 seconds whereas nitrate has a circulating half-life of 5-6 hours [134, 135]. In tissues, both nitrite and nitrate show half-lives of tens of minutes [136]. Both nitrite and nitrate are mostly excreted by the kidneys, but also small amounts could be detected in feces, sweat and exhaled breath [137-139].

1.2.3 The entero-salivary circulation of nitrate

The entero-salivary circulation of nitrate (illustrated in figure 1.1) fuels the intragastric formation of $^{\bullet}$NO from salivary nitrite described in 1994 [109, 110] but was several years before that nitrate and nitrite were identified in human saliva [140]. In turn, the sole occurrence of this recirculation, whose ultimate consequence is the maintenance of nitrite at high steady-state concentration in the blood, supports a biological role for nitrite. Studies with $^{15}NO_3^-$ in humans and rats, showed that nitrate is absorbed in upper small intestine to the systemic circulation, adding to nitrate originated from endogenous $^{\bullet}$NO oxidation [141, 142]. After a nitrate rich meal not only an increase in plasma nitrate is observed with a maximum 30 minutes after the intake and maintained by hours [143], but also nitrite levels in plasma increase [130]. Although about 75% of nitrate is secreted in urine, the remaining 25% is actively taken up by salivary glands, concentrated up to 20-fold (reaching 2-10 mM) and secreted into the oral cavity by a mechanism not fully understood [119, 123, 130, 141, 144]. Mammalian cells lack the enzymatic machinery to reduce nitrate back to nitrite. However, in the oral cavity, commensal facultative anaerobic bacteria use nitrate as an alternative electron acceptor instead of oxygen during respiration, effectively reducing nitrate to nitrite by nitrate reductases [119, 123, 124]. This way, salivary nitrite concentration increases from 50-300 μM under fasting to 1-2 mM after a nitrate load, as reviewed in [120]. Once swallowed, nitrite-rich saliva encounters the

acidic stomach and much of the nitrite is promptly protonated to HNO_2 (pKa ~3.3), which decomposes to form $^{\bullet}NO$ and other RNOS [109, 110, 145]. This complex chemistry originates new molecules that through several secondary reactions can results in additional nitrate and nitrite.

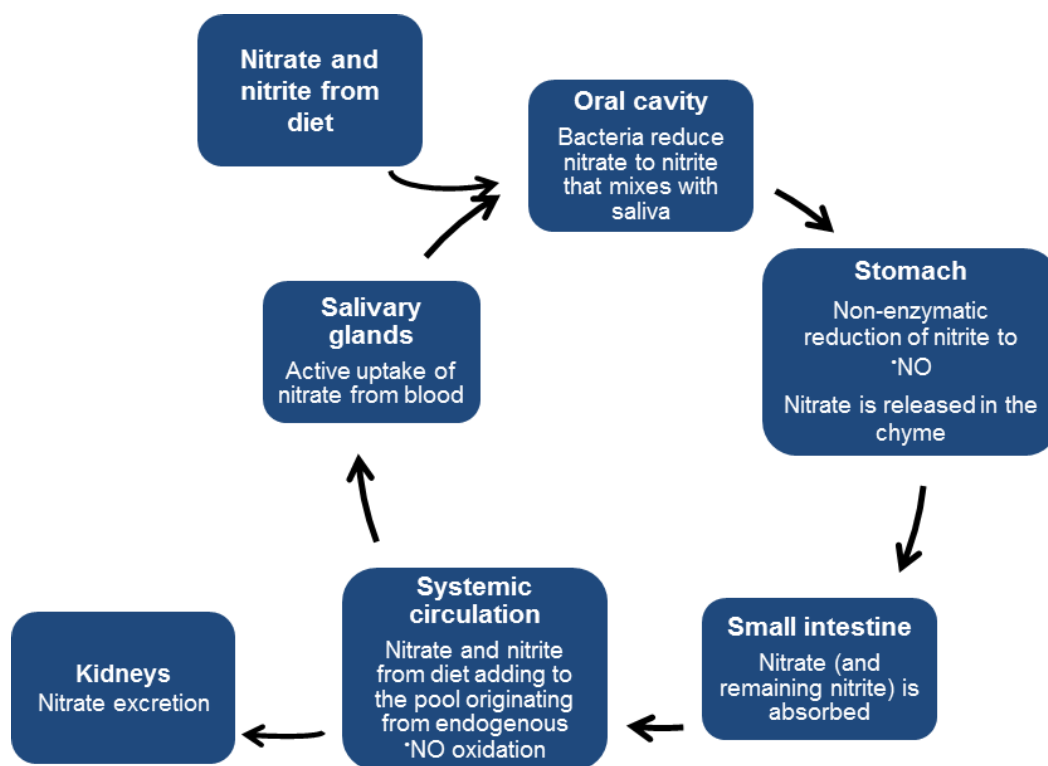


Figure 1.1 The entero-salivary circulation of nitrate in humans. Adapted from [121].

1.2.4 Biological effects

It is apparent that the recirculation shown above ultimately contributes to maintain a nitrate and nitrite pool *in vivo*. The tight regulation of nitrate and nitrite in the human body suggests that these compounds might exert relevant biological functions and are more than an inert decomposition products of $^{\bullet}NO$ metabolism. If one add the recent findings that nitrite can be reduced to bioactive $^{\bullet}NO$ *in vivo* by several mechanisms, it becomes evident the attention nitrite has been given beyond the more orthodox view as a toxic contaminant [146]. Nitrite reduction to $^{\bullet}NO$ can occur by spontaneously acidification

(protonation) [109, 110], upon one-electron reduction by ascorbate and polyphenols [147-150] or via reaction with a number of proteins possessing nitrite reductase activity such as heme proteins (deoxyhaemoglobin and deoxymyoglobin) [112, 115, 151], molybdenum-containing enzymes (xanthine oxidase) [114, 152], eNOS [116] and components of the mitochondrial electron transport chain (ubiquinol and CcOX) [117, 153-155]. The nitrite reduction by mammalian reductases endowed with different oxygen affinities, tissue distribution and rates of reduction is optimized under conditions of hypoxia and acidosis, constituting a physiological mechanism by which $^{\bullet}$NO production is sustained, particularly when catalytic $^{\bullet}$NO generation by NOS (the L-arginine pathway, which relies on oxygen) is compromised [108, 120, 156]. The reduction of nitrite to $^{\bullet}$NO and the consequent $^{\bullet}$NO-dependent modification of target proteins during physiological and pathological hypoxia in the cell [120] appears to contribute to a wide spectrum of biological responses during physiological hypoxic signalling, such as hypoxic vasodilation [151, 157], stimulation of angiogenesis [158], modulation of glucose metabolism [159], increase of exercise efficiency [160], regulation of mitochondrial function [115, 161, 162] and tolerance to I/R [114, 158, 163, 164].

Nitrite was also reported to be a signalling molecule and a regulator of protein expression in a $^{\bullet}$NO-independent fashion, through S-nitrosation, under physiological conditions [136]. Furthermore, the mitochondria has been reported as a target for nitrite-dependent S-nitrosation particularly at complex I, resulting in attenuation of ROS generation after I/R [161]. In addition, nitrite was shown to be involved in hypoxic mitochondrial biogenesis, in a rat model, associated with protective vascular remodelling [165].

Despite the implication of nitrite in diseases such as infant methaemoglobinemia ("baby blues") and gastric cancer (through the formation of N-nitrosamines) no unequivocal association has been established between nitrite consumption and these pathological conditions [102, 103, 108, 166].

1.2.5 Biochemistry of intragastric nitrite

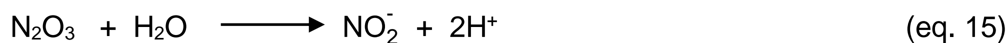
Considering the scope of this thesis, special attention will be given to the non-enzymatic reduction of dietary nitrite, as well as to the biological effects of its derivatives, $^{\bullet}NO$ and other RNOS, in the stomach.

As described in *The entero-salivary circulation of nitrate* section, dietary-derived nitrite is protonated in the gastric acidic medium to HNO_2 that, in turn, is readily decomposed to $^{\bullet}NO$. The decomposition equilibrium of HNO_2 to $^{\bullet}NO$, $^{\bullet}NO_2$ and N_2O_3 (eq. 12-14) [109, 110] can be modulated by alterations of the redox enviroment such as the presence of reductants (ascorbate (Asc), polyphenols (Ph-OH) and glutathione), transition metal centers and oxygen levels, abundance of biotargets, pH and relative hydrophobicity of the milieu [73, 167-169].

$$NO_2^- + H^+ \rightleftharpoons HNO_2 \quad \text{(eq. 12)}$$

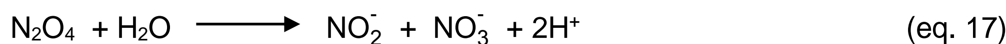
$$2HNO_2 \rightleftharpoons N_2O_3 + H_2O \quad \text{(eq. 13)}$$

$$N_2O_3 \rightleftharpoons {}^{\bullet}NO + {}^{\bullet}NO_2 \quad \text{(eq. 14)}$$



$$N_2O_3 + H_2O \longrightarrow NO_2^- + 2H^+ \quad \text{(eq. 15)}$$

$$2\,{}^{\bullet}NO_2 \longrightarrow N_2O_4 \quad \text{(eq. 16)}$$



$$N_2O_4 + H_2O \longrightarrow NO_2^- + NO_3^- + 2H^+ \quad \text{(eq. 17)}$$

In the absence of reductants, only 1% of nitrite is converted to $^{\bullet}NO$, being $^{\bullet}NO_2$ a relevant product. $^{\bullet}NO$ and $^{\bullet}NO_2$ may combine to produce N_2O_3 (eq. 7) that can be hydrolysed to nitrite (eq. 115) but $^{\bullet}NO_2$ can also dimerize to form N_2O_4 (eq. 16), that decomposes in nitrite and nitrate (eq. 17), propagating the cycle. In the presence of reductants most of nitrite is reduced to $^{\bullet}NO$ (eq. 18 and 19) [109, 145, 168, 170-172].

$$2HNO_2 + Asc \longrightarrow 2{}^{\bullet}NO + \text{dehydroAsc} + 2H_2O \quad \text{(eq. 18)}$$

$$HNO_2 + Ph\text{-}OH \longrightarrow Ph\text{-}{}^{\bullet}O + {}^{\bullet}NO + H_2O \quad \text{(eq. 19)}$$

Given the high pO_2 (~70 torr [173]) and the high fluxes of $^{\bullet}NO$ (c.a. thousands of ppb after a nitrate load [110]) found in the gastric compartment, $^{\bullet}NO$ auto-oxidation may play a relevant role in nitrite biochemistry. Likewise, the reaction of nitrite–dependent $^{\bullet}NO$ with $O_2^{\bullet-}$ (derived from the chemical one electron reduction of O_2 - e.g., by phenolic semiquinone radicals - or the activity of epithelial oxidases [174]), yielding $ONOO^-$, can modulate the chemical outcome of dietary nitrite.

The chemical reduction of nitrite to $^{\bullet}NO$ in the stomach has been shown to exert a wide range of protective effects. The first to be acknowledged was the antimicrobial effect of $^{\bullet}NO$ in *Escherichia coli* and *Candida albicans* [109, 175]. Later, Dykhuizen and co-workers demonstrated that 1mM of acidified nitrite was able to eradicate *Helicobacter pylori* (an ulcerogenic pathogen) cultured from gastric biopsies after 30 minutes of incubation [176], indicating that concentrations of nitrite easily achieved *in vivo* might had antimicrobial effects against different strains of pathogens responsible for gastrointestinal infections.

Nitrite-derived $^{\bullet}NO$ has been also implicated in important physiological processes in the stomach by increasing gastric mucosal blood flow (vasodilation) and mucus thickness [177-179]. Considering that, nonsteroidal anti-inflammatory drugs (NSAIDs) and *Helicobacter pylori* compromise the gastric mucosal integrity by decreasing mucus thickness through mucin production inhibition [180-184], nitrite-derived $^{\bullet}NO$ is regarded as a gastroprotective molecule, since a higher blood supply and mucus production can protect the gastric mucosa. In addition, Petersson and colleagues showed that the gastroprotective and blood pressure lowering effects of dietary nitrate were abolished by the use of antiseptic mouthwash, revealing the importance of nitrate reducing bacteria in the physiological effects of dietary-derived nitrate, nitrite and $^{\bullet}NO$ [185]. Reports of a pro-active role for nitrite-derived $^{\bullet}NO$ in the protection of gastric ulcers suggest that regular consumption of nitrite may prevent inflammatory processes in the stomach, correlating

increased dietary-derived $^{\bullet}NO$ in the gastric headspace to decreased acute gastric ulceration induced by diclofenac [186, 187]. Nitrite-derived $^{\bullet}NO$ diffuses to deeper layers of the gastric mucosa and induce smooth muscle relaxation [188].

The formation of RNOS from acidified nitrite and/or from $^{\bullet}NO$ secondary reactions, with the ability to induce post-translational modifications such as protein nitrosation and nitration, have been reported mostly from a pathological point a view. In this thesis, nitrite derived protein nitrosation in the gastric compartment will be addressed, bearing in mind the relevance of these modifications in regulation of protein function and signaling pathways.

1.3 Gastric physiology

1.3.1 Gastric anatomy and physiology

The essential concepts in gastric anatomy and physiology will be addressed in the current section [189-191].

The stomach is a wholly, ‘J’-shaped, intra-abdominal organ located between the esophagus and the duodenum. It is an active reservoir adapted for mechanical churning, and grinding, storage and slowly dispense partially digested food (chyme) into the intestine for further digestion and absorption. The stomach consists of three anatomic regions (fundus, body or corpus and antrum), limited in the proximal end by the cardia and in the distal end by the pyloric sphincter (Fig. 1.2). It can also be divided into two functional areas: the oxyntic glandular mucosa (fundus and corpus) and the pyloric glandular area (antrum).

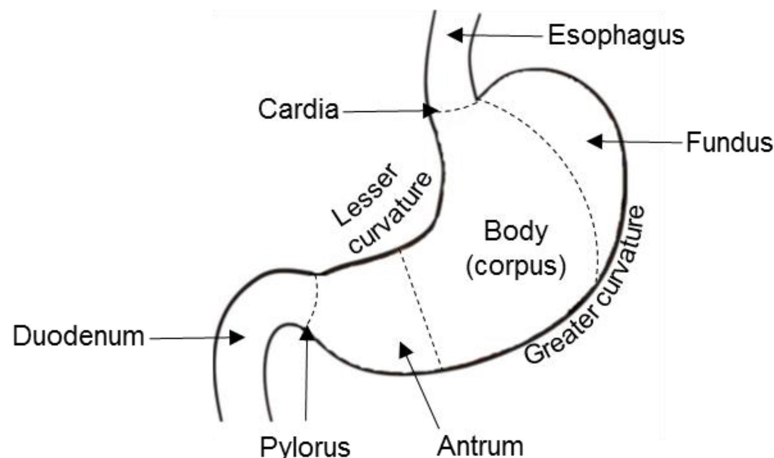


Figure 1.2 Schematic anatomy of the stomach. Adapted from [190].

The gastric wall is constituted by several distinct cell layers (from outside to inside): serosa, three muscle layers (longitudinal, circular and oblique), submucosa (connective tissue where the Meissner’s plexus can be found), *muscularis mucosae* and mucosa comprising the *lamina propria* (containing plasma cells, eosinophils, mast cells and lymphocytes, lymphatic and blood vessels) and columnar gastric epithelium, covered by a thick mucus layer. The luminal surface of the stomach presents rugae (longitudinal folds

which thickness depend on the degree of gastric distention) with invaginations called gastric pits, which allow gastric glandular secretions to reach the mucosal surface.

The gastric glands are tubular structures that consist on an isthmus (that connects the gland to the gastric pit), neck and base with specialized cells that secrete several messengers, hormones and neurotransmitters that regulate digestive functions and gastric motility, such as:

- Parietal or oxyntic cells, found throughout the stomach are the most common cell type in the gland neck and are responsible for hydrochloric acid (HCl) secretion;
- Chief cells , found mostly in the corpus are located mostly in the gland base, secrete pepsinogen (that is activated to pepsin by HCl);
- Mucous neck cells, the most common cell type in the isthmus and also found in the gland neck, are responsible for mucus secretion;
- Entero-endocrine cells, mostly G cells that secrete gastrin, D cells producing somatostatin and enterochromaffin-like (ECL) cells producing histamine;
- Stem cells and undifferentiated epithelial cells.

Gastric secretion is stimulated by the anticipation of food (cephalic phase) and by food arriving in the stomach (gastric phase). The exocrine messengers are those secreted into the gastric lumen like HCl and pepsin, and collectively known as gastric juice; the paracrine messengers are those produced and acting in the mucosa, like histamine; the endocrine messengers such as gastrin can act both locally and systemically.

The celiac artery supplies arterial blood to the stomach and venous blood drains into the hepatic portal vein. The stomach receives sympathetic innervation from the celiac plexus and parasympathetic innervation from the vagus nerve, integrating enteric and central nervous systems. Diet composition can not only modulate gastrointestinal homeostasis but also have implications in the gut-brain signalling axis, through diverse mechanisms [192, 193].

1.3.2 Gastric mucosal defence

The mechanisms responsible for maintaining gastric mucosal structural integrity despite the continuous exposure to noxious factors and substances, have been studied for more than 200 years, as reviewed in [194]. During the eighteenth and the nineteenth century, the hypothesis that a continuous circulation of alkaline blood through the mucosa neutralizing the acid was the most accepted theory [195, 196]. Over the years new and more convincing hypothesis for gastric mucosal defence have been formulated. In the 1970s and 1980s, the discovery of the role of prostaglandins (PGs) in the (NSAID)-induced gastric damage [197], and the concept of cytoprotection in the gastric defence system [198, 199] brought new interest to the topic.

Gastric mucosal injury may occur either if the intact mucosal defence is overpowered by noxious factors or the mucosal defence mechanisms are impaired. Endogenous substances such as 0.1 M HCl and pepsin that are able to digest tissue and exogenous factors such as drugs and bacteria can be responsible for severe damage in the gastric mucosa if the defence system fails [194].

The gastric mucosal integrity is maintained by a defence system that is supported by three major processes, namely:

- Mucus-bicarbonate-phospholipid barrier (premucosal defence)

A neutral microenvironment (pH ~7.0) is maintained at the epithelial cells surface by bicarbonate that is retained in the unstirred layer, in order to prevent proteolytic digestion via penetration of pepsin and the luminal acid [200-204]. The luminal surface of the mucus layer contains a film of strong hydrophobic phospholipids [200, 205]. The mucus gel is secreted by the gastric epithelial cells and is the product of mucin (MUC) genes. It contains about 95% water and 5% mucin glycoproteins that polymerize into large multimers forming the mucus gel [202, 203, 206]. The mucin polymer are long flexible strings constituted by alternated hydrophilic (glycosylated) and hydrophobic (cysteine-rich domains). The latter appear to fold into globular “beads” stabilized by dissulphide bonds

[207], adsorbing significant amounts of lipids and thus increasing the low affinity bonds among mucins and contributing to the viscoelasticity of the gel [208, 209]. Among the mucins, MUC5AC and MUC6 are the major components in the gastric mucus forming alternating layers, the outer layer, called the loosely adherent mucus, and the inner layer, the firmly adherent mucus. MUC5AC is secreted in the epithelial surface of the cardia, fundus and antrum and MUC6 is expressed in the neck cells of the fundus and in antrum glands [210-212]. Gastrointestinal hormones such as gastrin, secretin and prostaglandin E2 (PGE2) along with cholinergic agents stimulate the mucus secretion. Luminal acid and PGs among others stimulate bicarbonate secretion [203].

- Surface epithelial cells (mucosal defence)

The hydrophobic phospholipids covering the epithelial cells and the tight junctions between them act together as repellents for acid and water soluble toxins and prevent the back diffusion of acid and pepsin, as reviewed in [194]. These cells secrete mucus and bicarbonate (via the activity of carbonic anhydrase) and generate PGs, trefoil peptides, heat shock proteins and antimicrobial cathelicidins. PGs are made from arachidonic acid via catalysis by cyclooxygenase (COX) COX-1 and COX-2 and are key factors in the mucosal defence mechanisms by inhibiting acid secretion, stimulating mucus bicarbonate (PGE2) and phospholipids secretion, increasing mucosal blood flow (PGI2), and accelerating epithelial restitution as well as mucosal healing. Inhibitors of COX-1, such as NSAIDs, potentiate gastric mucosal injury, in part by decreasing bicarbonate secretion [194, 213].

Trefoil factor family peptides (TFFs) are low-molecular weight peptides that are secreted with mucins. TFFs are involved in the assembly and/or packaging of mucins [214, 215], increase of the mucous layer viscosity and promote mucosal protection and restitution, independent of COX-mediated PGs synthesis [216-218].

Hypoxia-inducible factor (HIF-1) and vascular endothelial growth factor (VEGF), PGE2 and survivin promote healing and the continuous cell renewal from mucosal progenitor cells, by increasing angiogenesis [219, 220]. Gastric surface epithelium takes

3-7 days to be completely replaced whereas the gastric glands can take months to be replaced [194, 221]. After surface injury, migration of preserved epithelial cells in the neck area of the gastric glands occurs within minutes. The migration of progenitor cells occurs hours later [194, 222].

- Mucosal microcirculation (submucosal defence)

The submucosal microcirculation delivers oxygen and nutrients to the mucosa and removes toxic substances and acid. It is also critical for the transport of bicarbonate, produced on the basolateral membrane of HCl-secreting parietal cells trough a phenomenon called alkaline tide, upward to the surface epithelium, in order to maintain the unstirred mucus pH gradient [194, 203]. The endothelial cells of the microvessels generate vasodilators such as prostacyclin (PGI2), •NO and hydrogen sulphide (H_2S) which protect the gastric mucosa from injury, and prevent leukocyte adherence to the microvascular endothelium [223], forming an endothelial barrier. Upon an irritant or an episode of acid back-diffusion, a rapid increase in the mucosal blood flow occurs enabling the removal and/or dilution of the toxic agent. In great part, the increase in mucosal blood flow in response to acid is mediated and modulated by •NO generated by endothelial NOS [194, 224-226].

The gastric mucosa and submucosal vessels are innervated by primary afferent sensory neurons and nerves forming a dense plexus at the mucosal base as reviewed in [194, 227]. Stimulation of the gastric sensory nerves leads to the release of neurotransmitters such as calcitonin gene-related peptide (CGRP) that protects the mucosa against damage through its vasodilatory, anti-inflammatory, anti-apoptotic, and antioxidant effects, some of which are mediated by •NO [228, 229]. Also, some hormones such as ghrelin and adrenal glucocorticoids appear to exert gastric protective and healing actions. Ghrelin enhances mucosal blood flow via •NO production and CGRP release and adrenal glucocorticoids maintain glucose homeostasis, gastric blood flow and mucus

secretion and attenuate enhanced gastric motility and microvascular permeability [230, 231].

1.3.3 Nitrite and nitric oxide in gastric physiology and beyond

The tight regulation of the gastric physiology and defence system described above allows the gastric mucosa to cope with constant exposure to foreign agents, including among others, microbes, exogenous toxins, foodstuff with different osmolarities and pH. Nitrite and $^{\bullet}$NO have been shown to be critical in maintaining gastric function and integrity, playing important roles in gastric physiology and pathology [172, 194, 226, 232].

Animal studies showed that the inhibition of endogenous production of $^{\bullet}$NO exacerbated acid- or ethanol- induced gastric damage [233, 234] and human studies proven the gastro protective role of $^{\bullet}$NO [235]. The production of gastric mucus by both $^{\bullet}$NO donors [236] and salivary nitrite-derived $^{\bullet}$NO [177, 179] was increased through a cGMP-dependent mechanism. Additionally, by increasing gastric mucosal blood flow, $^{\bullet}$NO has been considered a gastroprotective molecule [177, 179] and when released from the non-cholinergic non-adrenergic terminals $^{\bullet}$NO has been implicated in the regulation of gastric motility [237, 238]. Moreover, $^{\bullet}$NO is also involved in the modulation of inflammatory responses [239, 240], acts as an antimicrobial agent (mostly due to indirect effects) [109, 175, 176] and has been suggested to prevent the development of gastric ulcers [179, 186].

However, controversial effects have been also imputed to $^{\bullet}$NO concerning the integrity of the epithelial barrier. Acidified nitrite has been shown to decrease epithelial resistance via occludin disarrangement in an *in vitro* model [241].

Dietary nitrite-derived RNOS induce modification in biomolecules such as proteins and lipids with potential implications not only on local molecular pathways but also systemically [242-244]. *In vivo* studies in humans, showed that upon the consumption of nitrate- and ethanol-rich foods the bioactive ethyl nitrite is formed in the gastric

compartment. Ethyl nitrite is a potent vasodilator [245] and at physiological pH induces smooth muscle relaxation trough a cGMP-dependent pathway suggesting $^{\bullet}NO$ release and potential impact in gastric motility [246]. Reaching the blood vessels, ethyl nitrite could act as a $^{\bullet}NO$ carrier with systemic effects [246]. An important gastric protease, pepsin, can be nitrated by dietary nitrite in the stomach, acquiring a new activity preventing the progression of gastric ulceration [187].

Another class of bioactive molecules, the nitrated lipids were reported to be formed from acidified nitrite *in vitro* [247], therefore, due to the high concentrations of both lipids and nitrite from diet, lipid nitration *in vivo* in the stomach is expected to be facilitated [242]. Nitrated lipids modulate important anti-inflammatory pathways [244] and their formation in the stomach should have both local and systemic (upon absorption to the systemic circulation) effects [242].

1.4 Aims and strategy

This thesis aims at a better understanding of the implications of nitrite biochemistry on gastric physiology. Nitrite chemistry in the gastric compartment can be tuned by other diet components such as red wine. With this in mind, the experimental work was performed with three major objectives:

- The identification and quantification of post-translational modifications in the gastric compartment, namely protein nitrosation, induced by dietary nitrite, under physiological and inflammatory conditions.
- To assess the impact of dietary nitrite and its derivatives in gastric mucosa considering cell signalling and regeneration, under physiological and inflammatory conditions.
- To elucidate the impact on respiration, considering gastric mitochondria as targets for dietary-derived nitrite and $^{\bullet}NO$.

Accordingly, we have implemented a comprehensive experimental strategy involving *in vitro*, *ex vivo* and *in vivo* models.

In chapter 3, we sought to determine the effect of dietary nitrite and its reaction products on mucosa in terms of protein post-translational modifications. Protein nitrosation was found as a major modification and red wine exerted a modulatory role of these biochemical modifications, both in the mucus and underlying cells. A critical issue regarding protein modification by reactive nitrogen species is the use of a proper analytical approach. Thus, for this purpose a highly sensitive chemiluminescence methodology was used that is considered the golden standard approach for nitrosation analysis. Given the role that inflammation plays in the gastric compartment under disease conditions we have also implemented an inflammatory model with diclofenac was in order to assess the extent of nitrite-dependent nitrosation under pathological conditions.

In chapter 4, molecular biology approaches were used to assess the importance of dietary-derived nitrite and its related species in gastric defence mechanisms, particularly

via the expression stimulation of an important signalling peptide, TFF1, involved in mucosal protection and anti-tumorigenesis: following the same path as in the previous chapter, this study was performed under physiological and pathological conditions, and the modulatory role of red wine studied. The results reinforce the notion that dietary nitrite and its derivatives are relevant in gastric physiology contributing for the maintenance of gastric mucosal integrity.

Given the high production of $^{\bullet}NO$ form dietary nitrite in the stomach and its role as a major regulator of mitochondrial respiration with impact of cell bioenergetics, it is of high physiological relevant to understand how mitochondria in gastric cells in chapter 5, we studied the gastric mitochondrial function using high resolution respirometry, using both a conventional system consisting of isolated mitochondria and a new approach comprising the use of small pieces of gastric tissue, thus maintaining tissue architecture. Being potential targets for dietary nitrite and its derivatives such as $^{\bullet}NO$, gastric mitochondria were challenged with both nitrite and $^{\bullet}NO$, simulating the exposure after a nitrate/nitrite intake. The modulatory effect of red wine was also assessed. The results provide insight on how gastric mitochondria cope with the potential deleterious species and their impact in the respiratory function.

2

Methods and Materials

2.1 Chemicals, reagents and solutions

2.1.1 Chemicals

All chemicals were purchased from Sigma-Aldrich Chemicals except the following:

Sodium diclofenac (Voltaren® 100) Novartis was purchased at a local pharmacy.

Antibodies used are indicated throughout the methods section.

ECF substrate for Western Blotting was purchased from GE Healthcare.

Mowiol was purchased from CalBiochem.

2.1.2 Gases

Obtained from Linde, Portugal.

2.1.3 Reagents and solutions

- *Saline* was prepared by dissolving 0.9% NaCl in deionized water.
- *Phosphate buffer solution* (PBS) 0.05 M had the following composition (mM): 10 NaH_2PO_4, 40 Na_2HPO_4, and 100 NaCl, pH 7.4.
- *Phosphate buffer solution* (PBS for immunohistochemistry) 0.01M had the following composition (mM): 3 NaH_2PO_4, 7 Na_2HPO_4, and 100 NaCl, pH 7.4.
- *Simulated gastric juice* (SGJ) was prepared as described in the United States Pharmacopeia (vol. 25) and contained (mM) 7 HCl and 50 NaCl, dissolved in distilled water at pH adjusted to 2.
- *Krebs-Henseleit bicarbonate buffer* (KH), pH 7.4, had the following composition (mM): 120 NaCl, 4.7 KCl, 1.2 KH_2PO_4, 25 $NaHCO_3$, 1.2 $MgSO_4.7H_2O$, 2.5 $CaCl_2$ and 11.1 glucose.
- *Stabilizing solution for S-nitrosothiols* consisted of 10 mM N-ethylmaleimide (NEM) and 2 mM ethylenediamine tetra-acetic acid (EDTA), supplemented PBS.

- *Mowiol-DABCO stock solution* was used as a mounting medium and consisted of: 2.4 g of Mowiol, 6 g of glycerol, 6 mL of H_2O, 12 mL of 0.2 M Tris-HCl (pH 8.5) and 5% DABCO.
- *Radio-immunoprecipitation assay buffer* (RIPA), was the used lysis buffer and was composed of: 150 mM NaCl and 50 mM Tris-HCl, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, pH 7-8 and supplemented with 1/100 (v/v) protease inhibitor cocktail.
- *TBS-T buffer* consisted of 25 mM Tris–HCl pH 7.6, 150 mM NaCl, 0.1% (v/v) Tween 20.
- *Mitochondria isolation medium*: 200 mM mannitol, 70 mM sucrose, 10 mM HEPES, 0.5 mM EGTA and 1mg/mL free fatty acids BSA, pH 7.4 at 4 °C.
- *Isolated mitochondria respiration medium* containing 0.5 mM EGTA, 3 mM $MgCl_2*6H_2O$, 100 mM K-MES, 20 mM taurine, 10 mM KH_2PO_4, 20 mM HEPES, 110 mM Sucrose, and 1 g/l fatty acid free BSA (adapted MiR05; Oroboros, Innsbruck, Austria).
- *Biopsy preservation buffer* (BIOPS) contained (mM) 7.23 K_2EGTA, 2.77 $K_2CaEGTA$, 6.56 $MgCl_2$, 20 imidazole, 0.5 dithiothreitol, 50 K-MES, 20 taurine, 5.3 Na_2ATP, 15 Phosphocreatine, pH7.1.
- Biopsy respiratory medium containing 2.77 mM CaK_2EGTA, 7.23 mM K_2EGTA, 0.5 mM DTT, 1.38 mM $MgCl_2$, 100 mM K-MES, 20 mM taurine, 20 mM imidazole, 3 mM K_2HPO_4, 5 mM pyruvate, and 5 mg/ml fatty acid-free bovine serum albumin (BSA), pH 7.1.
- *Nitric oxide solution*: a saturated $^{\bullet}NO$ solution was prepared as described in [248], in deoxygenated saline buffer.
- *Potassium phosphate buffer 0.1 M:* 80.2 mM K_2HPO_4 and 19.8mM KH_2PO_4, pH 7.4.

2.1.4 Foodstuff

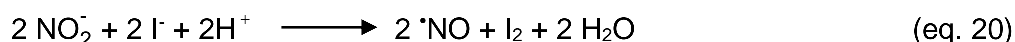
The red wine was *Touriga nacional* ‘‘Quinta de Cabriz” 2003 (13% v/v), Dão, Portugal and was obtained from a local shop.

2.2 Methods

2.2.1 Electrochemical measurements of nitric oxide

An ISO-NO Mark II Nitric Oxide electrode (World Precision Instruments, Ltd., Hertfordshire, UK) was used to record the time course of •NO production from sodium nitrite and from sodium nitrite/ascorbic acid or red wine mixtures, under simulated gastric conditions. The electrode consisted in a 2 mm diameter platinum electrode with an internal reference coated with a Teflon membrane permeable to gases, polarized at 0.8 V. Being a gas, •NO produced in the experimental solution diffuses through the membrane and is oxidized at the platinum surface, generating an electric current, monitored and recorded by Apollo 1000 (World Precision Instruments, LTD, Hertfordshire, UK).

The electrode was always calibrated before daily experiments, under aerobic conditions in a solution of H_2SO_4 and KI 1:1 (0.1 M) with increasing concentration of sodium nitrite at 37ºC, according to manufacturer's instructions. Since the concentration of both H_2SO_4 and KI are in excess relatively to nitrite, the latter is completely reduced to •NO, allowing the determination of the •NO concentration, following a 1:1 ratio as shown in the equation 20.



$$2\,NO_2^- + 2\,I^- + 2H^+ \longrightarrow 2\,{}^{\bullet}NO + I_2 + 2\,H_2O \qquad \text{(eq. 20)}$$

The measurements of •NO production from the reaction mixtures were performed in 10 mL of SGJ at pH 2 under stirring and aerobic at 37ºC. Once a stable baseline was achieved, the reaction was started by the addition of sodium nitrite. The reduction ability of red wine polyphenols was studied using the concentration indicated in the respective figures. All the solutions were freshly prepared. Control with vehicle (ultrapure water) was performed.

2.2.2 Tri-iodide based chemiluminescence

The quantification of nitrosated species was achieved after reductive cleavage of the nitrosated species by an iodide/tri-iodide containing reaction mixture and the subsequent

determination of the $^{\bullet}NO$ released into the gas phase by its chemiluminescent reaction with ozone (O_3). $^{\bullet}NO$ reacts with O_3 to form NO_2; a proportion of the latter arises in an electronically excited state (NO_2^*), which, on decay to its ground state, emits light in the near-infrared region [249] and can be quantified by a photomultiplier. Provided O_3 is present in excess and reaction conditions are kept constant, the intensity of light emitted is directly proportional to $^{\bullet}NO$ concentration (equation 21 and 22).

$$^{\bullet}NO + O_2 \longrightarrow NO_2^* + O_2 \qquad \text{(eq. 21)}$$

$$NO_2^* \longrightarrow NO_2 + \text{Light} \qquad \text{(eq. 22)}$$

This method is a high sensitive tool for bulk quantification of nitrosated compounds in biological samples, as described by Feelisch and colleagues [250]. In detail, reductive cleavage of the nitrosated species was achieved using a reaction mixture consisting of 45 mM potassium iodide (KI) and 10mM iodine (I_2) in glacial acetic acid at 56ºC and continuously bubbled with nitrogen (N_2) gas. The chemical output species of the reductive cleavage of nitrosated compounds is $^{\bullet}NO$ which is released into the gas phase and dragged by the nitrogen stream. The reaction mixture was kept in a septum-sealed, water-jacketed reaction chamber which design was similar to a commercially available unit (Sievers, Boulder, CO) built by Colaver (Colaver srl, Italy). The outlet of nitrogen gas stream containing $^{\bullet}NO$ passed through a scrubbing bottle containing 1M of ice cold sodium hydroxide (NaOH) in order to trap traces of acid and iodine before transfer to the analyser (CLD 88 Eco Medics, Switzerland) where the photomultiplier can be found. The $^{\bullet}NO$ signal output was registered in voltage (V) versus time using EDAQ Power Chrom software at 2 Hz. The peaks areas directly correlate with the amount of $^{\bullet}NO$ reaching the analyser and were calculated using the flow analysis (FIA) tool in the software.

Standards and samples aliquots (100 µL) were injected into the reaction mixture using Hamilton syringes. Considering that sodium nitrite is totally reduced to $^{\bullet}NO$ under these conditions, sodium nitrite standards freshly prepared were used at a range of

concentrations of 0.05 μM to 10 μM in order to obtained a calibration curve that allow to convert the samples peak areas into the amount of nitroso species present in the biological sample (Fig. 2.1).

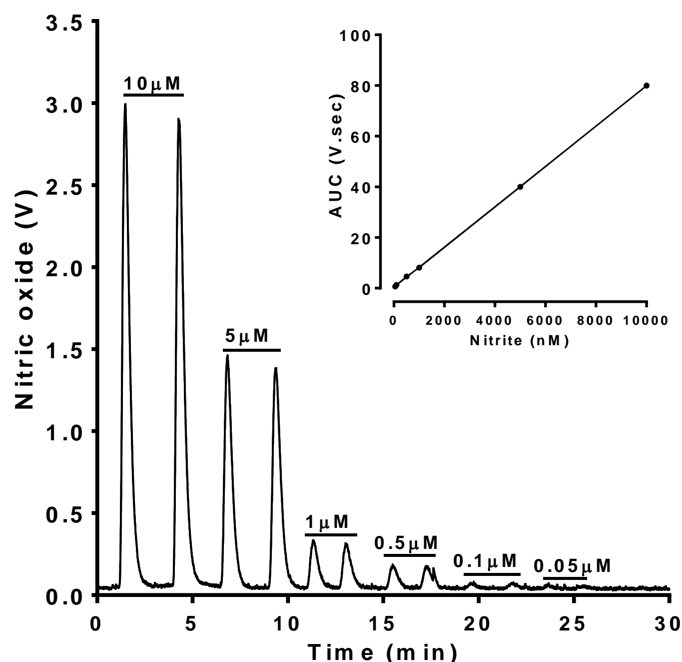


Figure 2.1 Typical •NO tri-iodide based chemiluminescence signals obtained after duplicate injections (100 μL) of nitrite standard solutions (from 50 nM to 10 μM) into the reaction mixture. Inset: Standard calibration curve from the same experiment obtained by plotting the geometric mean ± S.E.M. of the areas under curve (AUC) of individual peaks vs. nitrite concentration.

Nitroso compounds were expressed in nmol/mg of protein In order to differentiate between S-nitrosothiols and non-S nitrosated compounds, the samples were treated before analysis with group-specific reagents. Accordingly, the samples were divided into three aliquots and pre-incubated, respectively, with acid sulphanilamide (0.5%) for total nitroso compounds, acid sulphanilamide (0.5%) plus $HgCl_2$ (0.2%) for non-S or mercury-resistant nitroso compounds and sulphanilamide (0.5%) plus $HgCl_2$ (0.2%) plus $K_3Fe(CN)F_6$ (0.13%) for N-nitrosamines and heme nitrosyls quantification. Thus, it was possible to quantify all nitroso fractions by matching the sample aliquots. Before measurement the samples were kept on ice in the dark to avoid photolytic and thermolytic

decomposition. The results were normalized for protein amount quantified by the method of Lowry et al. [251] with bovine serum albumin as protein standard.

2.2.3 *In vitro* nitrosation of mucin

In vitro assays with partially purified mucin from porcine stomach were performed by dissolving the mucin powder in SGJ to a final concentration of 1 mg/ml. Incubations with sodium nitrite (0 – 10mM) and sodium nitrite (0.5 – 10 mM) plus ascorbic acid (0.5mM) were performed at 37ºC. The extent of nitrosation was determined by chemiluminescence at different time points after the nitrite load. Maximum nitrosation levels were achieved for 15-20 minutes mucin incubation with sodium nitrite (data not shown).

2.2.4 Biological samples

For all the studies regarding animal samples, male Wistar rats with 6 to 8 weeks old purchased from Charles River, Barcelona Spain, were used. All the experiments were performed in accordance with the European Community Council Directive for the Care and Use of Laboratory Animals (86/609/ECC) and approved by the local institutional animal care committee. The rats were kept under standard conditions of temperature and illumination. The day before the experiment the animals were deprived of food in order to be under fasting conditions (overnight) but with water *ad libidum* and were euthanized by cervical dislocation. For the *ex vivo* experiments (stomach strips and whole stomach models) the stomach was isolated in ice cold Krebs-Henseleit bicarbonate buffer pH 7.4, the mucosa was exposed by a cut through the stomach lesser curvature and then rinsed with ice cold saline.

2.2.5 *Ex vivo* nitrosation of stomach strips

The stomach corpus and antrum was divided with sharp scissors in 10 similar strips. The gastric strips were incubated under simulated gastric conditions and then kept in ice

cold NEM/EDTA (10/2 mM) supplemented PBS until use used for preliminary studies in the gastric mucus.

2.2.6 Whole stomach model in the diffusion chamber

Whole stomachs mounted in a diffusion chamber (CHM7 Ussing chamber, World Precision Instruments, Sarasota, USA) were used for mucus and mucosa assays. A previously established model [252] was used. Briefly, the organ was gently stretched and mounted in the chamber 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 in which the intra- and extra gastric environments were simulated: the mucosal side (filled with SGJ, in contact with gastric epithelium and mucus) and the serosal compartment (filled with KH buffer, in contact with gastric serosa). All the reagents (ascorbate, nitrite and red wine) were added to the mucosal compartment at the concentrations indicated in the respective figures.

2.2.7 Mucus and mucosa sampling for nitrosation quantification

The mucus and the mucosa were sampled for nitrosation quantification in both *ex vivo* and *in vivo* assays. Briefly, the isolated stomach or part of it was placed flat in an ice cold petri dish with, in ice cold, NEM/EDTA (10/2 mM) supplemented PBS. The mucus was gently removed from the surface of the stomach wall and collected to a tube with 1ml of NEM/EDTA supplemented PBS. After the removal of the mucus, a sample of gastric epithelium cells was collected and suspended in ice-cold NEM/EDTA-containing PBS, as in the mucus samples.

Finally, the samples were homogenized using a Potter-Elvehjem homogenizer. All the dilutions were made in ice cold NEM/EDTA-containing phosphate buffer and the

samples were kept in the ice and in the dark. Protein concentration was measured by the method of Lowry et al. [251] using bovine serum albumin as a standard.

2.2.8 pH-dependent nitric oxide release from nitrosated mucus glycoproteins

Using the whole stomach model described above the gastric mucus was challenged with nitrite under simulated gastric conditions. The mucus was collected to a tube with 1ml of NEM/EDTA (10/2 mM) supplemented PBS and handled as described above for nitrosation quantification.

The release of •NO from the nitrosated mucins was performed using the same experimental setup used for nitrosation quantification but instead of using the previous reaction mixture the samples were injected in, consisting of a tri-iodide solution at 56º C, the mucus was injected in phosphate buffer at 37ºC, at three different pH values: 5, 6.5 and 7.4 and the •NO release in ppb (parts per billion) was recorded over time using EDAQ Power Chrom software. The total amount of •NO released from the samples at any point in time was calculated using OriginLab software.

2.2.9 Mucus removal by mechanical and chemical means

The whole stomach model was used to determine the influence of the mucus removal in the extent of mucosa nitrosation at deeper cell layers. Prior to the incubation with sodium nitrite (1mM) as described above, the mucus was removed using two different approaches: a mechanical approach, were the mucus was gently scrapped from the surface of the stomach; and a chemical approach in which the organ was incubated with a mucolytic agent, a 2% N-acetylcysteine (NAC) solution, for 5 minutes. Sample collection and analysis was performed as described above.

2.2.10 *In vivo* nitrosation under physiological and acute inflammatory conditions

Rats were anesthetized with urethane (1.25 g/kg, intraperitoneal injection) and gastric acute inflammation was induced by the administration of 30mg/kg of diclofenac (Voltaren®, Novartis) by oral gavage (OG) as previously described [185]. The rats were left in their cages lying in a heating pad for 4 hours. Subsequently 1mM nitrite and/or a 10% red wine solution were given to the animals by OG. After 20 minutes the stomach was isolated and samples were collected for further studies.

2.2.11 Detection of TFF1 expression in the stomach by immunohistochemistry

The expression and distribution of the TFF1 in the gastric mucosa was investigated by immunohistochemistry. Part of the body region of the stomach was fixed with 4% paraformaldehyde (PFA) prepared in PBS, cryoprotected with a 10% to 30% sucrose gradient and cut into 10-12µm slices using a cryostat. Then the slices were permeabilized with 0.1 M PBS containing 0.25% Triton X-100 for 10 minutes. Blocking of non-specific binding was performed for 2 hours with PBS-T (0.01 M PBS buffer supplemented with 0.5% Tween) containing 0.3 M of glycine and 10% chicken serum (serum from the species that the secondary antibody was raised in). Then, the tissue sections were incubated with a diluted goat polyclonal antibody (Santa Cruz Biotechnology, raised against the C-terminus of the TFF1 of rat and mouse origin) in PBS-T supplemented with 10% chicken serum, overnight at 4 ºC in a humidified chamber. Next day the slices were incubated with a secondary antibody chicken anti-goat (Alexa flour 594, Santa Cruz Biotechnology) for 1 hour at room temperature in the dark. Counter staining of the nuclei was performed with incubation of the slices with Hoechst 33342 for 5 minutes. The slides were mounted with a coverslip over a drop of mounting medium (Mowiol) and then the coverslip was sealed with nail polish to prevent drying and movement under the microscope and stored in the dark at -20 or 4 ºC The slides were then observed under a

microscope with fluorescence filters (Zeiss Axioskop 2 plus and Axiovision Software, Carl Zeiss Microscopy, Germany). The fluorescence score was analysed by three blind observers in a scale from 0 (no fluorescence) to 5. The tissue morphology was evaluated by Haematoxylin & Eosin staining under a light microscope.

2.2.12 Histological analysis: haematoxylin & eosin staining

Slices (10-12 μm) were cut using a cryostat, washed briefly with deionized water and incubated with Mayer haematoxylin solution for 5 minutes, followed by 10 minutes wash with warm running tap water. Next the slices were rinsed in sequence with deionized water and 95% ethanol. Counterstaining with Eosin Y solution was performed for 30 seconds. Then, the slices were dehydrated through 95% ethanol and 2 changes of absolute ethanol (5 minutes each) and clear in 2 changes of xylene for 5 minutes each. Permanent mounting was performed with a xylene based mounting medium (DPX Mountant). The preparations were then observed under a light microscope (Zeiss Axioskop 2 plus and Axiovision Software, Carl Zeiss Microscopy, Germany).

2.2.13 Detection and analysis of TFF1 peptide in the stomach by Western Blotting

The cellular levels of TFF1 were assessed by western blot. Briefly, following incubation under the specified conditions, tissue samples were removed, washed twice with PBS and homogenized in ice cold RIPA lysis buffer supplemented with a protease cocktail inhibitor and left to rest in ice for 30 minutes. Lysates were subsequently centrifuged at 20000g for 10 min at 4 °C and supernatants were then collected and stored at −80 °C. Cellular protein content was quantified by the Bradford protein assay dye (Bio-Rad), using bovine serum albumin as the standard. Equal amount of protein (40-60 μg) were analysed by electrophoresis on a 12% SDS-polyacrylamide gel and were blotted to polyvinylidene difluoride (PVDF) membranes (Hybond-P Amersham, Buckinghamshire, UK) for 2 h at 250 mA. To avoid non-specific binding, membranes were blocked for 1 h at

room temperature with 5% (w/v) non-fat dried milk in TBS-T buffer. Membranes were then incubated overnight at 4°C with primary goat polyclonal anti-TFF1 (dilution 1:500) antibody (Santa Cruz). After three times 10 minutes washings with TBS-T, membranes were incubated with phosphatase alkaline-labeled secondary antibody anti-goat (dilution 1:15000, Abcam) for 2h at room temperature. Membranes were washed again three times with TBS-T. The bands were revealed with ECF and visualized by using a Typhoon FLA 9500 (GE Healthcare Life Sciences). Ponceau S staining was used as a loading control.

2.2.14 Mitochondrial isolation from stomach and liver

All the isolation procedures were performed in a 4ºC chamber. The gastric and hepatic tissue samples were placed on an ice cold Petri dish with isolation buffer and finely minced with forceps and scissors. A 2 minutes 0.02% protease incubation was used to soften the tissue and help liberating mitochondria. The protease was removed by diluting and washing twice with buffer. The excess of buffer was drawn off before transferring the tissue to an ice cold glass-Teflon Potter-Elvehjem tissue grinder (0.15 mm clearance). The tissue was homogenized at 1000 rpm for 2-5 minutes with slow vertical plunger movement in an ice bath. The homogenate was transferred from the homogenizer vessel into 1.5 ml microcentrifuge tubes that have been chilled in an ice bath. Mitochondria were isolated by differential centrifugation. All centrifugation steps were performed at 3-4°C using a microcentrifuge (Eppendorf 5417, Westbury, NY). A low speed spin (900 g) for 10 minutes firstly removed the myofibrillar portion. The supernatant containing the mitochondrial fraction was then transferred to clean, chilled microcentrifuge tubes and centrifuged at 10000g for 10 minutes to pellet mitochondria. The supernatants were carefully drawn away and the pellets from each tube were combined and resuspended in 1 ml of isolation buffer by gentle stirring and pipetting. A second high-speed centrifugation was performed at 9000 g for 10 minutes. The supernatant was discarded and the pellet gently resuspended [253]. The mitochondrial suspension was stored on ice and in the dark and used up to 1 hour without any noticeable loss of

function. Protein concentration was determined by the Bradford protein assay dye (Bio-Rad), using bovine serum albumin as the standard.

2.2.15 Biopsy collection from gastric mucosa and liver

Stomach and liver were removed from fasting wistar rats euthanized as described in the “Biological samples” section. Part of the corpus mucosa and the liver tissue were collected and placed immediately in ice-cold BIOPS solution and used for studies of mitochondrial function.

2.2.16 Preparation of permeabilized tissue

The permeabilized cells of gastric mucosal and liver tissue were prepared by the technique described in [254]. Briefly, the tissue biopsy samples were cut into small pieces (1-1.5 mm) in the ice-cold BIOPS solution and the pieces were gently stretched with thin tweezers, to facilitate the diffusion of the medium into the intercellular space. Next, the tissue was incubated at 4ºC, at mild stirring for 30 minutes in BIOPS solution containing 50 µg/ml saponin for permeabilization of the cell plasma membrane. The permeabilized mucosal tissue samples were then washed for 10 min in biopsy respiratory medium and this procedure of washing was repeated two more times to remove all metabolites from the cells.

2.2.17 Measurement of mitochondrial respiratory function

The rates of oxygen consumption were recorded using a high-resolution Oroboros oxygraph-2k (OROBOROS INTRUMENTS Corp. Austria). The 2 ml chambers were washed with 70% ethanol, rinsed 3 times with distilled water and then filled with respiration medium. The chamber is allowed to equilibrate with ambient gas phase at 37°C with a stirrer speed of 750 rpm for >30 minutes to allow air saturation of the respiration medium. Isolated mitochondria or permeabilized biopsies were added to the

chambers. The polyvinylidene fluoride stopper is inserted to close the chamber with a final volume of 2 ml. Oxygen concentration is recorded at 0.5 hz and converted from voltage to oxygen concentration using a two-point calibration. Respiration rates (O_2 flux) are calculated as the negative time derivative of oxygen concentration (Datlab Version 5.1.1.9, Oroboros Instruments). The O_2 flux values are corrected for the small amount of back-diffusion of oxygen from materials within the chamber, any leak of oxygen from outside of the vessel, and oxygen consumed by the polarographic electrode [255]. The protocol (see below) involved serial additions of various substrates, inhibitors, and uncouplers allowing a comprehensive assessment of mitochondrial function.

2.2.18 Protocol for assessment of mitochondrial respiratory function

- Isolated mitochondria

An aliquot of the mitochondrial suspension containing 100-400µg mitochondrial protein is added to each oxygraph chamber. After the stabilization of the baseline the following reagents were added: 10 mM glutamate and 2 mM malate, 2.5 mM ADP, 10 µM cytochrome c, 10 mM succinate, 0.5 µM rotenone, 2 µg/ µl oligomycin, 0.05mM titrations of carbonylcyanide-4-(trifluoromethoxy)-phenyllhydrazone (FCCP), 2.5 µM antimycin A and 0.5mM/2mM N,N,N',N'-Tetramethyl-p-phenylenediamine dihydrochloride plus ascorbate (TMPD/Asc).

- Saponin-permeabilized biopsies

A small biopsy (10-20 µm) was added to each oxygraph chamber. After the stabilization of the baseline the following reagents were added: 10 mM glutamate and 2 mM malate, 2 mM ADP, 10 µM rotenone, 10 mM succinate, 25 µM carboxyatractyloside, 5 µM antimycin A, 0.5mM/2mM TMPD/Asc, 10 µM cytochrome c, 5 mM sodium azide (NaN_3).

For the assessment of the uncoupled respiration the following protocol was used: 10 mM glutamate and 2 mM malate, 2 mM ADP, 10 mM succinate, 25 µM carboxyatractyloside, 0.05mM titrations of FCCP and 5 µM antimycin A.

2.2.19 Modulation of mitochondrial respiration by nitric oxide

Isolated mitochondria and biopsies from stomach and liver were challenged with •NO concentrated solutions in order to expose the organelles to •NO 10 and 20uM. The impact of •NO on mitochondrial respiration was assessed by its effect on the oxygen consumption rate (OCR) in a functional mitochondrial preparation with no substrate limitation. The duration of OCR was considered to be the time since the start of the inhibition until the total recovery of the respiratory rate.

2.2.20 Modulation of the gastric mitochondrial function by nitrite and red wine

- Stomach strips (*ex vivo*)

The acute effect of nitrite and its derivatives was assessed using stomach strips collected form the stomach corpus and antrum regions from fasting wistar rats. The gastric strips were incubated under simulated gastric conditions with 1mM nitrite, 10% red wine and 1mM nitrite plus 10% red wine for 20 minutes, in order to assess modifications in mitochondrial function. The following controls were performed: nitrite vehicle, 10% red wine solution, and 1.3% ethanol solution.

- *In vivo*

Four groups of fasting rats received by OG the following solutions: 1mM sodium nitrite, 1mM nitrite plus 10%red wine, 10% red wine and a control group received saline. The effect of nitrite in mitochondrial function was assessed 4 hours later, after isolation of the stomach, in permeabilized gastric biopsies as described above.

2.2.21 Measurement of reactive oxygen species and oxidants in homogenates of gastric mucosa

An adaptation of the method described by Ravindranath [256] was used. The method is based on the oxidation of 2',7'-dichlorofluorescin (H2DCF) to dichloroflourescein (DCF) by H_2O_2, although it should be noted that other oxidants may contribute to the oxidation of the probe. Before oxidation, H2DCF diacetate (H2DCFDA) is deacetylated to H2DCF by intracellular esterases. Tissue samples were collected as described above for the mitochondrial function studies and homogenized in ice cold potassium phosphate buffer 0.1 M in a smooth glass Potter-Elvehjem homogenizer for 3 minutes. An aliquot was reserved to determine protein concentration by the Bradford protein assay dye (Bio-Rad), using bovine serum albumin as the standard. H2DCFDA was dissolved in absolute ethanol at 1mM final concentration and stored at -20ºC. Immediately before the reaction, the stock solution of H2DCFDA was diluted 1:5 in potassium phosphate buffer. The homogenates were incubated in triplicate and in the presence of 10µM H2DCFDA in a black flat-bottom 96-well plate in the dark at 37°C for 30 minutes. Readings were made using a SPECTRA max GEMINI EM microplate spectroflourometer, (Molecular Devices, CA, USA) at the excitation wavelength of 488 nm and emission of 525 nm. The contribution of monoxidized DCFDA (that was found to be negligible) and the auto fluorescence of every sample was determined in parallel and subtracted. The measurements were expressed as fluorescence units/mg protein). H_2O_2 was used as a positive control. The fluorescence increased linearly with time from 0 to 30 min (at least). Autofluorescence from the samples was quantified and subtracted and was always <5% of the signal.

2.3 Statistical analysis

Unpaired and two-tailed Student's t-test was used in two-sample comparison. One-way ANOVA was used to compare three or more sets of unpaired measurements. Two-

way ANOVA variance followed by Bonferroni multiple comparison test was used to compare more than two groups. A probability value (*p* value) of less than 0.05 was considered significant and unless otherwise stated, all values are presented as mean ±S.E.M..

3

Protein post-translational modifications in the stomach: Nitrosation promoted by dietary nitrite and modulation with red wine

3.1 Introduction

During the past two decades, after $^{\bullet}NO$ formation from inorganic nitrite in the gastric compartment was demonstrated for the first time [109, 110], the biochemical relevance of dietary-derived nitrite in the stomach has been subject of many studies. In the gastric compartment, a high concentration of nitrite can be achieved (1-2 mM upon a normal nitrate load from diet [143]) due to the direct reduction in saliva and the contribution of the enterosalivary recirculation of nitrate [119, 141, 257]. At the acidic gastric pH, nitrite is protonated to nitrous acid that, in turn, decomposes to $^{\bullet}NO$ and other RNOS (eq. 12-14) [109, 110]. All species formed have emerged as new agents with a role in the gastric pathophysiology, including host defence against gut pathogens [175], regulation of gastric mucosal blood flow and mucus production [177, 258], and protection against gastric ulcer [186].

Endogenous or dietary reductants, such as red wine polyphenols, are known to be implicated in the nitrite chemistry in the gastric lumen, enhancing $^{\bullet}NO$ formation from nitrite at acidic pH, as shown by our group and others [147, 148, 188]. The reaction between nitrite and red wine polyphenols can be mechanistically described as a univalent reduction of nitrite to $^{\bullet}NO$ while the polyphenol is oxidized to the corresponding o-semiquinone radical (eq. 19) [148].

$^{\bullet}NO$ and RNOS can induce post-translational modifications in biomolecules like lipids and proteins, thus altering their structure and/or function [97]. RNOS, such as $^{\bullet}NO_2$, N_2O_3, $ONOO^-$ and HNO_2 (which generates NO^+), are nitrosating agents that can react with aromatic compounds, amines, amides, alcohols and thiols to form C-, N-, O-, and S-nitroso species, respectively [83, 84]. Nitrosation reactions in general and S-nitrosation in particular, have been the major focus of recent studies. In case of proteins, S-nitrosation is a dynamic post-translational modification affecting a broad range of functional parameters, including protein stability and subcellular localization, protein-protein interactions, known to be involved in the regulation of major signalling pathways [90-92],

such as controlling oxygen delivery to tissues, modulating the function or activity of transcription factors, enzymes, membrane receptors and ion channels [87]. Accumulated evidence suggests that in way analogous to other common post-translational modifications that are regulated enzymatically (e.g., phosphorylation) enzymatic mechanisms for regulating S-nitrosation may be operative *in vivo* but currently it is accepted that the formation and degradation of S-NO bond is largely dependent solely on chemical reactions without intervention of enzymatic catalysis [100]. Kinetically, S-nitrosation ($k = 4.6 \times 10^5$ M^{-2} s^{-1}) is preferred over N-nitrosation ($k = 4.6 \times 10^3$ M^{-2} s^{-1}) [259, 260] under similar experimental conditions [87] in particular at lower pH [85, 259] and in presence of bicarbonate [261], such as found in the gastric environment. N-nitrosation occurs more readily in amines than amides, resulting in N-nitrosamines being the major components of the N-nitroso fraction [262].

As described before, mechanistically, S-nitrosation may occur either due to the reaction between $^{\bullet}NO$ and a thyil radical that had been previously formed in the cysteine residue (reaction 9) [97] or, more importantly, by the action of nitrogen oxides (formally addition of a NO^+), such as N_2O_3 formed by the reaction of $^{\bullet}NO$ with oxygen or from acidified nitrite (reaction 10) with a thiol group [55, 97]. Also, two distinct thiols can undergo fast transnitrosation reactions (reaction 11), which may explain in part the liability of SNO [98, 99].

The gastric mucosa is daily challenged by exogenous and endogenous aggressors, such as diet components, drugs, microorganisms, HCl secretion and proteases, for which an efficient defence system to maintain mucosal integrity is required, as reviewed in [194, 263]. The gastric mucus layer is a first line of defence and represents both a chemical (due to its bicarbonate gradient) and a physical (due to its thick gel composition) barrier to luminal contents [180, 264-266]. Additionally, adequate blood flow is essential for proper mucus and bicarbonate secretion, response to luminal irritants, buffering acids and removing toxic compounds [258, 267]. Mucus is secreted by epithelial cells and its main

constituents are large gel-forming glycoproteins named mucins (constituted by alternated hydrophilic (glycosylated) and hydrophobic (cysteine-rich) domains) and water [207, 268]. The continuous mucus layer which covers the gastric mucosa can be separated into the outer layer, called the loosely adherent mucus, and the inner layer, the firmly adherent mucus [210, 211] in addition to degraded mucus in the lumen [202, 269].

The high cysteine content of the gastric mucins, combined with the low pH of the gastric compartment, favour S-nitrosation over the formation of other nitroso compounds [85]. Moreover, the hydrophobic microenvironment of the mucus, enhances the rate of reaction between $^{\bullet}NO$ and O_2 [56], favouring the conditions for local (mucus) nitrosation, especially S-nitrosation, when exposed to acidified nitrite and its derivatives.

Underlying the mucus, the cell layer is protected from the lumen contents via the mucus barrier effect. The gastric mucosa is constituted by many cell types which are responsible for most of the physiological processes of the stomach, namely mucus production and accumulation and gastric juice and digestive enzymes secretion. $^{\bullet}NO$ diffusing through the stomach wall [252] can produce modifications in key proteins involved in signalling pathways of gastric physiology.

In this chapter we sought to determine the effect of dietary nitrite and its reaction products on mucosa nitrosation as well as the modulatory role of red wine on these biochemical modifications. To this purpose we used a highly sensitive chemiluminescence methodology to quantify the formation of nitroso compounds in the gastric mucosa (both in the mucus and underlying cells) following exposure to nitrite under simulated gastric conditions and *in vivo*. An inflammatory model with diclofenac was used to assess the extent of nitrite-dependent nitrosation under pathological conditions.

3.2 Results

3.2.1 Nitrosation of mucin *in vitro* by nitrite under simulated gastric conditions

Mucin from porcine stomach was incubated with nitrite under simulated gastric conditions (1mg/mL in SGJ) for 15-20 minutes in the dark at 37ºC (Fig. 3.1). The formation of S-nitrosothiols and non-S nitroso compounds was observed in a nitrite concentration dependent fashion. Nitrosation was quantified by the tri-iodide based chemiluminescence assay. Nitroso derivatives increased in all fractions as a function of nitrite concentration. However, it is apparent that for the lower nitrite concentrations (0.1 – 0.5 mM), as those easily achieved in the stomach, S-nitrosothiols were formed in a greater extent than non-S nitroso compounds, as expected in view of the higher reaction rate constants of S-nitrosation over N-nitrosation. For the higher nitrite concentrations (1 – 10 mM) the formation of non S- nitroso compounds suffered a notorious increase.

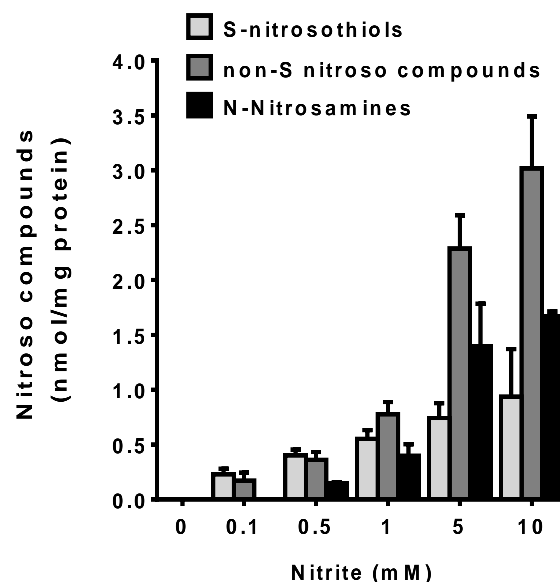


Figure 3.1 Nitrosation of mucin by nitrite under simulated gastric conditions. The values are expressed as mean ± S.E.M., n=2-3.

3.2.2 *Ex vivo* model of mucus nitrosation by nitrite under simulated gastric conditions

Preliminary studies of mucus nitrosation in stomach strips exposed to nitrite and red wine mixtures were performed in order to search for the nitrosation pattern and its dependence on nitrite concentration, thus optimizing the time for sample analysis after incubation with nitrite (data not shown).

Then, in a closer approach to what occurs in the gastric medium *in vivo* upon nitrite consumption, a whole stomach model was used as described before. A direct correlation between nitrite concentrations (0 – 10 mM) and the extent of mucus nitrosation can be observed (Fig. 3.2). There is a clear predominance of S- (86-88% of total nitrosation, in average) over non-S nitrosated compounds.

Interestingly, in the control (no nitrite addition), a fraction of S-nitrosothiols was found (0.017±0.007 nmol/mg of protein; n=4) but no other nitroso compounds were detected, suggesting a low background nitrosation under physiological conditions. For the remaining nitrite conditions, all fractions (S-nitrosothiols, non-S nitroso compounds and N-nitrosamines) were detected.

At lower nitrite concentrations (0.5 and 1 mM) the amount S-nitrosothiols formed was 0.912 ± 0.214 nmol/mg of protein, n=6 and 1.449 ± 0.213 nmol/mg of protein, n=7, respectively. Within the non-S nitroso fractions (0.134 ± 0.031 nmol/mg of protein, n=6 and 0.192 ± 0.018 nmol/mg of protein, n=7, respectively), N-nitrosamines represent 31% of the non-S nitrosation for 0.5 mM nitrite and 44% for 1mM nitrite, in average. These lower concentrations of nitrite are within the range found *in vivo* in the stomach after a nitrate load from a meal containing fresh leafy vegetables (1-2 mM) [143].

For higher nitrite concentrations (5 and 10 mM) the amount S-nitrosothiols formed was 2.470 ± 0.742 nmol/mg of protein, n=6 and 3.950 ± 1.301 nmol/mg of protein, n=5, respectively. Within the non-S nitroso fractions (0.374 ± 0.084 nmol/mg of protein, n=6 and 0.666 ± 0.264 nmol/mg of protein, n=5, respectively), N-nitrosamines represent

approx. 100% of the non-S nitrosation for 5 mM nitrite and 82% for 10 mM nitrite, in average.

The •NO produced at each nitrite concentration was quantified *in vitro* under simulated gastric conditions and, as expected [148], showed a direct correlation with the concentration of nitrite.

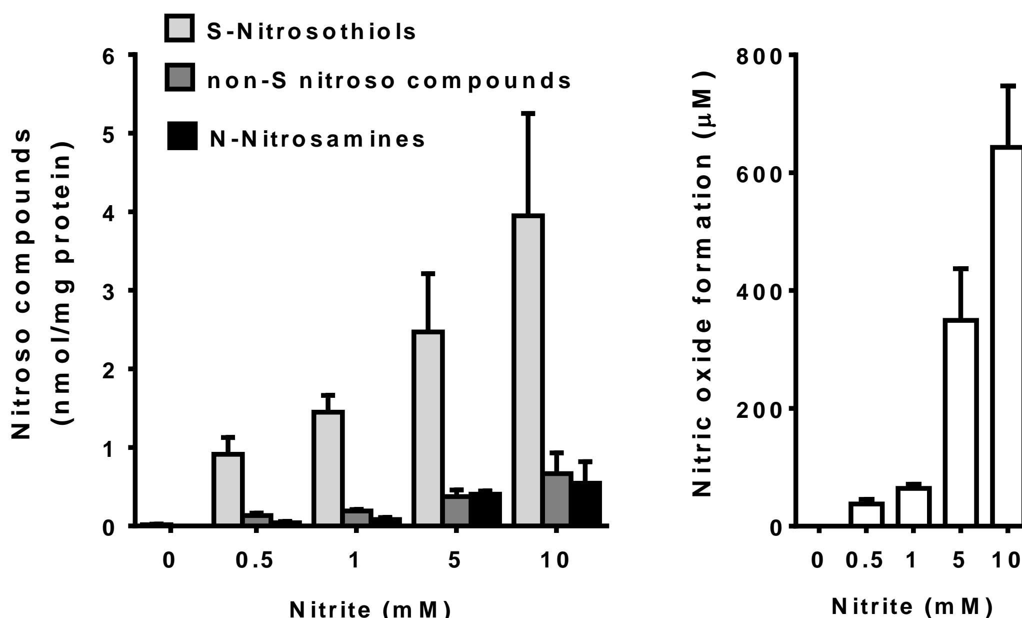


Figure 3.2 Pattern of gastric mucus nitrosation upon nitrite exposure under simulated gastric conditions *ex vivo* in a whole stomach model (left) and the correspondent •NO formation *in vitro* (right) in a similar medium. Values are mean ± S.E.M. (n = 3 – 7).

3.2.3 *Ex vivo* model of mucus nitrosation upon nitrite exposure in the presence of red wine under simulated gastric conditions

Red wine is a diet component containing two major fractions, ethanol and polyphenols, both of which have been shown before to be involved in the gastric nitrite metabolism [148, 245]. Thus, the effect of red wine as a potential modulator of nitrite-dependent mucus nitrosation was evaluated (Fig. 3.3). The formation of S-nitrosothiols, non-S nitroso compounds and N-Nitrosamines was assessed along with the determination of the concentration of •NO produced under the same conditions.

A 10% red wine solution was able to reduce nitrosation of the gastric mucus for both nitrite conditions. Particularly, S-nitrosothiols formation was impaired with statistical significance ($p < 0.05$), to 0.207 ± 0.084 nmol/mg of protein, n=4, with 0.5 mM nitrite and to 0.377 ± 0.086 nmol/mg of protein, n=3 with 1mM nitrite.

Determination of •NO revealed an increase in the presence of red wine under simulated gastric conditions.

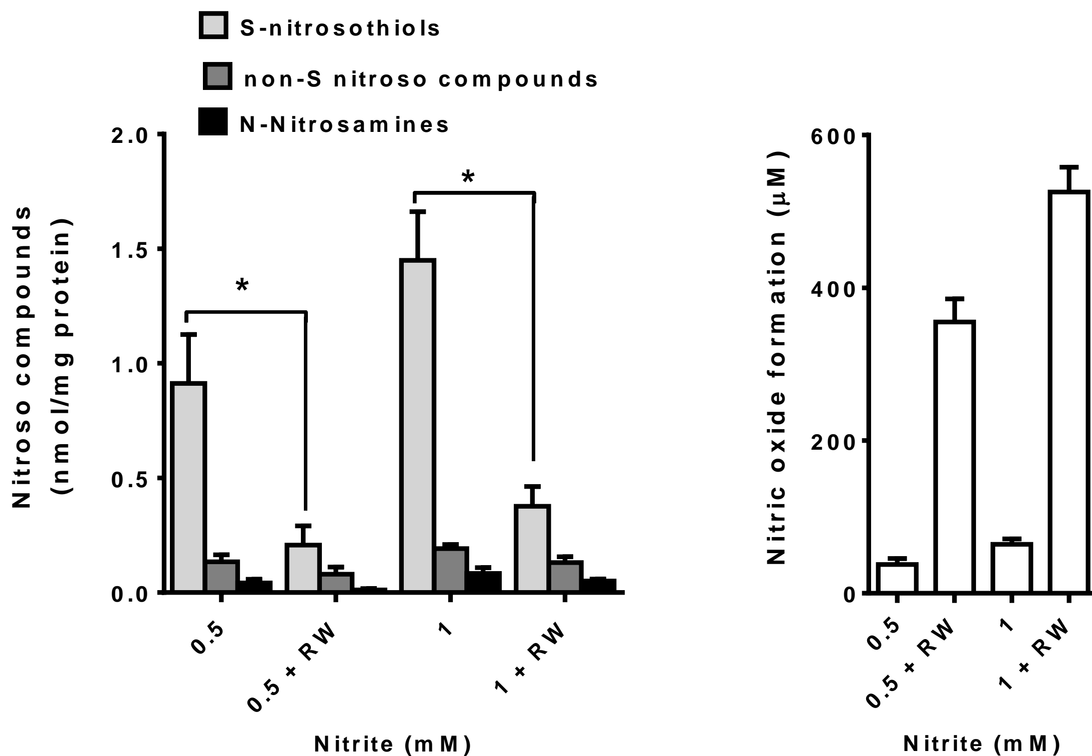


Figure 3.3 Gastric mucus nitrosation pattern upon exposure to nitrite and red wine (RW) mixtures under simulated gastric conditions (left). Right, *in vitro* •NO formation from acidic nitrite from the correspondent mixtures. Values are mean ± S.E.M. (n = 3 – 7), * $p<0.05$.

3.2.4 Gastric mucosa nitrosation upon exposure to nitrite under simulated gastric conditions

In order to understand the extent of the nitrosation throughout the gastric mucosa cell layers, samples of gastric cells under the mucus layer were collected and the nitrosation pattern determined following incubation of the stomach with nitrite under simulated gastric conditions.

In the control mucosa (not exposed to nitrite), nitrosation was not detected (Fig. 3.4). Upon exposure to nitrite, nitrosation (all fractions, S-nitrosothiols, non-S nitroso compounds and N-nitrosamines) was observed, increasing with the increase of nitrite concentration (0.5 – 10 mM). We observed a predominance of S-nitrosothiols formation over non-S nitrosated compounds, corresponding to 72-82% of the total nitrosation amount, in average.

For the lower nitrite concentrations (0.5 and 1 mM) the amount S-nitrosothiols formed was 0.080 ± 0.022 nmol/mg of protein, n=3 and 0.113 ± 0.037 nmol/mg of protein, n=4, respectively. Within the non-S nitroso compounds fractions (0.020 ± 0.004 nmol/mg of protein, n=3 and 0.043 ± 0.012 nmol/mg of protein, n=4, respectively), N-nitrosamines represent 30% of the non-S nitrosation for 0.5mM nitrite and 28% for 1mM nitrite, in average.

For the higher nitrite concentrations (5 and 10 mM) the amount S-nitrosothiols formed was 0.368 ± 0.170 nmol/mg of protein, n=3 and 0.399 ± 0.144 nmol/mg of protein, n=3, respectively. Within the non-S nitroso compounds fractions (0.102 ± 0.034 nmol/mg of protein, n=3 and 0.089 ± 0.030 nmol/mg of protein, n=3, respectively), N-nitrosamines represent about 52% of the non-S nitrosation for 5mM nitrite and 68% for 10 mM nitrite, in average.

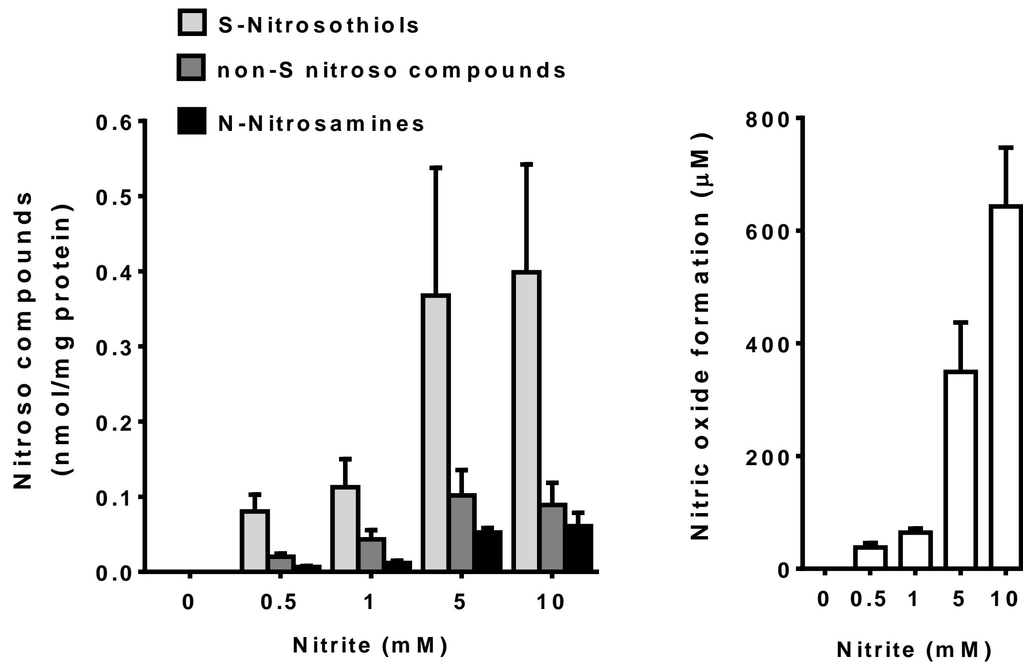


Figure 3.4 Pattern of gastric mucosa nitrosation upon nitrite exposure under simulated gastric conditions *ex vivo* in a whole stomach model (left) and the correspondent •NO formation *in vitro* (right) in a similar medium. Values are mean ± S.E.M. (n = 3-4).

3.2.5 Effect of red wine on the nitrosation pattern of gastric mucosa challenged with nitrite under simulated gastric conditions

Since an effect of red wine components was observed in gastric mucus nitrosation, we have also studied their impact in nitrosation of mucosa cells under 0.5 and 1mM of nitrite (Fig. 3.5). The formation of S-nitrosothiols, non-S nitroso compounds and N-nitrosamines was assessed along with the determination of the concentration of •NO produced under the same conditions.

Mixtures of red wine (10%) with nitrite (0.5 and 1 mM) leads to an overall decrease in nitrosation when compared to nitrite alone, with a more relevant impact in the S-nitrosothiols fraction. In particular, the mixture of 0.5 mM nitrite with red wine induced a statistically significant reduction in S-nitrosothiols formation (0.016 ± 0.010 nmol/mg of protein, n=4, $p < 0.05$).

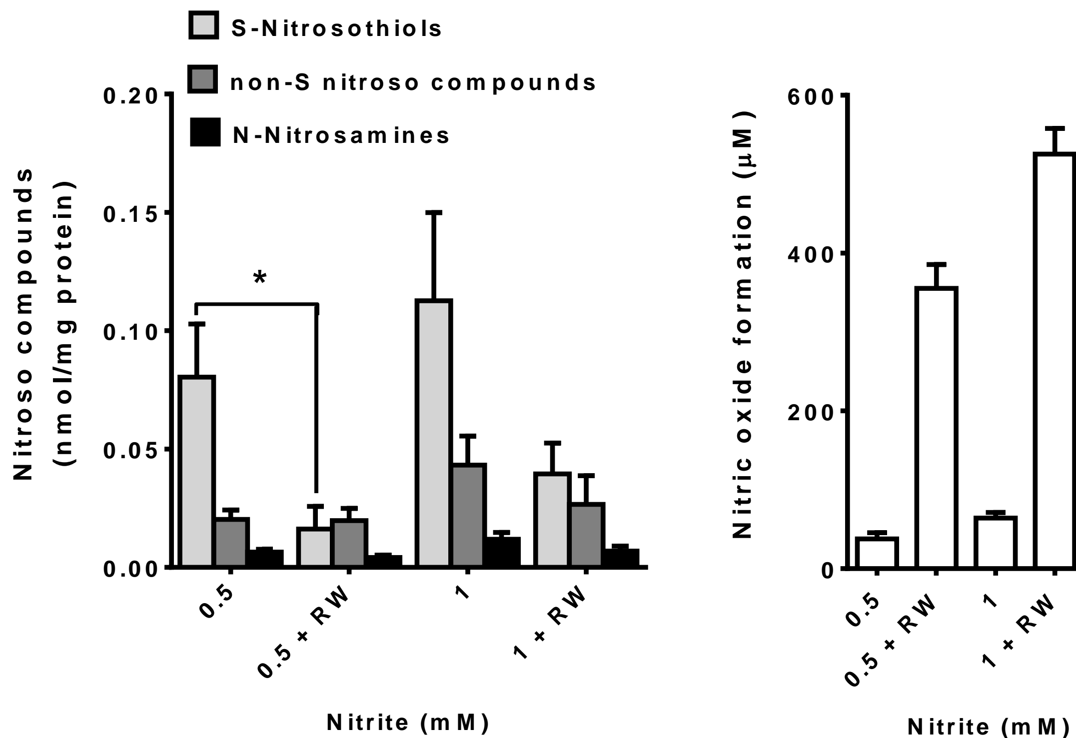


Figure 3.5 Effect of red wine (RW) on the nitrosation pattern of gastric mucosa challenged with nitrite under simulated gastric conditions (left). Right, *in vitro* ${}^{\bullet}$NO formation from acidic nitrite from the correspondent mixtures. Values are mean ± S.E.M. (n = 3), * p<0.05.

3.2.6 Influence of the mucus layer removal in the nitrosation pattern of the gastric mucosa

The importance of the mucus layer as a barrier against nitrosation of underlying mucosa cells was assessed by removing the mucus. Two removal methodologies were used: mechanical removal by scrapping and chemical removal by 2% N-acetylcysteine (Fig. 3.6).

Overall, it is evident that removal of the mucus led to a higher extent of nitrosation in the mucosal underlying cells that was more prominent in the S-nitrosothiols fraction. In the mucosa incubated with 1mM nitrite after mucus scrapping, all fractions of nitroso compounds were increased, when compared with normal (control) conditions. In the mucosa incubated with 1 mM nitrite after chemical removal of the mucus by 2% N-acetylcysteine an increase in S-nitrosothiols fraction (0.452 ± 0.133 nmol/mg of protein,

n=3, $p < 0.05$) was observed, but no significant changes in the non-S nitroso compounds was observed.

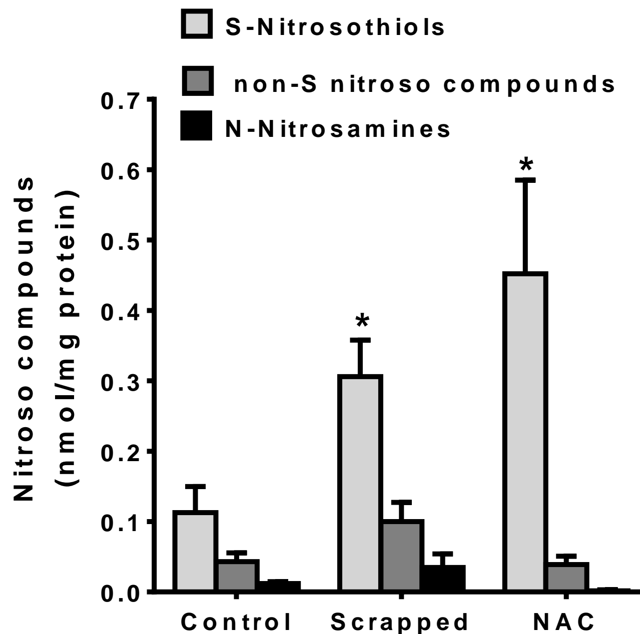


Figure 3.6 Effect of mucus gel removal on the nitrosation of the gastric mucosa exposed to nitrite (1mM). Mucus was removed by scrapping or by pre-incubation of the mucosa with 2 % N-acetyl-cysteine (NAC). Values are mean ± S.E.M. (n = 3), * $p<0.05$, relative to control.

3.2.7 Nitrosated mucus as a nitric oxide donor at physiological pH

In order to elucidate the fate of the nitrosated mucins in the gastrointestinal tract, the total release of $^{\bullet}NO$ over time from nitrosated mucus was assessed at different pH (Fig. 3.7). At physiological pH (7.4) a higher amount of $^{\bullet}NO$ was released over time, with statistically significance for 1 ($p < 0.05$) and 15 ($p < 0.01$) minutes when compared to pH 5. The maximal release of $^{\bullet}NO$ (peak high) directly correlates with the increase in pH. At physiological pH (7.4), a higher amount of $^{\bullet}NO$ was released ($p < 0.05$) when compared with both pH 5 and 6.5.

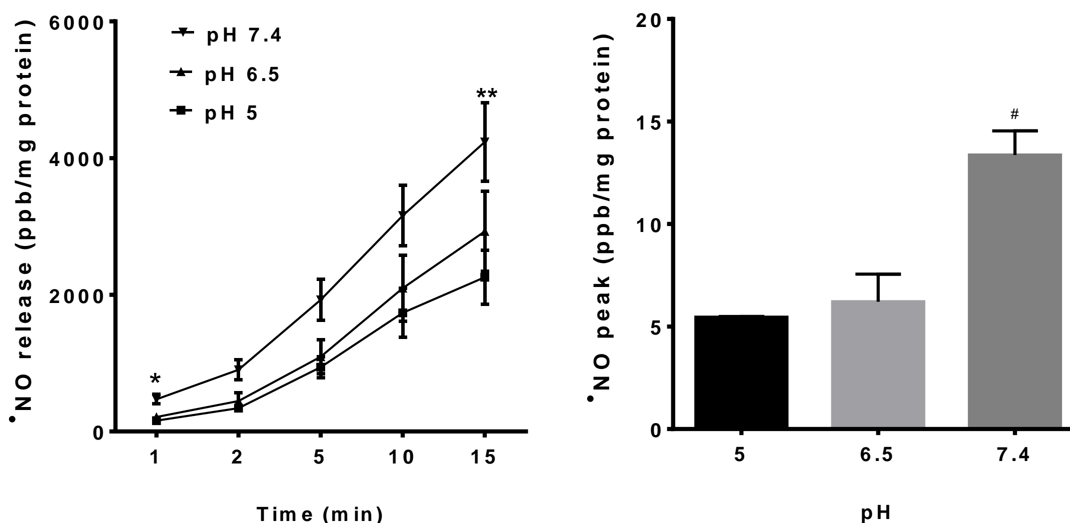


Figure 3.7 •NO release from nitrosated mucus with acidified nitrite: variation with pH. Left panel, total amount of •NO released for each time period (AUC, ppb/mg protein). Right panel, •NO peak, maximum amount of •NO measured (ppb/mg protein). Values are mean ± S.E.M. (n = 2-5), * $p<0.05$, ** $p<0.01$ (pH 7.4 versus pH 5); # $p< 0.05$ (pH 7.4 versus pH 5 and pH 6.5).

3.2.8 *In vivo* nitrosation induced by dietary nitrite

Nitrosation of the gastric mucus and epithelial mucosa can occur *in vivo* upon nitrite consumption. The results in figure 3.8 show that, overall, *in vivo* nitrosation was about 10-fold lower than *ex vivo* nitrosation. Upon administration by OG of sodium nitrite, nitrosated compounds were found in the gastric mucus layer. The major fraction of nitrosated compounds were S-nitrosothiols (0.060 ± 0.015 nmol/mg of protein, n=7) corresponding to about 77% of the total nitrosation. The minor fraction corresponds to non-S nitroso compounds (0.018 ± 0.009 nmol/mg of protein, n=7) about 23%, which comprises N-nitrosamines along with other nitroso compounds.

Of note, the pattern of nitrosation in the gastric epithelial mucosa was similar to that of the mucus layer. The major fraction of nitrosated compounds correspond to the S-nitrosothiols (0.014 ± 0.005 nmol/mg of protein, n=7) corresponding to near 76% and the other fraction (0.004 ± 0.003 nmol/mg of protein, n=7), about 24%, correspond to non-S nitroso compounds.

In the control animals, nitrosation was not detected under these conditions.

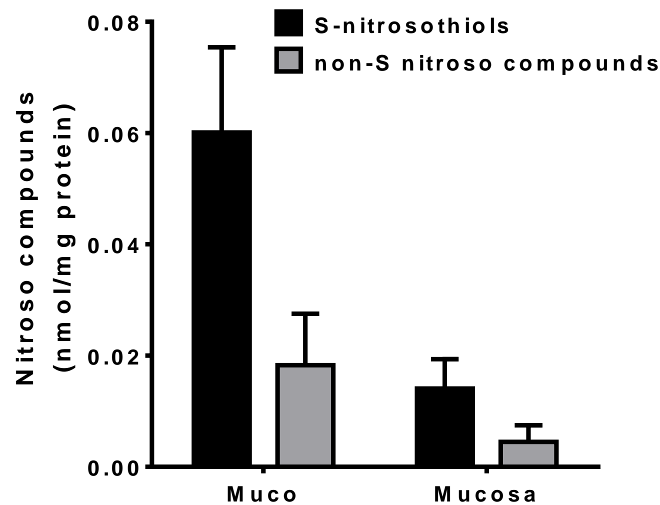


Figure 3.8 *In vivo* nitrosation in the presence of 1mM nitrite of the mucus and mucosa layers. Values are mean ± S.E.M., n=7.

3.2.9 Modulation of gastric nitrosation *in vivo* by red wine polyphenols

As shown in the previous chapter, the nitrosation patter upon exposure to nitrite from diet can be modulated by red wine. *In vivo*, (Fig. 3.9) the nitrite/red wine mixture exerted similar effects on nitrosation pattern similar to those ones obtained in the *ex vivo* experiments.

In the gastric mucus layer, a statistically significant reduction of S-nitrosation by nitrite plus red wine (0.019 ± 0.013 nmol/mg of protein, n=3) relatively to only nitrite (0.060 ± 0.015 nmol/mg of protein, n=7) corresponding to a 68.9% decrease, can be observed. In the gastric mucosal epithelium the red wine polyphenols have also inhibited the formation of S-nitrosothiols by about 86.7%. The control with the red wine solution showed the presence of a small amounts of nitroso compounds.

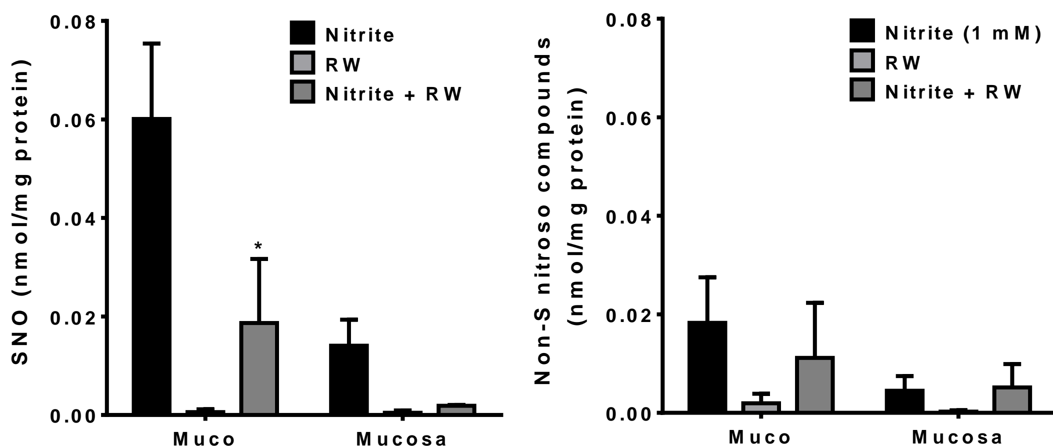


Figure 3.9 *In vivo* nitrosation of the mucus and mucosa layers, in the presence of 1mM nitrite, and modulation with red wine. Values are mean ± S.E.M., n= 3-7.

3.2.10 Nitrite-induced nitrosation under inflammatory conditions

An *in vivo* inflammatory model was implemented by administration of 30mg/kg of diclofenac to the stomach for 4h. *Per se*, this condition did not induced the formation of nitrosated compounds amenable to be detected under this conditions, except for a small amount of S-nitrosothiols in the mucus sample (data not shown). Figure 3.10 shows the amount of nitrosated compounds found in both mucus and mucosa layers under physiological and inflammatory conditions upon 1mM nitrite, being the incubation conditions considered significant as source of variance by two-way ANOVA analysis. An overall increase in the nitrosation pattern can be observed in both layers of the gastric mucosa across all fractions of nitrosated compounds. Statistically significant increase can be observed in S-nitrosothiols fraction with an increase of both of about 220% relatively to the fraction obtained with1mM nitrite under physiological conditions.

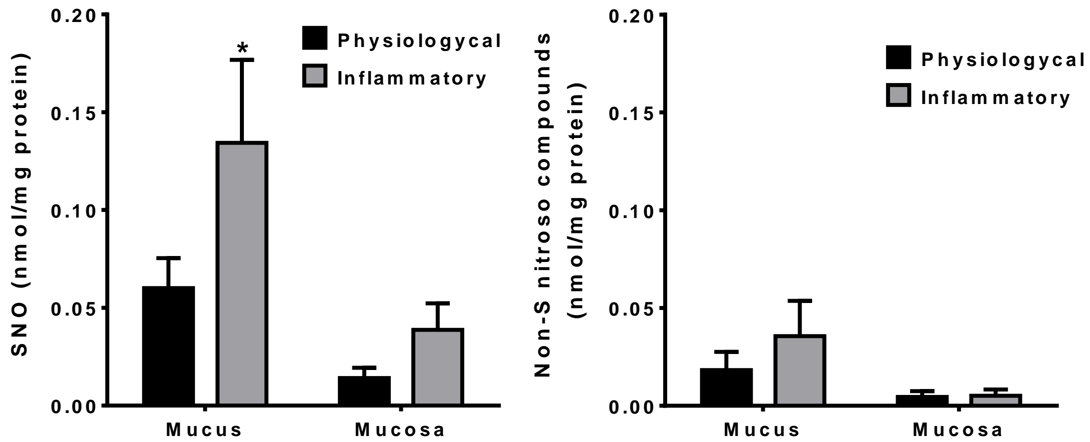


Figure 3.10 *In vivo* nitrosation in the presence of 1mM nitrite under physiological and inflammatory conditions. Values are mean ± S.E.M., n= 5-7.

3.2.11 Modulation of nitrite-induced gastric nitrosation with red wine under inflammatory conditions

The impact red wine on nitrosation pattern induced by nitrite under conditions of acute inflammation of the gastric lining was assessed. The nitrite/red wine mixture administrated under inflammatory conditions (Fig. 3.11) led to a reduction in the nitrosation extent, particularly in the S-nitrosothiols fraction, but with no statistically significant differences relatively to the case of nitrite alone.

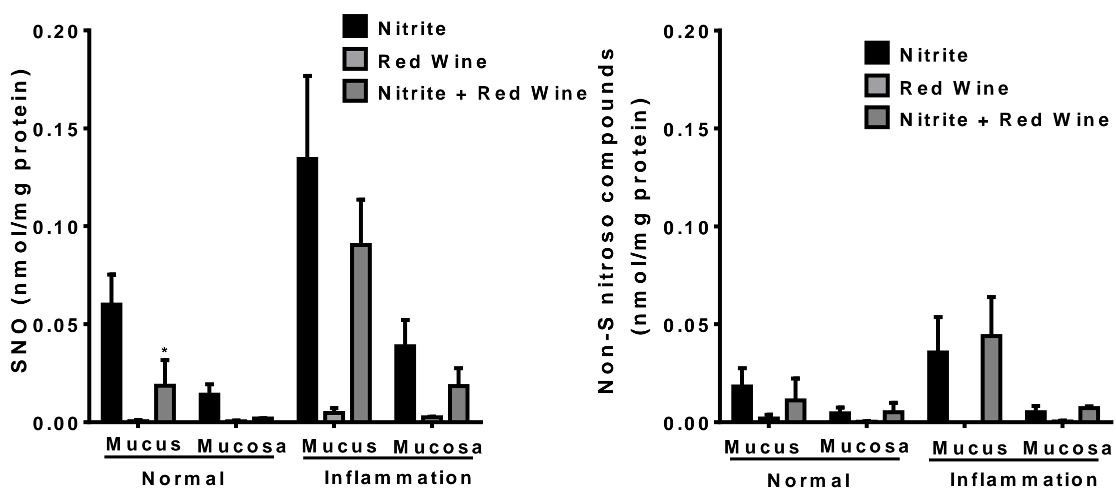


Figure 3.11 *In vivo* nitrosation in the presence of 1mM nitrite and modulation with red wine under physiological and inflammatory conditions. Values are mean ± S.E.M., n= 3-7.

3.3 Discussion

Nitrate and nitrite consumption in the diet unleashes a complex chemistry in the stomach, promoting the formation of •NO and reactive species that, in turn, may induce molecular modifications of endogenous and exogenous biomolecules with a functional impact, both locally and systemically [97, 109, 110, 187, 242]. Considering the potential formation of nitrosating species in the chemical mixture of nitrite under acidic and reductive conditions, we assessed if cysteine rich proteins in the mucus (mucins) could be preferential targets for nitrite-dependent nitrosation and, therefore, the mucus could act as a chemical barrier to nitrite and •NO-derived nitrosating species, protecting the underlying mucosa cells from potential harmful nitrosative stress.

A comprehensive approach using *in vitro*, *ex vivo* and *in vivo* models was implemented. The modulation of the chemical equilibria in the nitrosation pattern, both in the mucus and mucosa, was studied using red wine, in view of the redox properties of its polyphenolic fraction. Also, an *in vivo* acute inflammatory model was used in order to assess the gastric susceptibility to nitrite-dependent nitrosation under non-physiological conditions.

Initial studies involved the use of commercial porcine mucin in order to assess whether mucus proteins would be sensitive targets for nitrosation and, expectedly, the results showed that acidified nitrite was able to nitrosate mucin glycoproteins in a concentration dependent fashion. Although at lower nitrite concentrations, S-nitrosothiols formation, which is kinetically favoured, prevails over other nitroso compounds, [85, 166, 259, 260], the increase in all fractions is apparent with the increase of nitrite concentration.

Then, by using a more physiologically relevant approach - a rat whole stomach mounted in a diffusion chamber simulating the stomach compartment - it was observed that the gastric mucus glycoproteins exhibited a nitrosation pattern whose extent showed a direct correlation with the nitrite concentration for all the nitroso fractions analysed,

confirming the results previously obtained *in vitro*. In accordance with the fact that cysteine thiols are preferential targets for nitrosation [87, 166], the major fraction of nitroso compounds formed was the S-nitrosothiols for all nitrite concentrations. At a lesser extent other nitroso compounds were formed [262], among which N-nitrosamines emerged as the most relevant non-S nitroso species. Also, the underlying gastric mucosa cells were analysed for nitrite-derived nitrosation and the results showed a pattern qualitatively similar to that found in the mucus layer, but quantitatively lower. These results are consistent with the fact that $^{\bullet}$NO produced upon nitrite reduction at acidic/reductive conditions is able to cross the stomach wall, reaching deeper targets within the cell layers [252]. This is a relevant observation for it supports the notion that nitrite-derived chemistry in the stomach is not restricted neither to the lumen nor to the more superficial proteins of the mucus but protein modification and functional consequences can be found deep in the tissue. Removal of the gastric mucus layer improved the nitrite induced nitrosation of the gastric mucosa. Together, these results point to the fact that the mucus gel acts as barrier [180, 264-266] but is not fully effective against nitrite-derived reactive species.

The potential formation of carcinogenic N-nitrosamines in the stomach triggered by nitrite has been matter of concern by many made it be considered by many as a harmful agent (reviewed in [270, 271]), however, as highlighted in the general introduction studies failed to causally link nitrite intake and increased risk of gastric cancer. In fact, the physiological secretion of bicarbonate and ascorbic acid into the stomach is known to impair the formation of N-nitroso compounds, fostering the formation of $^{\bullet}$NO [272, 273]. Also, a decrease in the cases of gastric cancer has been observed with the increase in consumption of fruits and vegetables [274], probably due to their content in ascorbic acid and polyphenols [270]. Opposing to N-nitrosation, traditionally been linked to harmful effects, the formation of nitrosothiols has been proposed as a transducer of $^{\bullet}$NO signalling, as they can function as $^{\bullet}$NO donors. Accordingly, in 2003 the Joint FAO/WHO

declared that “Overall, the epidemiological studies showed no consistently increased risk for cancer with increasing consumption of nitrate”.

Red wine has two active components that are very likely to participate in the redox chemistry in the gastric compartment, namely ethanol and polyphenols. In fact, a reduction in the nitrosation extent of both gastric mucus and underlying mucosa was observed in the presence of these compounds. Our group shown previously that under gastric conditions ethanol is nitrosated to ethyl nitrite [245] and polyphenols can univalently reduce nitrite to $^{\bullet}NO$, resulting in the formation of the corresponding phenoxyl radical [147, 148]. In fact, we observed an increased production of $^{\bullet}NO$ in the presence of red wine. Under these conditions a radical:radical interaction between $^{\bullet}NO$ and the phenoxyl radical, yielding nitrosated species [275], is more prone to occur than the interaction of $^{\bullet}NO$ with O_2 yielding a nitrosative species that, in turn, would nitrosate endogenous proteins. Consequently, in the presence of red wine despite the increase in $^{\bullet}NO$ formation, a decrease in the nitrosation extent was expected. Hence, in the absence of red wine the nitrosation extent directly correlates to $^{\bullet}NO$ formation.

During digestion, the gastric mucus is removed and the mucins mixed with the diet components. At acidic pH, S-nitrosated mucins are relatively stable, but increasing the pH, simulating what occurs along the gastrointestinal tract, S-nitrosated mucins can act as $^{\bullet}NO$-donors, releasing $^{\bullet}NO$ in a pH depend manner, adding to the systemic effects of nitrite-derived $^{\bullet}NO$ [257].

An *in vivo* model was established by administrating 1mM nitrite via OG to Wistar rats. A nitrosation pattern similar to the *ex vivo* model was found in the mucus and mucosa layers, but at lower extent. Modulation of the nitrite chemistry in the gastric compartment was also studied by administration of a nitrite/red wine mixture, and as observed before, a reduction in the nitrosation pattern in both mucus and mucosa was observed, particularly for the S-nitrosothiols fraction. Together, the results obtained with

red wine illustrate the relevance of the diet content in the fate of nitrite in the gastric compartment and their impact in terms of nitrosative modifications.

Considering that different physiological/pathophysiological conditions may redirect the gastric chemistry such as, for instance, an achlorhydric stomach, where the neutral pH facilitates the formation of N-nitroso compounds (reviewed in [166]) an acute inflammatory model was established with diclofenac, a widely used NSAID. An increase in the nitrosation pattern was observed for both mucus and mucosa after the use of diclofenac when compared with physiological (normal) conditions. This increase is probably due to a higher number of targets for nitrosation as a consequence of the degradation of the gastric mucus and mucosa by diclofenac [197, 276]. The modulatory effect of red wine in the nitrosation pattern extent (as seen along this chapter) is impaired under inflammatory conditions. These results illustrate the importance of gastric mucosal integrity in the gastric protective system and the susceptibility for nitrosative stress.

The data discussed in this chapter supports that upon a nitrate/nitrite rich meal, the gastric mucus proteins undergo nitroso post-translational modifications, thus filtering in some extent nitrite-derived reacting species, that otherwise could potentially induce a nitrosative stress with pathobiological impact in the deeper layer of the gastric mucosa [166]. Accordingly, $^{\bullet}$NO-like effects of nitrite describe in the stomach such as regulation of blood flow and mucus generation [177] are plausibly to have a contribution from nitrosothiols. The associated ingestion of nitrite with other substances such as wine can orchestrate protein nitrosation in the gastric compartment and trigger the formation of fairly stable compounds, that may interfere with local signalling pathways or upon diffusion may can act as $^{\bullet}$NO carriers, exerting more systemic impact [257]. The redox-modulation by red wine, suggest novel actions for wine polyphenols *in vivo* via the balance of S- an N-nitroso compounds in the gastric wall.

4

Nitrite induced trefoil factor 1 expression in the gastric mucosa

4.1 Introduction

Dietary nitrite and its derivatives •NO and RNOS are now considered gastric physiological modulators with particular relevance in the regulation of mucus production and mucosal blood flow [177, 258], host defence against pathogens [175], and gastric ulcer protection [186]. From their interaction with other dietary components and endogenous biomolecules, molecular modifications can be induced with functional impact locally and systemically [109, 110].

In order to maintain mucosal integrity, the gastric mucosa has a well-established protection system revised in [194, 263] constituted by a mucus gel layer with a pH gradient, acidic secretion, a tight epithelium and an active mucosal blood flow [180, 264-266]. At the cellular level, the gastric mucosa cells produce active peptides involved in signalling mechanisms of protection, including the trefoil factors family of peptides (reviewed in [277-281]). Trefoil factor 1 (TFF1) is expressed in the normal stomach from the corpus to the pyloric sphincter, particularly in the superficial epithelium of the upper part of the pits, where new functional secreting mucosa is differentiated [282-284]. TFF1 features perinuclear accumulation within the cells cytoplasm and is secreted in the gastric juice [279, 283-285]. Ectopically, TFF1 can be found in gastrointestinal inflammatory disorders and several malignant carcinomas as reviewed in [285]. Interestingly, the absence of TFF1 is associated with the occurring of gastric cancer, pointing to a tumour suppression function [285-287].

TFF1 is implicated in the immediate recruiting of the restitution machinery in gastric mucosa upon an external aggression, promoting cell motility and regeneration (proliferation and differentiation) and loosening of the adherent junctions in order to restore mucosal integrity [285, 288]. TFF1 is also involved in the folding and packing of mucins that constitute the gastric mucus [289, 290] and has been described as a neuropeptide [291, 292].

Three molecular forms of TFF1 can be found in the gastric mucosa: monomer (~7 kDa), homodimer (~14 kDa) and a heterodimer, a complex formed by TFF1 and gastrokine 2 (GKN2) (~24 kDa), being the latter the most abundant [214, 289, 293-297]. The interaction with mucin MUC5AC, one of the most abundant in the mucus and secreted in the superficial gastric epithelium [298] is preferentially exerted by TFF1 homodimer and heterodimer [214, 289].

NSAIDs such as diclofenac are widely used pharmacological compounds known to cause gastrointestinal damage associated with hypoxia and inflammation via reducing of PGE2 synthesis and blood flow [276, 299]. As a response to hypoxia, an important regulator of transcription is activated, the hypoxia inducible factor-1 (HIF-1), that masters the cell response to hypoxic stress via a complex signalling network [300, 301]. At low oxygen tensions, the expression of trefoil factors, particularly TFF1 and TFF2, is increased via HIF-1, thus maintaining mucosal integrity [302]. HIF-1 activity is regulated through post-translational modifications and •NO has also been described as a HIF-1 stabilizer in both hypoxia and normoxia [303-305].

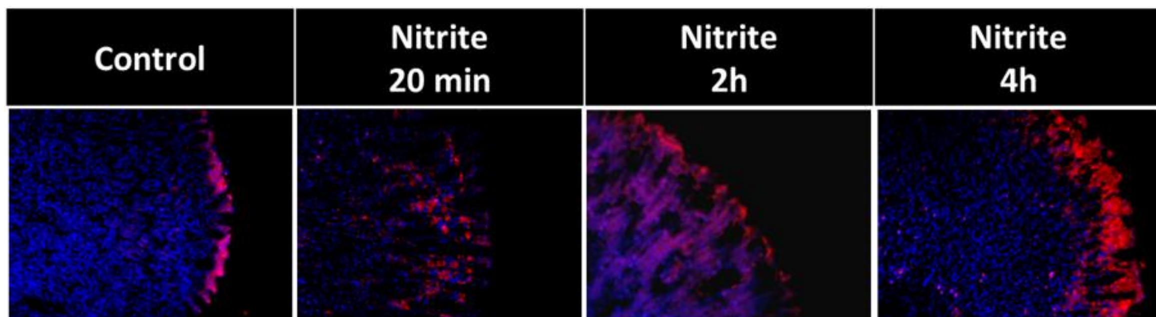
The results presented in this chapter reinforce the notion that dietary nitrite and its derivatives are relevant in gastric physiology contributing for the maintenance of gastric mucosal integrity via the stimulation of an important signalling peptide, TFF1, involved in the mucosal protection and anti-tumorigenesis.

4.2 Results

4.2.1 Nitrite-induced TFF1 expression in the gastric mucosa

The expression of TFF1 upon nitrite stimulus over time was assessed by immunohistochemistry and western blot analysis. A solution consisting of 1mM nitrite was given to the fasting rats by OG during 20 minutes, 2 hours and 4 hours.

In the immunohistochemistry assay (Fig. 4.1), the expression of TFF1 can be observed in the gastric epithelium in control conditions and upon 1mM nitrite exposure an increased expression in a time dependent fashion. Three unbiased observers analysed the TFF1 expression found by immunohistochemistry in the gastric mucosa. In figure 4.1 it can be observed an increase over time on the TFF1 expression particularly for the 2 hours incubation period ($p < 0.01$) and more significantly ($p < 0.001$) for the 4 hours incubation period. Hematoxilin & eosin staining was used to confirm the localization of TFF1 in the gastric mucosa and as a control for tissue damage (data not shown).



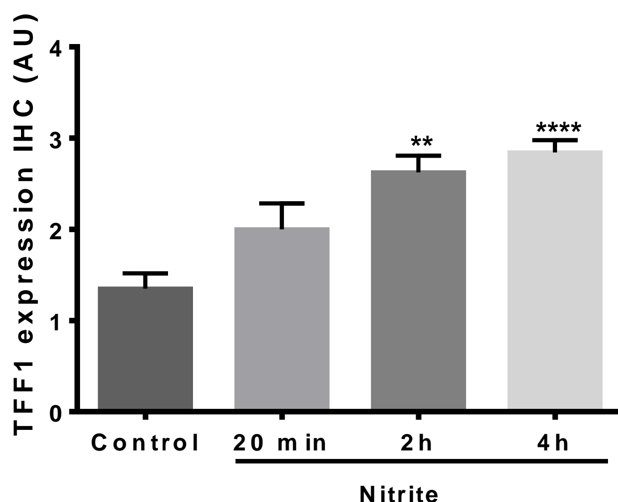


Figure 4.1 TFF1 expression in the gastric mucosa in the presence of nitrite over time: Immunohistochemical localisation of TFF1 (red) in the gastric mucosa superficial epithelium. Nucleic acids, blue. Magnification 200x (up). Below, analysis of TFF1 expression Values are mean ± S.E.M. (n = 7-9) ** p<0.01 and **** p<0.0001 relatively to control.

Considering that TFF1 can be found in the gastric mucosa in three forms (complex, dimer and monomer) a western blot analysis (Fig. 4.2) was performed in order to identify the TTF1 form responsible for the increased expression in the presence of nitrite. It was observed an increase over time of all the TFF1 forms, but more robustly the complex form. A 4 hour incubation with nitrite showed a significant increase in both the complex ($p < 0.05$) and the dimer forms relatively to control.

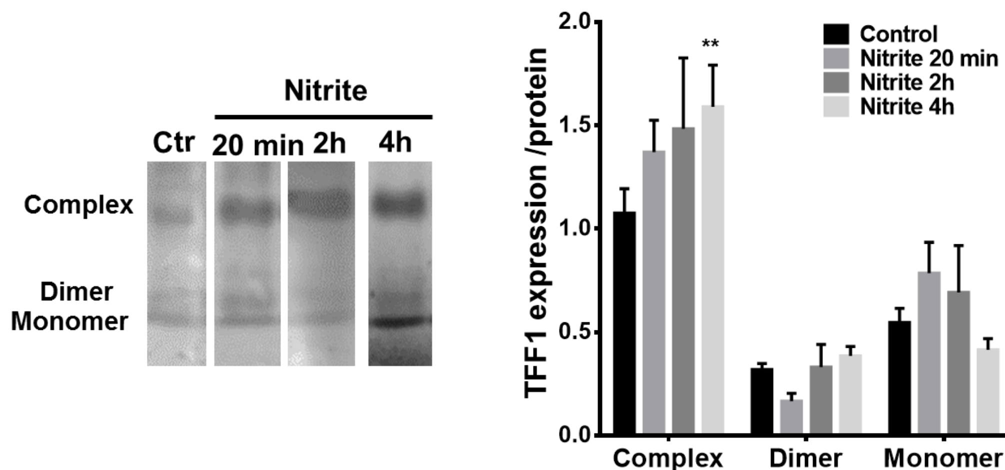


Figure 4.2 Western blot analysis of nitrite-induced TFF1 expression in the gastric mucosa. Left, western blot under non-reducing conditions. Control (Ctr) and 1 mM nitrite for 20 minutes, 2 and 4 hours. Values are mean ± S.E.M. (n = 4-9). ** $p<0.01$.

4.2.2 Nitrite induced TFF1 expression under inflammatory conditions

A blind analysis of the results for TFF1 expression obtained by immunohistochemistry, showed that under normal conditions TFF1 is expressed in the gastric mucosa, at the surface of the epithelium as described above and in the literature and also the expression increased in the presence of nitrite. Then, nitrite dependent TFF1 expression under inflammatory conditions was assessed after administration of diclofenac 30 mg/Kg by OG for 4 hours.

Using immunohistochemistry analysis (Fig 4.3) an increase in TFF1 expression under inflammatory conditions relatively to physiological conditions was observed with statistical significance ($p < 0.05$). 1 mM nitrite was administrated under physiological and inflammatory conditions for 4 hour period. Under physiological conditions (as shown

above) nitrite is able to stimulate TFF1 expression in the gastric mucosa with statistical significance ($p < 0.001$). Likewise, the inflammatory conditions *per se* also induced an increased expression but nitrite exerted no further significant changes in the expression of TFF1 under inflammatory conditions.

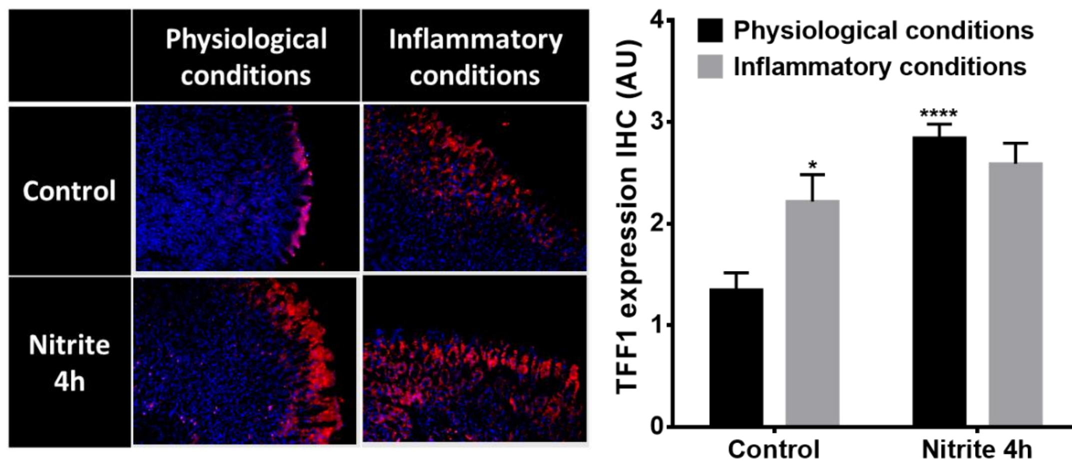


Figure 4.3 TFF1 expression in the gastric mucosa in the presence of nitrite under physiological and inflammatory conditions: Immunohistochemical localisation of TFF1 (red) in the gastric mucosa superficial epithelium. Nucleic acids, blue. Magnification 200x (left). Right, analysis of TFF1 expression. Values are mean ± S.E.M. (n = 7-9) * p<0.05 and **** p<0.0001 relatively to control (physiological conditions).

Western blot analysis (Fig 4.4) show similar results to immunohistochemistry. Diclofenac induces an increase in TFF1 expression compared to physiological control in all three TFF1 forms. However nitrite is not able to significantly modify the peptide expression under inflammatory conditions in neither forms.

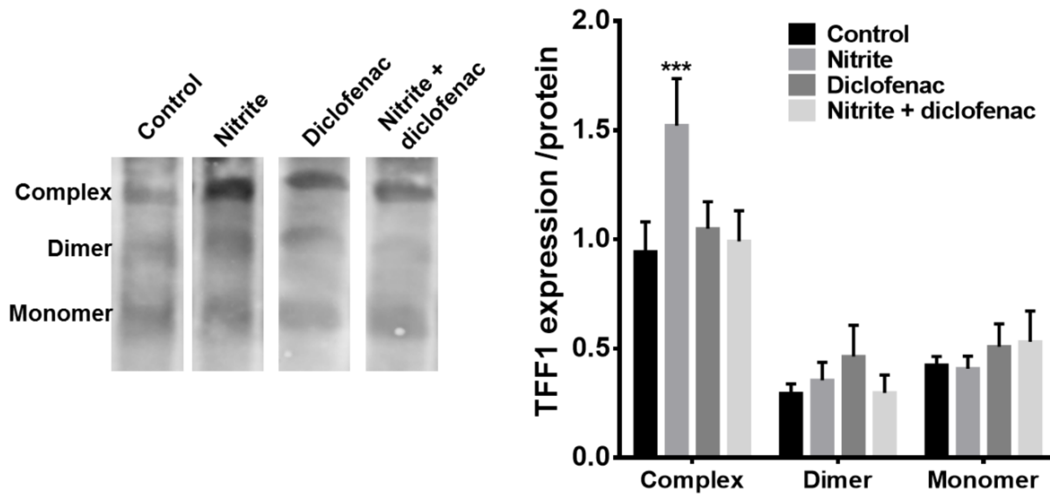


Figure 4.4 Western blot analysis of nitrite-induced TFF1 expression in the gastric mucosa under physiological and inflammatory conditions. Left, western blot under non-reducing conditions. Values are mean ± S.E.M. (n = 4-9). *** p<0.001, relatively to control.

4.2.3 Modulation of the nitrite induced TFF1 expression by red wine under physiological and inflammatory conditions

Considering the impact of red wine components, namely polyphenols in the nitrite chemistry in the gastric compartment, the effect of red wine and nitrite/red wine mixtures in TFF1 expression in the gastric mucosa was assessed both under physiological conditions and the diclofenac inflammatory model.

Immunohistochemistry analysis (Fig. 4.5) show that neither a nitrite/red wine mixture nor red wine alone has a significant impact in TFF1 expression when compared to nitrite alone and the control, respectively. Nevertheless when used diclofenac to induced inflammation, an increase in TFF1 expression was observed for the nitrite/red wine mixture.

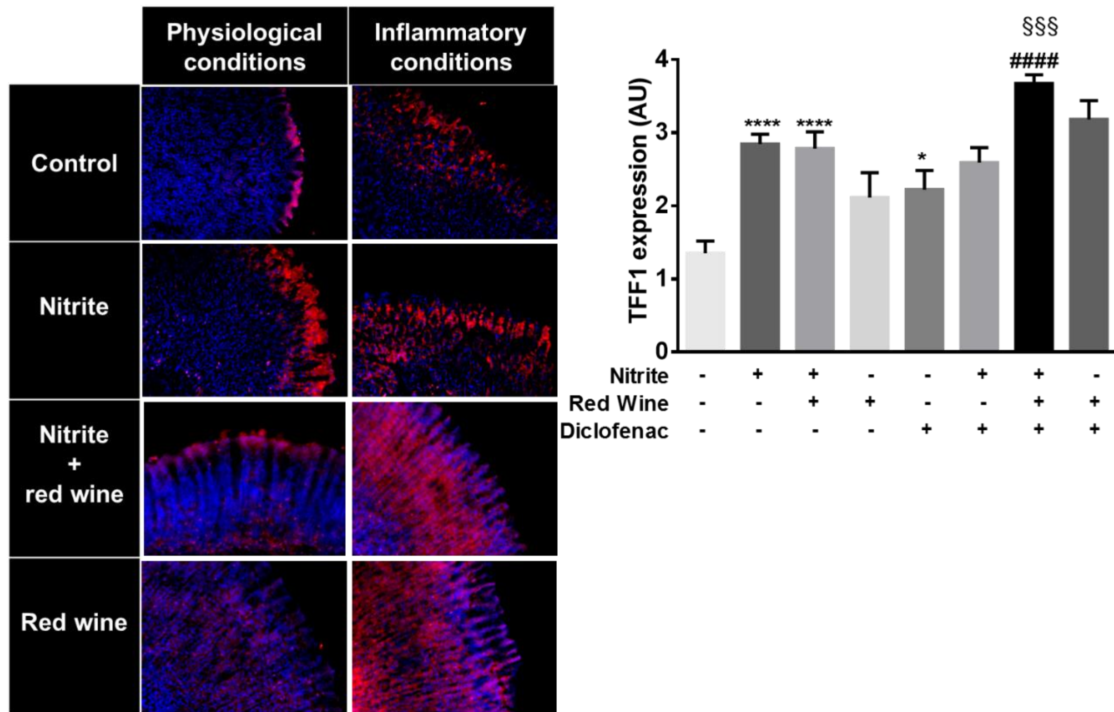


Figure 4.5 TFF1 expression in the gastric mucosa in the presence of nitrite under physiological and inflammatory conditions and modulation with red wine: Immunohistochemical localisation of TFF1 (red) in the gastric mucosa superficial epithelium. Nucleic acids, blue. Magnification 200x (left). Right, analysis of TFF1 expression Values are mean ± S.E.M. (n = 2-9) * $p<0.05$ and **** $p<0.0001$ relatively to control (physiological conditions), #### $p<0.0001$ relatively to diclofenac, §§§ $p<0.001$ relatively to nitrite + diclofenac.

Using western blot assay (Fig 4.6) to better understand the impact of red wine in TFF1 expression some differences were found comparing physiological and inflammatory conditions. Regarding the TFF1 complex form the 10% red wine solution alone and the nitrite/red wine mixture induced a slightly increase in the TFF1 expression relatively to the control, albeit without statistical significance. In the case of TFF1 dimer no differences were observed in the presence of red wine or red wine/nitrite mixture relatively to the control. Finally, in the case of the monomer form of TFF1 the results show a decrease in the expression in the presence of nitrite/red wine mixture but with no statistically significant difference.

The impact of red wine components in TFF1 expression in the gastric compartment was also assessed under inflammatory conditions. Contrary to what occurred under physiological conditions and in tune with the observed in immunohistochemistry a mixture

of nitrite and red wine significantly increased TFF1 monomer in the gastric tissue ($p < 0.05$).

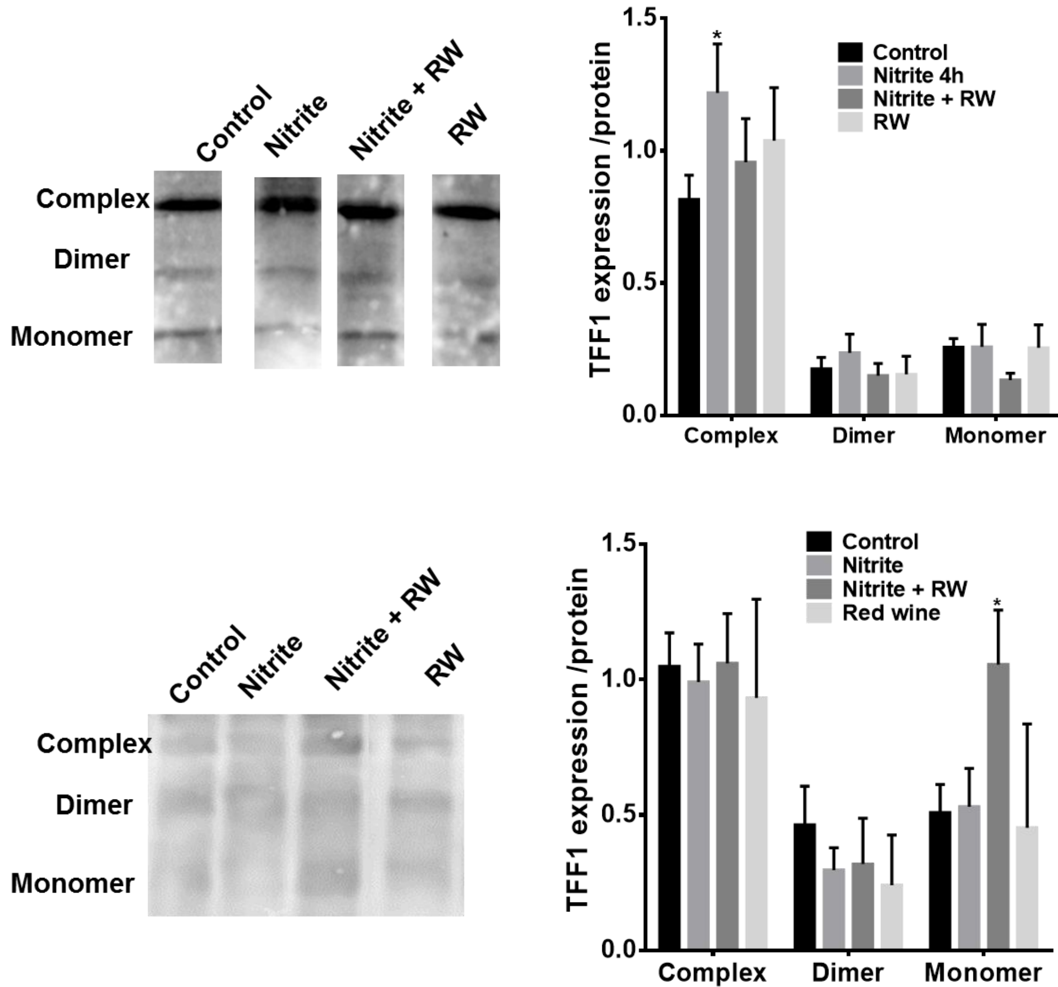


Figure 4.6 Western blot analysis of nitrite induced TFF1 expression in the gastric mucosa under physiological (up) and inflammatory conditions (below): modulation with red wine. Left, western blot under non-reducing conditions. Values are mean ± S.E.M. (n = 2-9). * p<0.05, relatively to control.

4.3 Discussion

Dietary nitrite and its derivatives, such as •NO, are implicated in several pathways that underlie the gastric physiology [175, 177, 186, 258]. The modulation of gastric environment during inflammation or the interference with local nitrite chemistry by red wine [148, 188, 245] can lead to alterations in gastric signalling pathways. Firstly, we assessed the impact of nitrite in the expression of a trefoil family peptide involved in gastric protection and regeneration, the TFF1, under physiologic and inflammatory conditions. Secondly, the effect of red wine under the same previous conditions was studied. TFF1 is physiologically expressed in the gastric epithelium in association with mucin secretion, being key in mucus proteins folding, packing and function [289, 290].

Overall, the results in this chapter support that dietary nitrite can induce TFF1 expression in the gastric mucosa and that red wine modulate such an expression, particularly under inflammatory conditions.

Using an *in vivo* animal model and nitrite amounts that mirror the ones ingested upon a nitrate/nitrite rich meal [143], we found that 1mM nitrite is able to induce the expression of TFF1 in a time dependent fashion in the gastric mucosa in particular the heterodimer (TFF1-GKN2) form of TFF1 [296, 297, 306].

Dietary derived nitrite increases mucus secretion [177] in the stomach contributing for an efficient defence system [263, 307]. The trefoil factor peptides are expressed mostly by cells which synthetize and secrete mucins [284, 289]. TFF1 is synthetized in surface mucous cells of the normal gastric epithelium typically in association with one of the major gel-forming mucins, MUC5AC [284, 289, 298]. Therefore, these result show that dietary nitrite enhances mucosal protection via TFF1 induced expression in the normal gastric mucosa.

The use of diclofenac to induce inflammation and gastric damage *in vivo*, allowed the study of the impact of dietary derived nitrite in the TFF1 expression under acute inflammatory conditions. NSAIDs like diclofenac are known to cause gastrointestinal

damage by reducing gastric PGE2 levels and blood flow leading to hypoxia and inflammation [276]. In response to NSAID- induced hypoxia, HIF-1 activation has been reported to occur in both, rat and human models [304, 305]. HIF-1 activation mediates TFF1 and TFF2 expression in gastric epithelial cells initiating the gastric mucosal restitution process [302]. The diclofenac model implemented has permitted to detect that an increased TFF1 expression also occurs under inflammation.

Several cell models have supported the involvement of $^{\bullet}$NO in the modulation of HIF-1 activity [303, 308-312]. Additionally, *in vivo* models have shown the involvement of iNOS-derived $^{\bullet}$NO in HIF-1 activation and subsequent TFF expression in gastric restitution upon NSAID induced damage [304]. Our immunohistochemistry results support that dietary nitrite is able to induce TFF1 expression under both physiological and inflammatory conditions, but only with statistical significance in the first case. In turn, the western blot results show that the major contributor for the TFF1 increase is the monomer form.

Modulation of the nitrite chemistry in the gastric compartment by red wine components such as polyphenols has been described by our group, as well as by others [147, 148]. Red wine polyphenols promote a univalent reduction of nitrite to $^{\bullet}$NO resulting in a higher $^{\bullet}$NO production in the stomach [148, 313]. Here, we have observed that red wine *per se* or red wine in a mixture with nitrite induced no significant changes in the TFF1 expression under physiological conditions relatively to the nitrite alone. However, under inflammatory conditions, the nitrite/red wine mixture lead to a significant increase in TFF1 expression comparing with both control and nitrite alone. To this increase contributed mostly the TFF1 monomer, as can be observed in figure 4.6.

In sum, we can conclude that dietary nitrite, at physiologic concentrations, can increase TFF1 expression in the gastric mucosa in a time dependent manner. However, under inflammatory conditions, a condition in which mucosal blood flow is compromised and hypoxia is operative, the action of nitrite is less obvious in terms of increasing TFF1

expression, in part because inflammation *per se* induces an increase of TFF1 expression. The activity of nitrite is modulated by the presence of red wine. Of particular relevance is that under inflammatory conditions the simultaneous presence of nitrite and red wine induces an increased expression of TFF1, significantly higher than that promoted by nitrite alone. Overall, these results might contribute to open novel approaches to modulate gastric inflammation via the diet.

5

Influence of dietary nitrite on gastric mitochondrial function

5.1 Introduction

The gastric mucosa is a complex and active system that endures daily challenges from diet components and its metabolites. A well-established protective system along with a rapid self-regeneration are key to the maintenance of gastric mucosal function and integrity [194]. The energy required to these processes is in part derived from gastric mitochondria, and of particular relevance is the gastric acid secretion by parietal cells (which are endowed with high mitochondrial content) [191, 314] stimulated by food intake and the consequently maintenance of the acidic environment [315].

Mitochondria is a well characterized target for •NO and the most sensitive and widely studied target for •NO is the terminal enzyme of the electron transport chain (ETC), cytochrome c oxidase (CcOX). At low concentration, •NO binds reversibly to CcOX, leading to a partial inhibition of mitochondrial respiration [41]. The binding is competitive with oxygen and, thus, •NO may regulate tissue oxygen gradients, along with the formation of signalling reactive oxygen species (ROS) [15]. •NO has been also implicated in mitochondrial biogenesis through a cGMP-dependent mechanism [316]. Nitrite might trigger similar effects to those exerted by •NO since proteins from the ETC, namely complexes III and IV, might reduce nitrite to •NO particularly under hypoxic conditions [117, 153-155]. The univalent reduction of nitrite will ensure •NO generation and signalling to a wide spectrum of biological responses such as hypoxic vasodilation, stimulation of angiogenesis, modulation of glucose metabolism, increase of exercise efficiency, regulation of mitochondrial function and tolerance to ischemia/reperfusion (I/R), as reviewed in [156]. However, it has been claimed that nitrite may exert intrinsic signalling effects in mitochondria, particularly during reperfusion injury, via nitrosation of mitochondrial complex I [161], by stimulation of hypoxic mitochondrial biogenesis via nitrite-dependent activation of AMP kinase, Sirtuin-1, PPARg-coactivator-1a, as well as via upregulation of mitochondrial transcription factors [165].

As described in previous chapters, in the stomach, high amounts of nitrite and nitrite-derived redox species including •NO, can be found after a nitrate/nitrite rich meal via the nitrate-nitrite-•NO pathway and the enterosalivary recirculation of nitrate [119, 120, 130, 257]. Other dietary components, particularly those with reduction properties, such as polyphenols contained in red wine, are known to have implications in the nitrite chemistry, enhancing •NO formation under the gastric acidic conditions that, in turn, is able to cross cellular membranes [147, 148, 188, 252, 313] and modify key proteins involved in signalling pathways.

Gastric mitochondria, are therefore, potential targets for dietary-derived nitrite and •NO. Hence and this chapter aims to provide insight on how gastric mitochondria deals with the high concentration of these species, avoiding deleterious implications for gastric physiology and what is the impact in the respiratory function. For this purpose, an experimental setup was implemented in which mitochondria isolated from gastric mucosa and mucosal tissue were challenged with both •NO and nitrite, simulating the exposure after a nitrate/nitrite intake.

5.2 Results

5.2.1 Characterization of the gastric mucosa mitochondrial function

Isolated mitochondria from rat gastric mucosa were studied in comparison with liver mitochondria since the later has been used extensively in mitochondrial studies and may be considered a standard against which mitochondria metabolism can be compared. Isolation of functional mitochondria from the gastric mucosa is a low yield and difficult process. A protocol of substrates and inhibitors was used in order to assess rate of the oxygen consumption associated with the activity of each mitochondrial complexes (Fig. 5.1). Baseline respiration in the absence of exogenous substrates is significantly lower for gastric mitochondria as compared with liver ($p < 0.05$). State 2 (respiration in the presence of glutamate and malate) and state 3 (saturating levels of ADP) respiration specific for complex I are similar in both samples. At this point, outer mitochondrial membrane integrity was assessed by the addition of exogenous cytochrome c (not shown in the figure). Since cytochrome c cannot penetrate an intact outer mitochondrial membrane, the absence of a stimulatory effect on O_2 consumption rates (OCR) is indicative of a high quality preparation. Samples with cytochrome c induced OCR increments over 20% were discarded [317, 318]. State 3 respiration for both complexes I and II obtained by succinate addition, evidenced a higher OCR ($p < 0.05$) in the case of gastric mitochondria. In the presence of rotenone, a complex I inhibitor used to abolish electron input through complex I and obtain a complex II-dependent respiration, gastric mitochondrial inhibition was less pronounced than that of liver mitochondria.

State 4 respiration is conveniently achieved by the inhibition of ATP synthase with oligomycin, blocking the proton channel and eliminating ATP synthesis. The residual oxygen consumption in the absence of ADP phosphorylation is mostly attributable to proton leak across the inner mitochondrial membrane or uncoupled respiration. Gastric mitochondria has a higher rate of oxygen consumption under oligomycin-inhibited respiration than liver mitochondria ($p < 0.05$). An uncoupled respiratory state was

achieved with stepwise titrations of FCCP in order to dissipate the proton gradient across the inner mitochondrial membrane and a higher FCCP-induced OCR in gastric than in liver mitochondria ($p < 0.05$) was observed. Non-mitochondrial respiration was measured adding antimycin A that inhibits cytochrome c reduction in the complex III. Isolated gastric mitochondria evidenced higher non-mitochondrial O_2 consumption than liver ($p < 0.05$). Stimulation of complex IV as an isolated step was achieved adding artificial electron donors to reduce cytochrome c, namely ascorbate plus TMPD, shown no difference between samples.

The respiratory control ratios (RCR) were calculated for both gastric and liver mitochondria. The results showed that gastric mitochondria have a lower RCR than liver mitochondria (4.36 ± 0.365, n=40 and 6.81 ± 0.870, n=20, respectively, $p < 0.05$) but both preparations showed good quality.

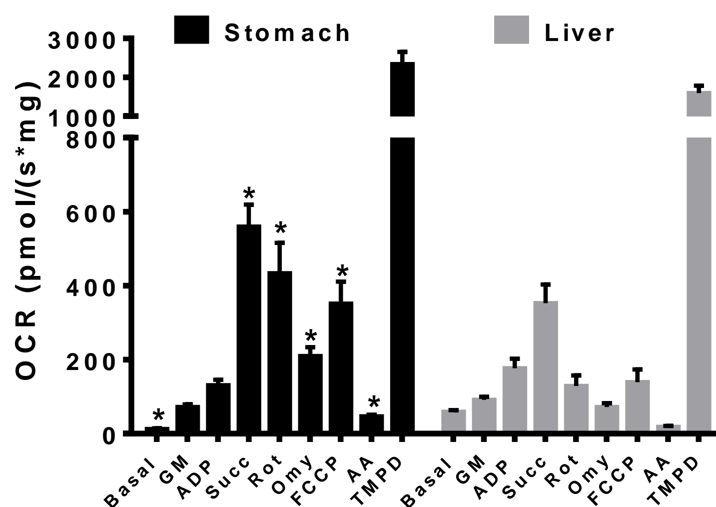


Figure 5.1 Characterization of the respiratory function in isolated mitochondria from stomach and liver. Oxygen consumption rates (OCR) during a stepwise protocol for functional assessment of isolated mitochondria. Basal, baseline mitochondrial respiration; GM, substrates glutamate and malate; ADP, saturating ADP; Succ, succinate; Rot, rotenone; Omy, oligomycin; FCCP, uncoupler; AA, antimycin A; TMPD, TMPD and ascorbate injection. Values are mean ± S.E.M. (n = 15-25 for the stomach and n = 10-15 for the liver). * $p<0.05$, relatively to liver.

In order to better illustrate the complexity of the mitochondrial respiration within the tissue, gastric and liver saponin-permeabilized biopsies were used and the analysis of the

respiratory chain function is summarized in table 5.1. The protocol of substrates, inhibitors and uncoupler used is described in the Methods and Materials chapter (section 2.2.18). For the gastric mucosa, the respiratory rates OCR_{GM}, OCR_{Succ}, OCR_{CcOX} and OCR_{FCCP} mean values were lower than in liver biopsies. In the gastric sample complex I-dependent respiration exceeded complex II-dependent respiration, pointing to a limitation in the electron flow at the level of complex II, where in liver biopsies the opposite was observed. The gastric mucosa presents lower RCR (2.420 ± 0.140 versus 3.223 ± 0.441, $p < 0.01$) which can be attributable to lower OCR_{GM} and OCR_{Succ}, although the lower proton leak.

Table 5.1: Characterization of respiratory parameters of rat gastric mucosa and liver biopsies. The rates of respiration (OCR) are expressed in pmol O_2/sec/mg protein. OCR_0—basal respiration without ADP or ATP; OCR_{GM}—ADP-stimulated respiration in the presence of glutamate and malate (indicating the function of the respiratory chain complex I); RCR_{GMt}—respiration control ratio calculated as OCR_{GM}/ OCR_0; OCR_{Succ}—ADP stimulated respiration in the presence of rotenone and succinate (characterize function of complex II); OCR_{CAT}—respiration after inhibition of succinate-stimulated respiration by carboxyatractyloside; RCR_{Succ}—ratio of OCR_{Succ}/ OCR_{rot}; Proton leak—measured indirectly as a state 4 respiration equal to difference between the respiration rates with carboxyatractyloside and antimycin A; OCR_{CcOX}—the respiratory equivalent of CcCOX (complex IV) activity calculated as [$OCR_{CcOX} = OCR_{TMPD} – OCR_{TMPD+NaN3}$] where OCR_{TMPD} and $OCR_{TMPD+NaN3}$ are TMPD-stimulated respiration rates before and after addition of NaN_3; OCR_{FCCP}— respiration in the presence of an uncoupler; RCR, total respiratory control ratio calculated as ADP stimulated OCR of complex I+II / OCR_{CAT}. The values are mean ± S.E.M. and n = number of specimens used. ** p<0.01, *** p<0.001, **** p<0.0001, relatively to liver.

Parameter	Stomach (N = 15 - 17)	Liver (N = 4 - 8)
OCR_0	6.174 ± 0.329	7.736 ± 0.609
OCR_{GM}	7.646 ± 0.528****	13.191 ± 1.384
RCR_{GM}	1.275 ± 0.041*	1.651 ± 0.100
OCR_{Succ}	7.234 ± 0.371****	14.820 ± 1.428
OCR_{CAT}	5.563 ± 0.411	6.997 ± 1.119
RCR_{Succ}	2.334 ± 0.106**	3.425 ± 0.401
OCR_{AA}	3.271 ± 0.155	4.352 ± 0.666
Proton leak	1.849 ± 0.277	2.568 ± 0.612
OCR_{CcOX}	9.935 ± 0.565****	21.302 ± 5.161
OCR_{FCCP}	14.719 ± 2.280****	24.466 ± 4.464
RCR	2.404 ± 0.139**	3.223 ± 0.441

5.2.2 Nitric oxide impact in gastric mitochondrial respiration

The gastric mucosa is daily confronted with high concentrations of dietary nitrite-derived $^{\bullet}$NO that can cross cellular membranes and is known to compete with oxygen for cytochrome c oxidase, leading to the inhibition of mitochondrial respiration.

Isolated mitochondria and tissue biopsies from gastric mucosa and liver (the later used for comparison) were challenged with a 10 and 20µM $^{\bullet}$NO solution (concentration that can be achieved in the gastric compartment upon a nitrate/nitrite rich meal) and the percentage and duration of the OCR inhibition were quantified.

In isolated mitochondria (Fig. 5.2, A and B), the $^{\bullet}$NO bolus induced transient OCR inhibition with a maximum of about 75% for gastric mitochondria and 55% for liver mitochondria, for both $^{\bullet}$NO concentrations used, with statistically significant difference for 10µM. The duration of the OCR inhibition shown to be dependent on $^{\bullet}$NO concentration (about 6 minutes for 10 µM and 7 minutes for 20 µM) but with no significant difference between gastric and liver mitochondria.

Using tissue biopsies of gastric mucosa and liver as a more physiological approach, we assessed the impact a $^{\bullet}$NO bolus in OCR (Fig. 5.2, C and D). Overall, both OCR inhibitions and its duration increased with $^{\bullet}$NO concentration for both tissues, and lower OCR inhibition percentages and duration were observed when compared with isolated mitochondria in the same conditions. Considering 10 µM $^{\bullet}$NO the gastric biopsies show lower OCR inhibition than liver biopsies (39.14 ± 6.66 %, n=6 and 44.32 ± 9.82 %, n=6, respectively). The duration of the OCR inhibition was slightly lower in the gastric biopsies than in liver biopsies (8.61 ± 1.80 min, n=6 and 8.79 ± 1.96 min, n=6, respectively). However, no significant differences were observed between gastric and liver mitochondria in both percentage of OCR inhibition and OCR inhibition duration.

Increasing the $^{\bullet}$NO concentration to 20uM, the tendencies observed before in both parameters become statistically different ($p < 0.05$). The inhibition of OCR by 20uM $^{\bullet}$NO was 46.95 ± 4.85 % (n=7) and lasted for 9.79 ± 0.87 min (n=7) in the gastric biopsies,

whereas in the case of liver biopsies was 65.61 ± 5.19 % (n=7) and lasted for 12. 91 ± 1.52 min (n=7). Therefore, upon a challenge of 20uM of •NO, gastric mitochondria are able to cope more efficiently with •NO, maintaining a higher respiration rate, as reflected in a lower percentage of OCR inhibition and a faster recovery from the OCR inhibition than liver mitochondria.

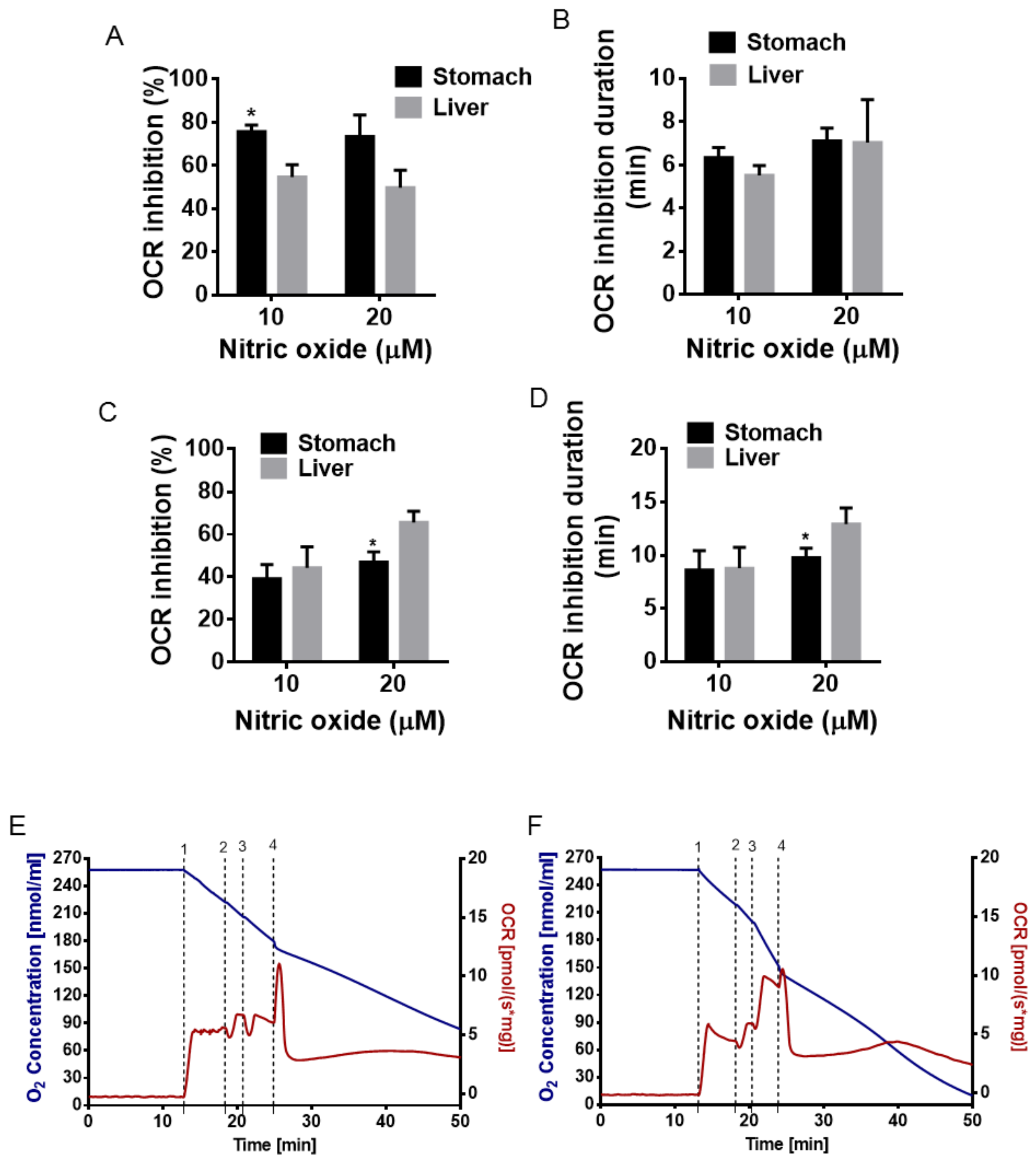


Figure 5.2 Effect of •NO in mitochondrial respiration. A and B, Isolated mitochondria from stomach mucosa and liver. C and D, fresh collected biopsies of gastric mucosa and liver. E and F, Representative recording of mitochondrial respiration on fresh collected biopsies of gastric (E) and liver (F) tissue. Additions: 1.Mitocochondria, 2. 10mM succinate, 3. 2mM ADP, 4. 20uM •NO. C, comparing gastric and hepatic mitochondrial respiratory rate inhibition upon •NO injection. Values are mean ± S.E.M. (n = 6-7). * $p<0.05$.

5.2.3 Impact of nitrite and red wine on gastric mitochondrial function *ex vivo*

Considering the effect of a •NO in the respiratory function of gastric mitochondria, the impact of nitrite, simulating the amounts found in the gastric compartment after a nitrate/nitrite rich meal, was assessed. Also, a nitrite plus red wine mixture was used considering the modulatory effect of the latter in the nitrite chemistry in the stomach.

As a first approach, rat stomach strips were exposed to 1mM sodium nitrite, a 1mM nitrite plus 10% red wine mixture and a 10% red wine solution, under simulated gastric conditions for about 20 min. The following controls were performed: nitrite vehicle, 10% red wine solution, and 1.3% ethanol solution. The respiratory parameters were assessed in saponin-permeabilized biopsies from the strips and represented in figure 5.3 A and B. Although some parameters show some variances, no statistically significant differences were observed between conditions except for the uncoupled respiration obtained with the addition of FCCP, in which lower OCR_{FCCP} was observed for all conditions compared with the control (control versus nitrite $p < 0.001$, control versus nitrite plus red wine $p < 0.01$ and control versus red wine $p < 0.05$). Along with the respiratory parameters, the overall oxidants production in the gastric mucosa cells was assessed by DCF assay. Gastric mucosa strips homogenates generated fluorescence when incubated with H_2DCF-DA. The results obtained shown no differences between groups under the experimental conditions.

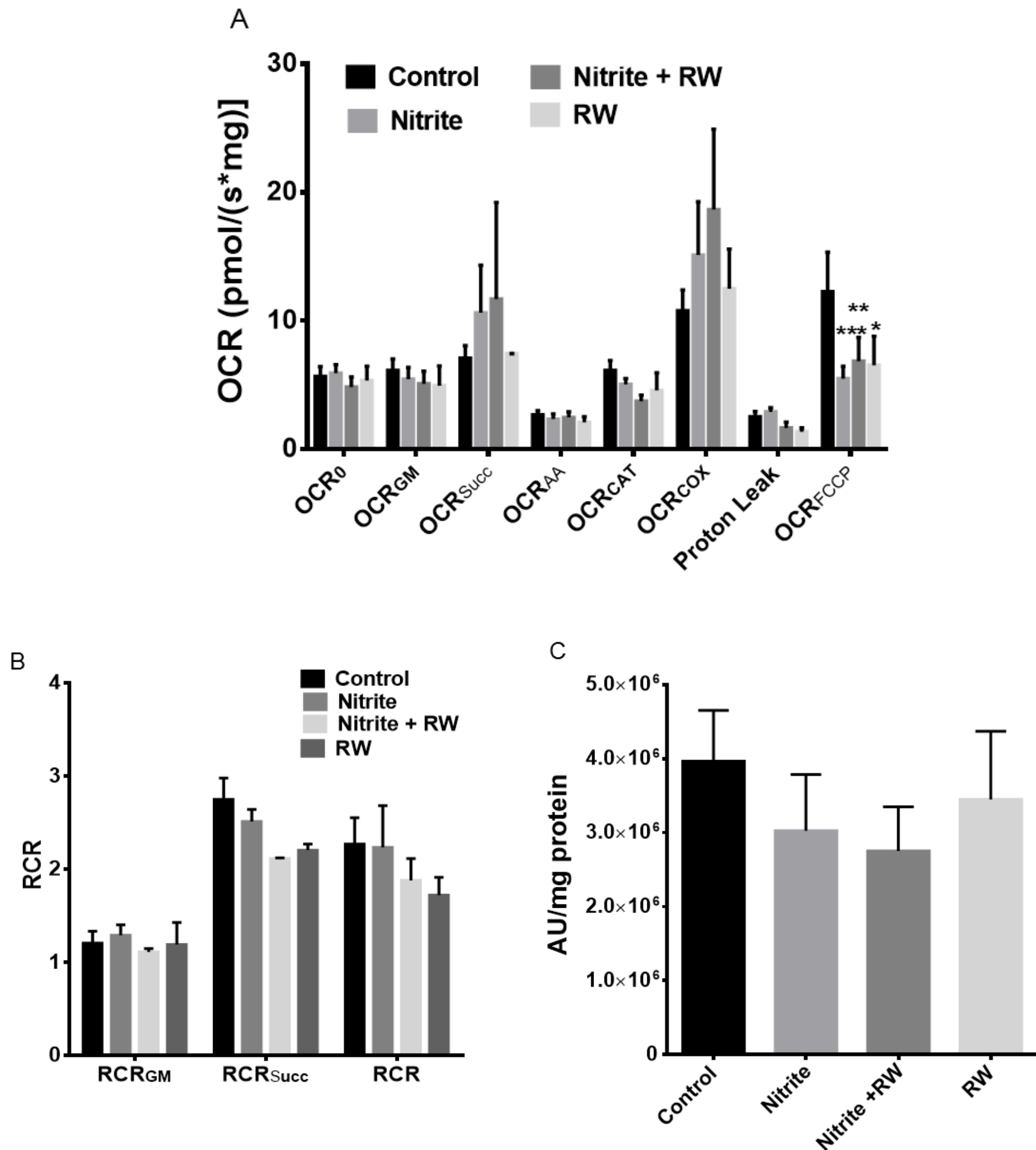


Figure 5.3 Characterization of mitochondrial respiratory parameters in gastric corpus mucosa biopsies of nitrite and/or red wine challenged gastric strips. A. OCR_0—basal respiration without ADP or ATP; OCR_{GM}—ADP-stimulated respiration in the presence of glutamate and malate (indicating the function of the respiratory chain complex I);; OCR_{Succ}—ADP stimulated respiration in the presence of rotenone and succinate (characterize function of complex II); OCRAA – respiration after inhibition with antimycin A; OCR_{CAT}—respiration after inhibition of succinate-stimulated respiration by carboxyatractyloside; OCR_{CcOX}—the respiratory equivalent of cytochrome oxidase (complex IV) activity calculated as [$OCR_{CcOX} = OCR_{TMPD} – OCR_{TMPD+NaN3}$] where OCR_{TMPD} and $OCR_{TMPD+NaN3}$ are TMPD-stimulated respiration rates before and after addition of NaN_3; Proton leak—measured indirectly as a state 4 respiration equal to difference between the respiration rates with carboxyatractyloside and antimycin A; OCR_{FCCP}— respiration in the presence of an uncoupler. B. RCR_{GMt}—respiration control ratio calculated as OCR_{GM}/ OCR_0 ; RCR_{Succ}—ratio of OCR_{Succ}/ OCR_{CAT}; RCR – total respiratory control ratio calculated as ADP stimulated OCR of complex I+II / OCR_{CAT} C. DCF fluorescence (arbitrary units) per mg of protein. The values are mean ± S.E.M. and n = 4 - 6, * p<0.05, ** p<0.001, *** p<0.001.

5.2.4 Impact of nitrite and red wine on gastric mitochondrial function *in vivo*

Nitrite (1mM) and/or red wine (10% solution) were administrated i*n vivo* by OG to Wistar rats, for 4 hours. Afterwards, saponin-permeabilized biopsies were obtained from each group and used to assess the respiratory parameters. The results are shown in figures 5.4 A and B.

The biopsies obtained from the 1mM nitrite group showed an increase in several respiratory parameters namely, OCR_0, OCR_{GM}, $OCR_{Succ,}$ OCR_{AA} and OCR_{CcOX} ($p < 0.05$) and decrease in the OCR_{CAT} and the proton leak (consequently). However, the groups challenged with nitrite/ red wine mixture and a 10% red wine solution alone showed a profile similar to the control for most parameters except for OCR_{CcOX} ($p < 0.05$ and $p < 0.01$) and OCR_{FCCP} which were markedly decreased when compared to control ($p < 0.0001$). OCR_{Succ} and OCR_{CAT} also showed small decreases.

The RCR, as representative of the phosphorylation capacity of the mitochondrial biopsies, showed a significant increase in the nitrite challenged group ($p < 0.01$) and a decrease in the other two groups with statistical significance for the red wine group ($p < 0.05$). Considering the complex I and II separately, the RCR_{GM}, which translate the activity of complex I, shows an increase in the nitrite and nitrite plus red wine groups (the latter with statistical significance $p < 0.05$) relatively to the control, however, RCR_{Succ}, which translate the activity of complex II, shows an decrease in the two groups with red wine, compared to control.

A thermodynamic approach was used as an alternative to estimate mitochondria respiratory efficiency, applying the following equation:

$$q = \sqrt{1 - \frac{state\ 4\ cat}{state\ 3u}}$$

The q value represents the thermodynamic coupling of mitochondria [319], state 4 cat is the equivalent to state 4 respiration in presence of carboxyatractyloside and state 3u is the fully uncoupled respiration after FCCP. The q value increased from 0.7820 to 0.818

with nitrite and decreased significantly to 0.561 with nitrite and red wine mixture and to 0.489 with red wine alone. Since there was no difference in FCCP-induced respiration, the improvement in thermodynamic efficiency was mostly due to lower OCR_{CAT}, in the nitrite challenged group.

As before, along with the respiratory parameters, the oxidant environment in the gastric mucosa cells was assessed by DCF assay. The results obtained showed a decrease of oxidants in the gastric cells exposed to nitrite and nitrite/red wine mixture which correlates with the results for RCR_{GM}.

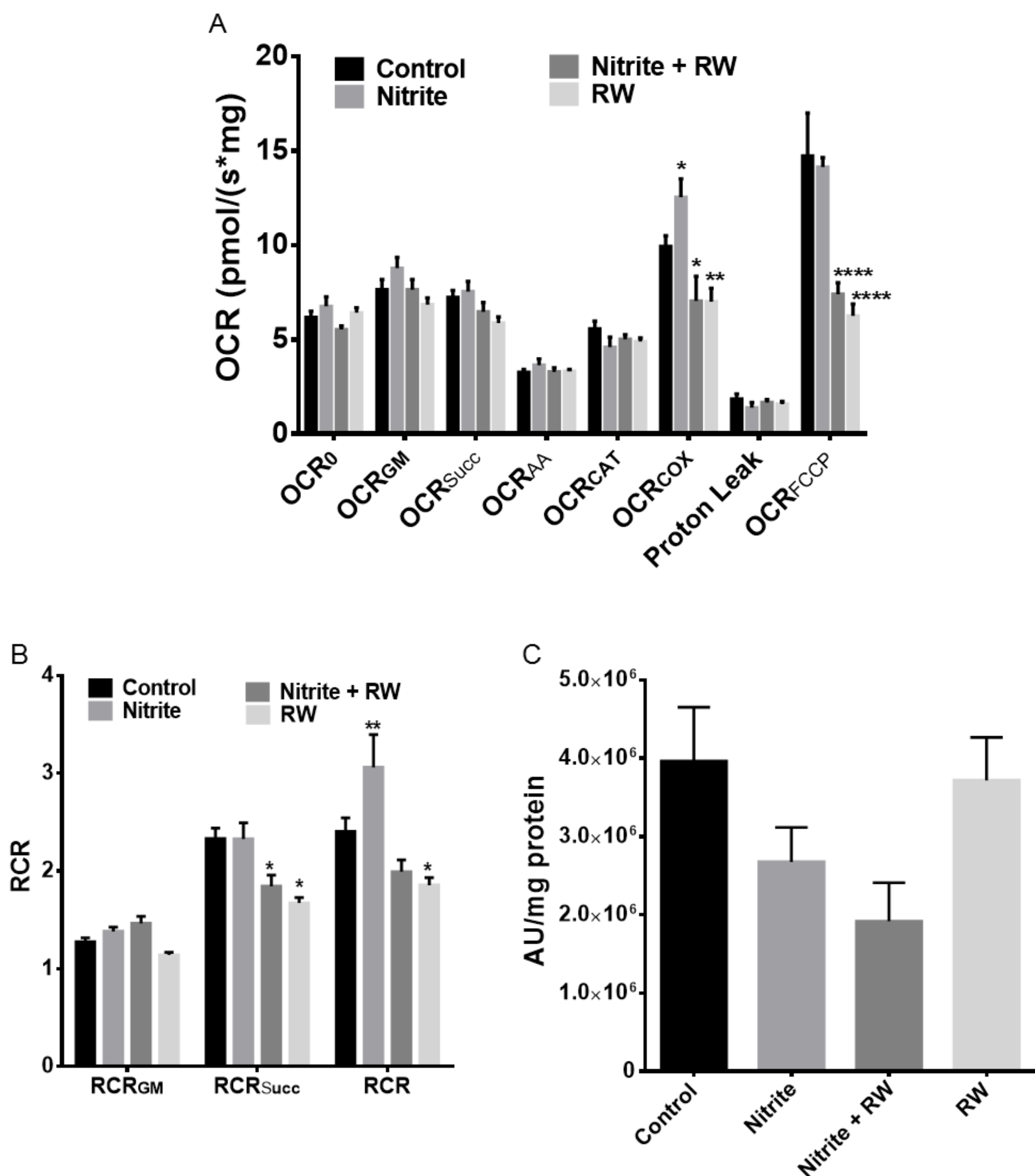


Figure 5.4 Characterization of mitochondrial respiratory parameters in gastric corpus mucosa biopsies of nitrite and/or red wine challenged rats. A. OCR_0—basal respiration without ADP or ATP; OCR_{GM}—ADP-stimulated respiration in the presence of glutamate and malate (indicating the function of the respiratory chain complex I);; OCR_{Succ}—ADP stimulated respiration in the presence of rotenone and succinate (characterize function of complex II); OCRAA – respiration after inhibition with antimycin A; OCR_{CAT}—respiration after inhibition of succinate-stimulated respiration by carboxyatractyloside; OCR_{CcOX}—the respiratory equivalent of cytochrome oxidase (complex IV) activity calculated as [$OCR_{CcOX} = OCR_{TMPD} – OCR_{TMPD+NaN3}$] where OCR_{TMPD} and $OCR_{TMPD+NaN3}$ are TMPD-stimulated respiration rates before and after addition of NaN_3; Proton leak—measured indirectly as a state 4 respiration equal to difference between the respiration rates with carboxyatractyloside and antimycin A; OCR_{FCCP}— respiration in the presence of an uncoupler. B. RCR_{GMt}—respiration control ratio calculated as OCR_{GM}/ OCR_0 ; RCR_{Succ}—ratio of OCR_{Succ}/ OCR_{CAT}; RCR – total respiratory control ratio calculated as ADP stimulated OCR of complex I+II / OCR_{CAT} C. DCF fluorescence (arbitrary units) per mg of protein.The values are mean ± S.E.M. and n = 5 - 17, * p<0.05, ** p<0.001, *** p< 0.001, **** p<0.0001.

5.3 Discussion

Nitrite is now a well-established mediator of several beneficial tissue responses but the underlying molecular pathways involved are still unclear. Several studies identified the mitochondria as an important target of nitrite, through the modulation of specific mitochondrial proteins such as complex I and IV, in both physiological (physiological hypoxia) and pathological (I/R) conditions [160, 161, 320]. Of note, it was shown years ago that nitrite can be metabolized to $^{\bullet}NO$ by mitochondrial proteins [117, 155]. Gastric mitochondria are particularly exposed to nitrite and $^{\bullet}NO$, since diet contribute with high amounts of these species, hence found in the gastric compartment under physiological conditions upon consumption of nitrate/nitrite rich products [109, 119, 120, 130]. The ability of $^{\bullet}NO$ to cross cellular layers and reach inner cells and subcellular compartments [16, 252] such as the gastric mitochondria, raised the question if dietary nitrite could impact gastric mitochondrial function and how gastric mitochondria overcome potential $^{\bullet}NO$ effects.

Gastric mitochondrial respiration is particularly relevant in gastric acid secretion by parietal cells [314] and a disturbance in the energy metabolism has been associated with gastric pathology and carcinogenesis [254, 321-327]. The formation ROS and RNOS [328-331] and the regulation of HIF-1α [332], which is responsible for sifting oxidative phosphorylation to glycolysis, have been also linked to cancer cell metabolism. Substrates are oxidised in the mitochondria and the generated electrons enter the ETC, creating a transmembrane proton gradient used to generate ATP, essential for cellular function. However, the membrane potential can be dissipated in other ways different from phosphorylation, resulting in oxidation not being totally coupled to ATP production. The back leakage of protons through the slightly permeable inner mitochondrial membrane has been found responsible for up to 25% of resting energy outflow [333].

The gastric mucosa mitochondrial respiratory profile was assessed in both isolated mitochondria and saponin-permeabilized biopsies, using liver mitochondria as a well-

established model for comparison. Isolation of gastric functional mitochondria revealed to be a difficult process leading to a low yield preparation. Similar problems have been described for intestinal mitochondria [334]. Overall, the results obtained by using a stepwise protocol of substrates and inhibitors support that, the profile of oxygen consumption is similar in both isolated mitochondria from the rat stomach and liver, beside the differences in OCRs. A residual oxygen consumption in the absence of ADP phosphorylation (blocked by oligomycin) point to an intrinsic higher proton leak across the inner mitochondrial membrane or uncoupled respiration due to damaged mitochondria in the gastric sample [335, 336]. Using gastric and liver fresh saponin-permeabilized biopsies we observed that the respiratory rates OCR_{GM}, $OCR_{Succ,}$ OCR_{CcOX} and OCR_{FCCP} mean values were lower than in liver biopsies which refers to less tissue content of mitochondria in the gastric sample [254]. The RCR values obtained were lower than others found in the literature [254], particularly the RCR for complex I (RCR_{GM}), which is consistent with samples with high ATPase activity and residual muscle fibers, which results into a higher state 2 and 4 respiration [335-337].

Nitric oxide is known to compete with oxygen for CcOX (complex IV), leading to a reversible inhibition of mitochondrial respiration [41, 338] with physiological implications particularly in ischemia [320]. Considering that gastric mitochondria is a likely target for dietary-derived •NO, the impact of a •NO burst in mitochondrial oxygen consumption was determined in both isolated mitochondria and tissue biopsies using •NO amounts easily found in the gastric compartment upon a nitrate/nitrite rich meal [110, 252]. As described before, these high amounts of •NO and its derivatives seem to have a more physiological than pathological role, which indicates that gastric mucosa cells have ways to overcome the potential deleterious •NO effects. As previously mentioned, liver mitochondria and biopsies were used as a comparison. Although isolated mitochondria from gastric mucosa showed more susceptibility to the •NO challenge than liver mitochondria, gastric biopsies were more prone to cope with the •NO burst, presenting lower oxygen consumption

inhibition and shorter duration of such inhibition, comparing with liver biopsies under the same conditions. These results support the fact that gastric mucosa cells are well adapted to the high $^{\bullet}NO$ amounts produced from dietary nitrite in stomach, enduring the exposure to $^{\bullet}NO$ without compromising cellular viability [252].

Gastric strips were also exposed to nitrite and nitrite plus red wine mixtures under simulated gastric conditions and the mitochondrial respiratory parameters were evaluated. It was previously shown that red wine components modulate nitrite chemistry in acidic pH enhancing $^{\bullet}NO$ formation [147, 148, 188]. Despite some variation in respiratory parameters such as OCR_{CcOX} and OCR_{FCCP}, no significant difference in the oxygen consumption, RCR or in the overall production of ROS under these conditions was observed. From these results we question if either the gastric mitochondrial respiration is not susceptible to modulation under these conditions or the experimental model was not adequate to illustrate the impact of dietary nitrite in the gastric mitochondrial function.

The *in vivo* results brought a clearer insight on the impact of nitrite intake on gastric mitochondrial function. Nitrite induced some increase in the OCR_0, OCR_{GM} and OCR_{Succ} and significant increase in OCR_{CcOX} ($p < 0.05$), that along with a decrease in OCR_{CAT} of about 17% and proton leak of about 25% support the increase in RCR and in the q value, indicating a better coupling between respiration and oxidative phosphorylation after nitrite. The decrease in proton leak and state 4 respiration with carboxyatractyloside was reported before in skeletal muscle of human subjects receiving nitrate, along with decreased whole body oxygen consumption during exercise [160, 162]. Taken together, these results suggest that the nitrate-induced increase in mitochondrial efficiency can be explained by reduced leakage/slippage of protons across the inner mitochondrial membrane. The group challenged with nitrite plus red wine, conditions that direct nitrite chemistry towards the formation of $^{\bullet}NO$, shows an increase in the respiration associated with complex I, when compared to the control, as observed by the increase in RCR_{GM} ($p <$ 0.05). Both groups with red wine, presented a significant decrease in the OCR dependent

on complex IV and for uncoupled respiration along with significant decrease of complex II-associated respiration when compared to control. Studies suggest that oxygen consumption by CcOX can be inhibited to a certain degree without compromising ATP production in the electron transport chain [339-341]. The group challenged with only red wine also presented a significant decrease in RCR ($p < 0.05$) relatively to the control. The reason underlying this red wine effect is still unclear. Although, low doses of the phenolic content of red wine have been reported to increase mitochondrial capacity in cells [342], polyphenols have an uncoupler-like chemical structure and a transient accumulation of red wine polyphenols in the mitochondrial membranes can result in a non-specific uncoupling state with consequent disturbance of their integrity and increase proton leak [343].

Also, a decrease in ROS production was observed in the nitrite group and a more accentuated one in the nitrite plus red wine group. These results correlate with the increase in the complex I dependent respiration illustrated by RCR_{GM}.

Taken together, these results highlight the relevance of everyday diet in physiology, particularly in the gastric compartment. In addition to the beneficial $^{\bullet}$NO-like effects attributable to dietary-derived nitrite in amounts easily achieved by a vegetables rich diet, mitochondrial function seem to also benefit with the nitrate-nitrite-$^{\bullet}$NO pathway.

Mitochondria have traditionally been called the “powerhouse” of the cell but it is now known that its function extends beyond ATP generation. Mitochondria is key to cellular homeostasis via pathways that include the production of ATP, the generation of ROS for signalling and the regulation of the apoptotic cascade. The role of dietary-derived nitrite and nitrite-derived $^{\bullet}$NO in regulating mitochondrial function in the gastric tissue may then have major effects in gastric physiology and disease.

6

General discussion and final conclusions

As the endeavour for a healthy life and disease fighting continues to be a global matter of concern, there has been increasing interest in gaining a more comprehensive understanding of how different aspects of life style, in particular diet, may impact on human health. In this regard, nitrate and nitrite, consumed in vegetables as part of a normal diet, are permanent constituents of blood in animal species and have been identified as bioactive compounds capable of influence biological processes, resulting in improvements for human health.

Paradoxically, until recently nitrite has been considered to be either a relatively inert by-product of metabolism or a toxic dietary compound. And yet, it is paradoxical that humans, as well as other species, by consuming high amounts of nitrate in green leafy vegetables, are endowed with an enterosalivary recirculation of nitrate which ultimate goal is to maintain nitrite in the blood at a relatively high steady-state concentration. This notion, among others, clearly point towards a biological role for nitrite.

The work developed and presented in this thesis addressed the relevance of dietary nitrate and nitrite and their derivatives such as $^{\bullet}NO$ in gastric physiopathology, giving particular attention to the molecular and cellular modifications induced by nitrite in the gastric compartment with potential functional outcomes, both locally and systemically. For that, we have implemented a comprehensive strategy involving *in vitro*, *ex vivo* and *in vivo* models to illustrate the complex biochemistry triggered by nitrite consumption, focussing on protein nitrosation, gastric defence and mitochondrial function.

The impact of nitrate/nitrite consumption on gastric and general health has been under discussion in the scientific community for more than five decades. However, only more recently a physiological relevance has been attributed to nitrate and nitrite *in vivo*, altering the perception that these species were only metastable metabolites of $^{\bullet}NO$ oxidation [107, 108]. In fact, the demonstration that, upon a nitrate/nitrite rich meal high concentrations of $^{\bullet}NO$ are formed in the human stomach in a pH and nitrite concentration-dependent rate and that several enzymes acquire nitrite reductase activity when the

oxygen tension is low, brought the attention of the biomedical community for nitrite, as a health promoting molecule [109-111, 119]. Nitrite is now considered a key player in the hypoxic signalling as a storage for •NO [111] and has been pharmacologically used as vasodilator, bronchodilator and intestinal relaxant among others [108].

Other diet components can modulate the chemical equilibria of nitrite in the stomach. Foods and beverages including red wine rich in polyphenols have been shown to boost the •NO production from nitrite at acidic pH in the human stomach [147, 148, 188]. The association of the diet-derived polyphenols with redox properties and their health benefits is well established from the epidemiologic point of view [344]. The interaction between nitrite and polyphenols in the gastric lumen has been claimed as a mechanism in support of the beneficial contributions of polyphenols. In fact, from this interaction results •NO, which is able to diffuse towards deep regions of the gastric mucosa [252, 345], participating in distinct physiological mechanisms such as smooth muscle relaxation [188], regulation of mucosal blood flow and mucus thickness [177, 179] and also in the eradication of pathogens such as *Helicobacter pylori* [176].

Gastric nitrosation upon dietary nitrite

In chapter 3, we addressed protein post-translational modifications in the gastric compartment, namely nitrosation, promoted by dietary nitrite and modulated by red wine. The chemistry of nitrite under acidic and reductive conditions in the gastric compartment leads to the formation of potential nitrosating species that are able to modify endogenous proteins and proteins from diet. Considering that mucins, the major components of the gastric mucus, are cysteine-rich glycoproteins we assessed whether mucins could be preferential targets for nitrite-dependent nitrosation and, therefore, if the mucus could act as a chemical barrier to nitrite and •NO-derived nitrosating species, protecting the underlying mucosa cells from potential harmful nitrosative stress. The methodological approach included *in vitro*, *ex vivo* and *in vivo* models and also an *in vivo* acute

inflammatory model used to assess the gastric susceptibility to nitrite-dependent nitrosation under non-physiological conditions. The results obtained support the hypothesis that upon a nitrate/nitrite rich meal, gastric endogenous proteins like mucins can undergo post-translational modifications, namely nitrosation, with particular relevance for S-nitrosation, in a nitrite concentration-dependent fashion. Thus, the gastric mucus is able to chemically filter nitrite-derived reactive species, that otherwise could potentially induce detrimental nitrosative stress in the deeper layers of the gastric mucosa, as confirmed by the chemical and mechanical removal of the mucus layer. However, analyzing the underlying mucosa cell layer, we found also a nitrosation pattern dependent on nitrite intake, which supports the notion that the nitrite-derived chemistry in not restricted to the gastric lumen, but also have functional consequences deeper in the tissue. Therefore, the barrier function of the mucus gel is not fully effective against nitrite-derived reactive species. The presence of diet components with redox properties like red wine and the inflammatory conditions of the gastric mucosa could modulate the nitrosation pattern and extent, in the mucus and mucosa cells layers. Red wine increased $^{\bullet}NO$ formation but led to a decrease in the nitrosation extent in both mucus and underlying mucosa, suggesting novel actions for red wine polyphenols *in vivo*. On the other hand, a disturbance of the gastric physiological conditions by inducing acute inflammation, led to an increase in the nitrosation extent, probably due to the increased number of targets for nitrosation as consequence of the inflammatory process.

Together, the results presented in chapter 3 showed the formation of fairly stable nitrosated compounds in the stomach, particularly S-nitrosothiols that may interfere in local signalling pathways, exerting an $^{\bullet}NO$-like effect or upon diffusion may act as $^{\bullet}NO$ carriers and act systemically. They also illustrate the importance of gastric mucosal integrity in the gastric protective system and the susceptibility to nitrosative stress.

Dietary nitrite and the gastric defence system

The maintenance of mucosal integrity is crucial for the gastric defence system. In chapter 4 we addressed the impact of dietary nitrite intake in the expression of TFF1, a trefoil peptide expressed physiologically in the gastric epithelium, associated with mucin secretion, folding, packing and function and involved in gastric protection and regeneration. Dietary nitrite and its derivatives, including •NO, have been implicated in several pathways underlying gastric physiology, namely increase in blood flow and mucus secretion. Our data, using an *in vivo* model, showed that nitrite resulting from a nitrate/nitrite rich meal could increase the expression of TFF1 and, therefore, contribute to the maintenance of the gastric mucosa integrity. The modulation of the nitrite chemistry by red wine, shifting the equilibrium towards the formation of •NO, revealed to exert a particular impact in TFF1 expression under acute inflammatory conditions. Hence, a novel contribution for dietary nitrite in gastric physiology has been described via the expression of a key regulator of mucosal protection and regeneration, the TFF1. Under inflammatory conditions, situation in which the mucosal blood flow is compromised and hypoxia begins to set, dietary nitrite-derived •NO increases the expression of TFF1 in order to initiate the regeneration pathway of the stomach epithelium.

Dietary nitrite and the gastric mitochondrial function

As stated previously, the integrity of gastric mucosa layers is essential for gastric physiology and diet-derived active components such as nitrite and red wine polyphenols can have repercussions in this equilibrium. The gastric mucosa is regularly defied with multiple stimuli from food stuff to bacteria and toxic species and the mucosa cells need to be equipped with protection mechanisms to cope with these challenges. In chapter 5 we addressed the implications of nitrite intake in gastric mitochondrial function, considering that high amounts of nitrite and •NO can be found in the stomach upon a nitrate/nitrite rich meal. •NO is able to cross the gastric layers and reach inner cells and subcellular

compartments, meaning that mitochondria are potential targets for nitrite and $^{\bullet}NO$. In this regard, it is important to note that $^{\bullet}NO$ and O_2 compete for cytochrome c oxidase in mitochondria, thus regulating respiration. Thus, by increasing $^{\bullet}NO$ steady-state over that of O_2, a situation may develop in which respiration is strongly inhibited, compromising ATP synthesis and cell viability. Therefore, and given the high amounts of $^{\bullet}NO$ generated in the stomach, it is of interest to understand mitochondrial respiration under such conditions. In the gastric mucosa, the energy derived from mitochondrial function is mostly used for acid secretion by parietal cells and mitochondrial dysfunction is associated with gastric pathology and carcinogenesis. When exposed to $^{\bullet}NO$, gastric saponin-permeabilized biopsies shown to be well adapted to cope with the potential inhibition of mitochondrial respiration by $^{\bullet}NO$ without compromising cell viability. In an *in vivo* model, the results shown that nitrite intake increases mitochondrial respiration efficiency via a lower leakage/slippage of protons across the inner mitochondrial membrane. The concomitant intake of red wine with nitrite resulted in ambiguous effects but the concept that dietary components can actively modulate mitochondrial respiration and cellular function is particularly relevant in gastric physiology and disease.

The overall conclusions drawn from this work can be summarized as follows:

- Dietary nitrite can trigger post-translational modifications such as nitrosation of structural proteins in both gastric mucus and mucosa layers.
- The gastric mucus layer acts at some extent as an active filter for the nitrosative chemistry as mucins are mostly nitrosated to fairly stable and active S-nitrosothiols.
- The modulation of nitrite biochemistry by diet components such as red wine and the modification of the gastric conditions by acute inflammation tune the nitrite-derived nitrosation pattern.
- The redox modulation by red wine suggests novel actions for wine polyphenols *in vivo* via the balance of S- an N-nitroso compounds in the gastric wall.

- Dietary nitrite triggers the expression of TFF1 *in vivo*, an important peptide in mucosal protection and regeneration.
- Gastric mitochondrial respiration shown a lower susceptibility to $^{\bullet}NO$ inhibition as compared with liver mitochondria.
- Dietary nitrite improved mitochondrial efficiency, particularly by decreasing the proton leak.
- Red wine components, likely polyphenols, act as modulators of dietary nitrite biochemistry in the gastric compartment.

7

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