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Influence of dietary antioxidants on luminal nitrite chemistry under conditions simulating the gastro-oesophageal junction.

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MBChB

A thesis submitted in fulfilment of the requirement for the Degree of M.Sc (Med.Sci.) by research

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April 2009

Abstract

The second half of the twenties century saw a sharp worldwide decline in the both incidence and mortality of gastric cancer. Despite this the condition remains the world's second leading cause of cancer mortality, second only to lung cancer. Although most gastric cancers arise in the antrum and body (non-cardia) of the stomach, the incidence of proximal tumours of the cardia and distal oesophagus have increased dramatically over the last fifty years throughout the world. This major change in the pattern of the disease suggests that gastric cancer (cardia versus non-cardia) is not one but two separate disorders with regard to cause and pathogenesis. Over the last fifty years there has been concern about luminal nitrite, derived from dietary nitrate, as a risk factor for upper gastro-intestinal malignancies. This has arisen from evidence that the salivary nitrite is rapidly converted to nitrosating species and (in the presence of ascorbic acid) nitric oxide (NO), which are both potentially mutagenics.

Thus this study aimed to investigate the influence of ascorbic acid (AA), sodium thiocyanate (NaSCN), oxygen (O_2) and pH (1.5, 2.5 and 3.0) on the nitrite chemistry in simulated gastric juice. Another aim of the present study was to investigate the influence of a range of water-soluble dietary phenolics antioxidants on the nitrite chemistry to compare their effect with that of ascorbic acid. The capacity of dietary phenolics and ascorbic acid to reduce the acidified nitrite to nitric oxide was also investigated under both aerobic and anaerobic conditions.

These studies were performed in a newly designed closed bench-top model reproducing the chemical environment occurring at the human gastro-oesophageal junction. Each of the experiments was performed with and without NaSCN at different pH values (1.5, 2.5 and 3.0) under both aerobic and anaerobic conditions. The studies were focused on the measurement of NO formation and O_2 consumption by electrochemical detection,

according to the antioxidant present in the system (ascorbic acid, ferulic acid, caffeic acid, gallic acid or chlorogenic acid, in a range of concentrations).

Nitric oxide production increased with increasing ascorbic acid concentration, and was greatest at the lowest pH of 1.5. The absence of oxygen in the system markedly increased nitric oxide levels in the presence of ascorbic acid, while the addition of NaSCN enhanced nitric oxide production and oxygen consumption. A different pattern of nitric oxide production was seen with the dietary phenolics compared with ascorbic acid. In addition, two different patterns of nitric oxide response were seen with ferulic acid and caffeic acid and another with gallic acid and chlorogenic acid. Ferulic and caffeic acids produced only a small initial increase in nitric oxide, which was not sustained under either aerobic or anaerobic conditions. In contrast, gallic and chlorogenic acids produced a much more marked rise in nitric oxide, which remained elevated under both aerobic and anaerobic conditions. The only phenolic experiment in which an equivalent concentration of nitric oxide to that with ascorbic acid was observed was with high concentration of gallic acid under anaerobic conditions.

These studies indicated that nitrite in the simulated GOJ environment is converted to varying extents to nitric oxide and factors influencing this include luminal pH, thiocyanate, oxygen tension and presence of antioxidants. We have also found that the capacity of antioxidants to convert acidified nitrite to nitric oxide varies as does the temporal profile of the nitric oxide concentration generated by them.

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Abbreviations

AA	─ Ascorbic acid
CA	─ Caffeic acid
CC	─ Pearson correlation coefficient
CGA	─ Chlorogenic acid
DHA	─ Dehydroascorbic acid
DTT	─ Dithiothreitol
EDTA	─ Ethylenediaminetetraacetic acid
FA	─ Ferulic acid
GA	─ Gallic acid
GIT	─ Gastro-intestinal tract
GOJ	─ Gastro-oesophageal junction
GORD	─ Gastro-oesophageal reflux disease
HCl	─ Hydrochloric acid
HLPC	─ High performance liquid chromatography
HNO_2	─ Nitrous acid
NaSCN	─ Sodium thiocyanate
RNS	─ Reactive nitrogen species
NO	─ Nitric oxide
NO_2	─ Nitrite
N_2O_3	─ Dinitrogen trioxide
ROS	─ Reactive oxygen species
NOSCN	─ Nitroso thiocyanate
mg	─ Mill gram
Min	─ Minutes
mL	─ Mill liter
MPA	─ Metaphosphoric acid
SA	─ Sulfamic acid
TVC	─ Total vitamin C
V	─ Voltage
µM	─ Micromoles
%	─ Percentage

Dedication

Unreservedly - to my parents, Fatma Elmesmari and Atyia Elmesmari.

Acknowledgement

I have been extremely privileged to have been able to carry out my postgraduate studies within the Division of Cardiovascular and Medical Sciences at Glasgow University.

This thesis would not have been completed without the support and encouragement of my family, many friends and colleagues; and to all of them I convey *many thanks indeed*.

I would like to primarily thank Professor Kenneth E. L. McColl, my Supervisor, whose expert knowledge and leadership assisted me greatly, and which I learned a lot from. His excellent advice, thoughtful comments and knowledgeable guidance during my research study and writing were invaluable.

My special thanks go to Dr Emilie Combet, for giving her time, assistance and professional guidance in regard to both the laboratory studies and advice on writing of my work.

A debt of gratitude is owed to the Libyan Government for the financial support, also. I am greatly beholden to my home country, Libya, from which I received this opportunity to complete my postgraduate studies at Glasgow University

My gratitude to Ms. Dorothy Ronney, who always provided me with great help and encouragement throughout the study period.

Many thanks to Mrs Valerie Fyfe for her most welcomed assistance in the technical lab analysis.

I extend my gratitude to professor Fakhri Akhleifa for his encouragement and supportive comments and to Professor Alan Jardine, for always being there as my Adviser.

I have enjoyed working with my many colleagues and wish to express my appreciation to them for their advice and encouragement, especially Anne McGarrity, Mohammad Derakhshan, Myzoon Ali, Alison Faichney and Judith Harriman.

And finally my sincere gratitude goes to my family Of course without my parents, Fatma Elmesmari and Atyia Elmesmari, this thesis could not have been written…. *Thank you*.

Declaration

All investigations in this thesis were undertaken by the author under the supervision of Professor Kenneth E.L. McColl within the Division of Cardiovascular and Medical Sciences, University of Glasgow from May 2007 until April 2009.

Chapter 1 - Introduction

1.1 Anatomy and physiology of stomach

The stomach is a **J**-shaped muscular bag located in the left upper part of the abdominal cavity mainly in the left hypochondrial, epigastric and umbilical regions, which has two openings: the cardia and the pylorus [1-3]. These openings join the stomach with the oesophagus proximally and with the duodenum distally to form the gastro-oesophageal junction and gastro-duodenal junction respectively (Figure 1.1A). The former lies 2.5 cm to the left of mid line at the level of T 10 vertebra, and the latter lies to the right of mid line at the level of L 1vertebra [1-3].

The stomach is completely invested by peritoneum to form the lesser and greater omenta that contain the vascular and lymphatic supply [3].

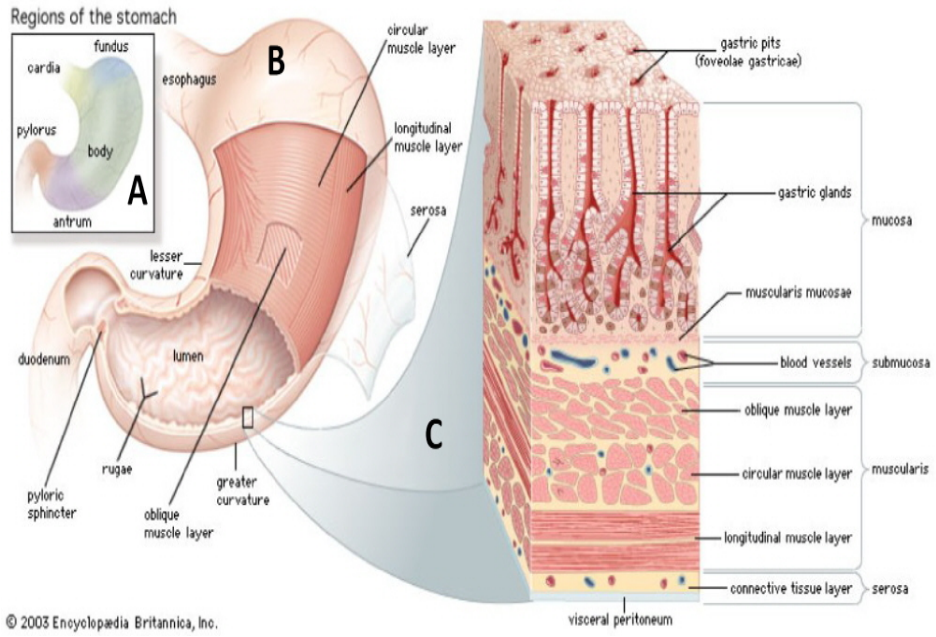


Figure 1.1:Anatomy and histology of the stomach is described the stomach and its subdivision (A), cut section shows the muscular layers and internal anatomy (B) and stomach wall histology (C) [4].

The stomach has four anatomically distinct areas, each containing different types of histological cell. The cardia, distal to the gastro-oesophageal junction (GOJ), predominantly consists of simple tubular glands lined by mucus secreting cells, numerous endocrine cells, and a few acid secreting cells [1, 5-7]. The fundus is the uppermost part of the stomach, occupying the area above the opening of oesophagus into the stomach (above the gastro-oesophageal junction). The body is the largest part of the stomach, representing about two third of gastric area. The glands of the fundus and body consist of a range of secretary cells:

- parietal cells mainly in the upper part of the gland, which in addition to acid also produces intrinsic factor and blood group substances;
- chief cells mostly located in the lower part of gland**,** which are the main source of pepsinogen and proteolytic enzymes. In addition to these cells the body mucosa also contains some mucus neck cells and endocrine cells, the latter producing histamine in response to gastrin [1, 5-7].

The pyloric antrum represents the lower third of the stomach and has a funnel shaped appearance. Its wide part connects to the stomach whereas the narrowing portion connects the stomach with the pyloric canal. The gland of the pylorus consists of cell secreting mucus and gastrin hormone and occasionally parietal cells [1, 5-7].

The stomach wall is composed of four different layers, which are arranged from inside to outside. These are the mucosa, sub mucosa, muscularis layer, and serosa (Figure 1.1C). The stomach mucosa is an entirely tubular glandular form, with a variety of secretary cells, these cells differ from one to another anatomical sub-division described before. In general, the gastric mucosa has two components - the surface epithelium and its pits (foveolae) and the glandular structure. The surface epithelium and the pits appear throughout the stomach wall and are lined by mucus-secreting columnar cells which also secrete bicarbonate and sodium ions. The glandular component differs in the thickness and type of the cells from

one region to another. For example, the foveolae zone of cardiac and pyloric is the half thickness of the mucosa, while the glandular component in the fundus and body is much thicker than the foveolar zone [5-8].

The stomach sub mucosa is composed entirely of a thick sheet of connective tissue, which provides elasticity to the stomach. It contains many vascular and lymphatic vessels as well as sub mucosal nerve plexus [6-8]. In addition to the standard pattern of wall structure which is present throughout the digestive tract, longitudinal and circular muscle layers, the stomach wall has a third layer of oblique muscles, or muscular layer [5-8]. The serosa is the outer layer of the stomach wall and consists of supporting connective tissue, the inner layer of fibrous tissue provides structural support while the epithelial tissue "mesothelium", secrets a watery lubricant fluid [6-8].

1.2 Stomach pathologies

The stomach, like any other organ in the body, can experience both benign and malignant tumours. Adenocarcinoma are much more common than any other malignant tumours (such as carinoid tumours, malignant stromal tumours and lymphoma) or benign tumours [8-10].

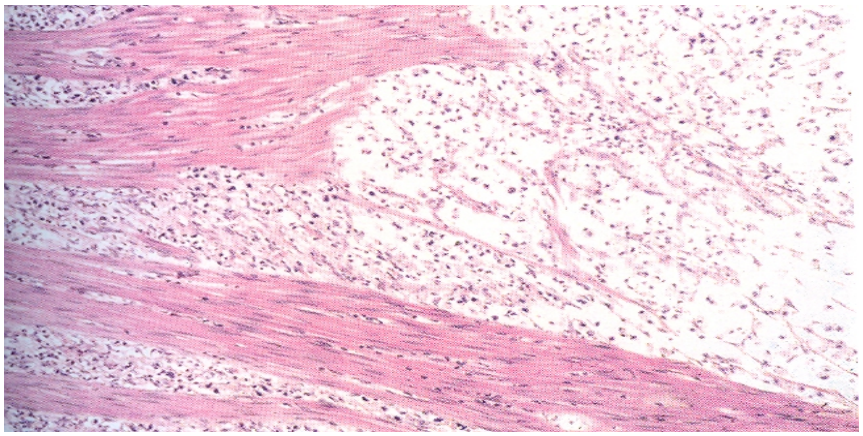
Adenocarcinoma have been divided histologically into two main types; the diffuse and intestinal types [11]. The diffuse type adenocarcinoma consists of sheets of neoplastic cells with minimal gland formation which leads to a significant thickening of the gastric wall. The development of the diffuse cancer is correlated with genetic factors, is more common in the younger population, and has a male to female ratio of 1:1. Unfortunately this type of adenocarcinoma carries a bad prognosis and survival rate is lower than the intestinal type tumours [8-12] (Figure 1.2A).

Intestinal type adenocarcinoma, on the other hand, shows glandular formation similar to that of colonic adenocarcinoma. This intestinal sub-type of adenocarcinoma is also associated with the presence of metaplastic intestinal epithelium, tends to be more common in countries presenting high-incidence of gastric cancer, and significantly correlates with environmental factors such as *H. pylori*-associated chronic gastritis. Furthermore, the intestinal type is more common in males than females; with a ratio of 2:1, and the mean age of patients is 55 years [8-12]. However, some tumours show features of both types and are more difficult to categorise [11] (Figure 1.2B).

Recently, gastric cancer has been sub-classified according to the intra- gastric anatomical site. It either arises from the proximal region of the stomach (cardia) or more distal part (non cardia) [13-15]. Most gastric cancer arises in the antrum and body (non cardia) of the stomach, however, the incidence of proximal tumours of the cardia and the gastro-oesophageal junction has increased dramatically over the last 25 years [16-18].

These changes in the pattern of stomach malignancies throughout the world is not merely to be related to changes in tumour classification, change in diagnostic methods or an increased general awareness of this disease aetiology and epidemiology [14, 19]. It is possible to conclude that gastric cancer (cardia versus non-cardia) is not one but two separate disorders with regard to cause and pathogenesis [14].

(A)



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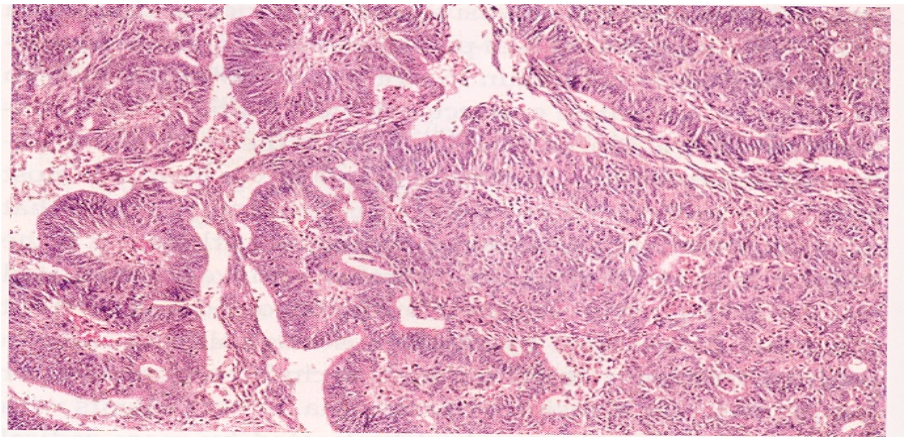


Figure 1.2: Histological classification of gastric adenocarcinoma, the diffuse type (A), and intestinal type (B) [(20)] .

1.3 Gastric and oesophageal adenocarcinoma

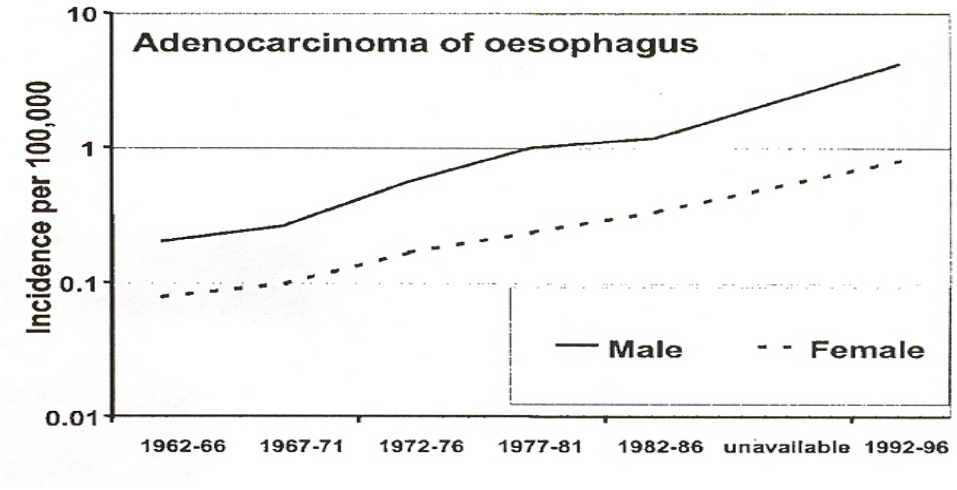
1.3.1 Epidemiology

In spite of a steady decline in the overall incidence of gastric cancer, observed in most countries in the last decades, incidences of cardia cancer have increased in recent years [16-18, 21]. Meanwhile, the overall incidence of oesophageal cancer (including oesophageal squamous cell carcinoma) has decreased throughout the last thirty years; while adenocarcinoma of the distal oesophagus has increased dramatically in most Western countries as well as in some other countries over the worlds [16-18, 21]. Thus gastric cancer and oesophageal cancers still remain the second and the sixth leading cause of cancer-related death, respectively [19, 22] (Figure 1.3A and B).

Gastric and oesophageal adenocarcinoma, similar to most other GI malignancies, are the diseases of older age groups in most countries. The mean age at diagnosis is 63 years and the risk increases with increasing age [19, 22]. The diseases predominantly affect men and white patients twice as often as black patients [14, 19, 23]. Furthermore, in relation to sub-site distribution, Roder (2002) states that the percentage of gastric cancers sited in the cardia, as opposed to more distal specified sub site, was 31.7 % for males and 18.8 % for females, with higher percentages applying to male than females in each region [24].

The geographical variation of oesophagus and cardia adenocarcinoma differs between countries. In developed countries, the incidence of distal stomach cancer has decreased, while stomach cancer in the most proximal cardia region and GOJ has increased substantially. According to a review of literature, the highest incidence has been reported in the United Kingdom, Australia, and United States. However, low incidence has also been reported in Eastern Europe and Scandinavia [14, 19].

(A)



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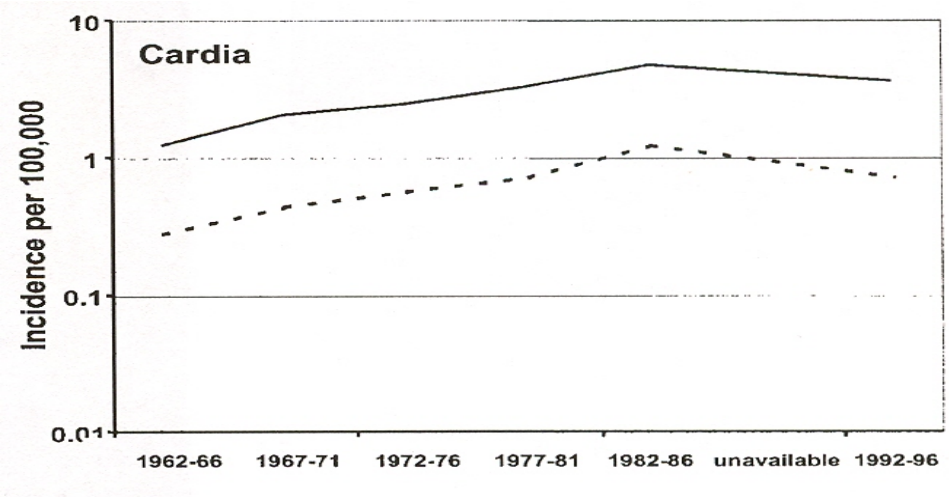


Figure 1.3: Age- standardised incidence of adenocarcinoma of oesophagus (A) and gastric cardia (B) cancer Solid black line adenocarcinoma in male; dashed line adenocarcinoma in female [(18)].

1.3.2 Aetiology

The reasons for the sharp increase in the incidence of adenocarcinoma of the oesophagus and the gastric cardia over the past fifty years are still unknown. However, one explanation for these similar trends may be that the adenocarcinoma of the oesophagus and the gastric cardia share at least some aetiological factors [18, 25-27].

Cancer of the oesophagus and gastric cardia differs from cancer of the more distal stomach [21, 28-31] (Figure 1.4). The cancer of the distal stomach develops against a background of atrophic gastritis and hypochlorhydria, usually due to *H. pylori* infection and is more common amongst those from lower socioeconomic classes [29, 31]. In contrast, the cancer of GOJ and cardia occurs in patients with healthy acid secreting mucosa, normal secretary function, who are usually *H. Pylori* negative, and are more in the professional social class population [21, 28-31].

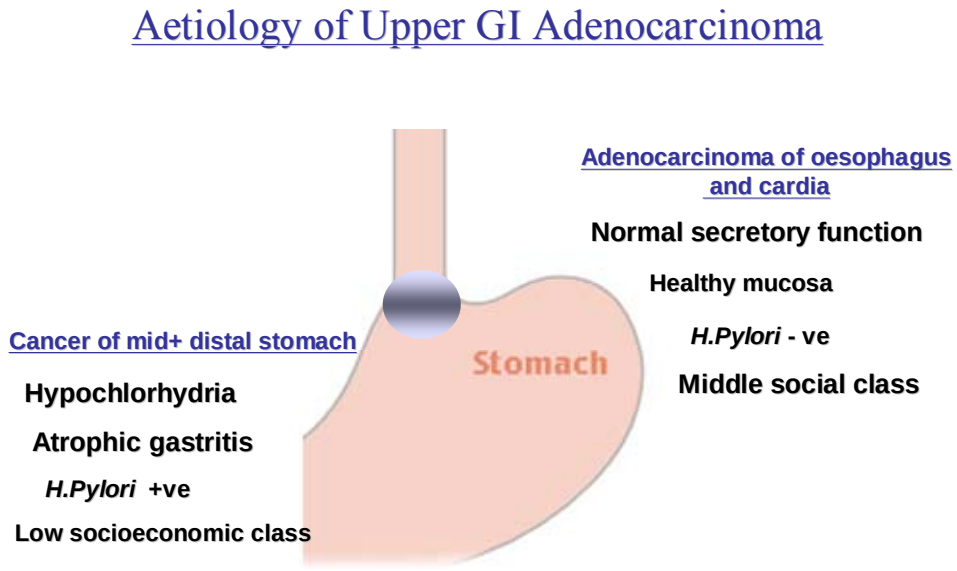


Figure 1.4:. Aetiology of upper GIT adenocarcinoma (cardia versus non cardia).

1.3.3 Risk factors of gastric and oesophagus adenocarcinoma

The pathogenesis of gastric cancer in general, and adenocarcinoma of the oesophagus and gastric cardia in particular, are complex with multiple steps and multi-factorial processes. However, the disease is the end-result of cellular and molecular changes triggered by endogenous and environmental factors [32, 33]. The mutagen responsible remains unknown, but important clues have been established and are discussed below [34].

- **Gastro-oesophageal reflux**

Gastro-oesophageal reflux is a strong risk factor in the development of adenocarcinoma of the oesophagus [19, 35]. Chronic exposure to gastric and bile acid leads to progressive damage and changes to the squamous mucosa of the oesophagus and causes its replacement by metaplastic columnar mucosa. The longstanding damage caused by gastric and bile reflux to the oesophageal squamous mucosa transforms it to gastric columnar mucosa, then into intestinal mucosa followed by dysplasia and finally adenocarcinoma [14, 36]. However, the sequence of events leading to oesophageal adenocarcinoma is not mandatory for the development of the disease, although large cohort studies have established a strong association between reflux disease and oesophageal adenocarcinoma [37].

The aetiology of adenocarcinoma of gastric cardia remains unknown; however, it may have a similar aetiology to oesophageal adenocarcinoma [18, 25-27]. Many studies have shown there are weak associations between the reflux disease and adenocarcinoma of cardia [35, 37]. However, recently several studies have established that cardia cancer might arise from the distal segment of the oesophagus mucosa when this segment is exposed to acid but the oesophageal sphincter does not allow the acid to reflux into the main body of the oesophagus[14, 38].

This explanation suggests that cardia cancer might resemble the oesophageal adenocarcinoma, and therefore may have at least two different aetiological factors.

- ***Helicobacter pylori* infection**

Recent studies have indicated that infection with *H. pylori* might be inversely associated with the risk of adenocarcinoma of the oesophagus and gastric cardia [39]. However, this association between the *H. pylori* infection and adenocarcinoma of the cardia is much weaker than that of between *H. pylori* infection and oesophageal adenocarcinoma [40, 41]. The postulated mechanism for the protective effect might be that infection could reduce the occurrence of acidic reflux by introducing atrophic gastritis, which in turn would reduce the harmful effects of acidity in the oesophagus [42]. Moreover, the eradication of *H. pylori* infection in individuals might lead to an increase in the incidence of oesophageal adenocarcinoma. However, the causal relation between the two remains unclear [19].

The association between *H. pylori* infection and gastric cancer may vary by the anatomical site; many studies have demonstrated that *H. pylori* infection is associated with a risk of non-cardia cancer [31, 40, 43]. However, the association with adenocarcinoma of gastric cardia is very complex and some studies have pointed out that *H. pylori* infection is not involved in the aetiology of cardia cancer [40, 41]. In contrast, other studies have confirmed there is an inverse association between the *H. pylori* infection particularly *cagA*$^+$ strains, and adenocarcinoma of gastric cardia [39, 44, 45]. Furthermore, recent studies have examined the relationship between the intra-gastric location of cancer, *H. pylori* infection, and atrophic gastritis [44]. These studies have confirmed that there is a strong association between *H. pylori* infection and atrophic gastritis and cancer of non cardia. However, a significant negative association between *H. pylori* infection and cardia cancer has also been detected. Individuals who are seropositive for *H. pylori* are at an increased risk for gastric cardia cancer particularly if they had atrophic gastritis [44].

This view is consistent with the fact that cardia cancer is a complex disease and has at least two distinct aetiologies [46]. One type resembles non-cardia cancer being associated with *H. pylori* infection and atrophic gastritis. The other type resembles the oesophageal adenocarcinoma and this type is shown to have a negative association with *H. pylori* infection and is more likely to be due to short segment gastro-oesophageal reflux disease [14].

Moreover, there is geographical variation between the *H. pylori* infection and gastric cardia cancer. For example, there is a strong negative association between *H. pylori* infection and cardia cancer in Western countries, while there is a positive association between *H. pylori* infection and cardia cancer in China and other Eastern countries [40].

- **Luminal chemistry of the gastric cardia**

Recently many studies have pointed out that the luminal chemistry of the cardia region is different from the rest of the stomach. For example, the post-prandial acidity of gastric juice is decreased in the body of the stomach due to the buffering effect of food. However, the GOJ and gastric cardia are escape the buffering effect of meals and remain highly acidic compared to the body of the stomach. Thus the acidity of gastric cardia would facilitate the chemical reaction occur at low pH [47]. The formation of nitrosating species and *N*-nitroso compounds from salivary nitrite, derived from dietary nitrate, in the acidic environment of the stomach is maximal at the cardia region [48-50]. These *N*-nitroso compounds are potential pre-carcinogens for the oesophagus and stomach in animals models [51]. Ascorbic acid, actively secreted in gastric juice, provides protection by converting nitrosating species to nitric oxide [52-54]. The luminal generation of nitric oxide (the reaction between nitrosating species and ascorbic acid) is maximal at GOJ and gastric cardia [50, 55-57]. Nitric oxide is known to be mutagenic through its ability to react rapidly with oxygen to form N_2O_3, which can damage DNA directly via deamination of bases or indirectly through generation of *N*-nitroso compounds [58-61]. Moreover, nitric oxide can

inhibit a number of DNA repair enzymes [62] . Furthermore at this anatomical site the nitrite:ascorbic acid ratio is higher than any other region of the stomach [48]. There is also a substantial concentration of thiocyanate, which is an important catalyst in this chemistry [48]. All of these observations indicate that the GOJ and cardia region of stomach are likely to be regions of high nitrosative stress; mechanisms may contribute to a high incidence of metaplasia and neoplasia at these anatomical sites.

1.4 Luminal nitrosative chemistry

1.4.1 Nitric oxide

Nitric oxide is an important free radical playing a significant role in various biological events. In general, nitric oxide has beneficial regulatory functions in cardiovascular, immune and nervous system [63-65]. Nitric oxide is involved in the regulation of gastric mucosa blood flow, mucus secretion and gastric motility, as well as being implicated in gastrin and gastric acid secretion [66-68]. Furthermore, it has an important antimicrobial activity against a large variety of pathogenic micro-organisms [69-71]. However, imbalance of this biological molecule secretion is implicated in a number of different diseases such as hypertension, arteriosclerosis, diabetes and neurodegenerative disorders [63, 65, 72, 73].

Nitric oxide is produced from the amino acid L- arginine by the enzyme NO synthase and there are three isoforms of nitric oxide synthase enzyme[74]. Nitric oxide is generated at low concentration (constitutive nitric oxide synthase) by endothelial type (e NOS) neuronal type (n NOS) to modulate neuromuscular and vascular function. While the inducible form NO synthase (i NOS) produced nitric oxide at high concentration in order to influence the immune and inflammatory response [74, 75]. The nitric oxide generated by the inducible form has been implicated in the aetiology of mutagenesis and neoplasia related to chronic inflammation [76].

Over the last decades, in addition to enzymatic production another alternative pathway (non enzymatic) has been described, which generated large concentration of nitric oxide in the stomach [57, 77]. Nitric oxide arises from the reduction of nitrite, derived from entero-salivary recirculation of dietary nitrate, to nitrosating species and nitric oxide (in presence of ascorbic acid) under the acidic condition of the stomach [53-55, 57].

1.4.2 Dietary nitrate and entero-salivary recirculation.

Over the last two decades, there has been concern about the role of luminal nitrite in the aetiology of human gastro-oesophageal junction and gastric cardia malignancies.[49, 78] This arises from the fact that the acidic environment of the stomach converts nitrite in the saliva to nitrous acid and nitrosating species [28, 49, 78]. These nitrosating species can generate potentially carcinogenic *N*-nitroso compounds through their ability to react with secondary amines and amides [28, 49, 78]. The major source of nitrite in gastric juice of the healthy stomach is swallowed saliva, which in turn is derived mainly from the entero-salivary recirculation of dietary nitrate[79, 80].The main dietary source of nitrate is green leafy vegetables; after ingestion nitrate is rapidly absorbed from the small intestine into the blood and it reaches its maximum plasma concentration after sixty minutes from ingestion. Most of the nitrate is excreted in the urine, while only about 20-30% of all nitrates (from dietary source or produced endogenously) is re-circulated and taken up by the salivary glands and secreted into the mouth [79, 81-84]. The nitrate-reducing bacteria on the dorsum of the tongue reduce at least 25% of that nitrate to nitrite (Figure 1.5) [83, 85, 86]. Approximately 1500 ml of saliva is swallowed every 24 hours, containing high concentrations of nitrite as a consequence of the reduction of dietary nitrate. Under fasting conditions, the nitrite concentration of saliva is approximately 50 μM and this increase to 200 μM after ingestion of a nitrate-rich meal [79, 83, 87, 88].

1.4.3 Acid catalysed nitrosative stress

When the nitrite-rich saliva reaches the stomach and encounters the acidic gastric juice, nitrite is rapidly converted to nitrous acid and nitrosating species such as N_2O_3, NO^+ [28, 49, 78]. The latter reacts with thiocyanate (SCN), which is derived from the diet (milk and vegetables) and also secreted by salivary glands, at a considerable concentration, to form the particularly potent nitrosating species NOSCN [89-92]. In addition, thiocyanate is secreted directly into the gastric juice [89]. Thiocyanate concentration in saliva is much higher in smokers than non-smokers as a consequence of the detoxication of cyanide compounds in tobacco [93]. These nitrosating species can react with a variety of organic nitrogenous compounds to regenerate potentially carcinogenic *N*-nitroso compounds [49, 78].

Over the last two decades, *N*-nitroso compounds have been used as carcinogens in animal models of the GOJ and gastric cardia of the stomach [94]. In addition, these nitrosating species can trigger oxidative stress in gastric mucosa by inducing more consumption of local antioxidants; this mechanism may be relevant to upper gastrointestinal tract malignancies [48, 53].

The main factor protecting against the formation of the *N*-nitroso compounds, formed by the reaction between the nitrosating species and intra-gastric amines and amides, is ascorbic acid [52, 54, 95-97]. Ascorbic acid actively competes with secondary amines and amides for nitrosating species, preventing nitrosation [54, 98-100]. In this reaction, ascorbic acid reacts with nitrosating species and the latter is reduced to nitric oxide and the former oxidized to dehydro ascorbic acid [52-54, 101]. Stoichiometrically, one molecule of ascorbic acid should convert two molecules of nitrite in acidic conditions to nitric oxide [102].

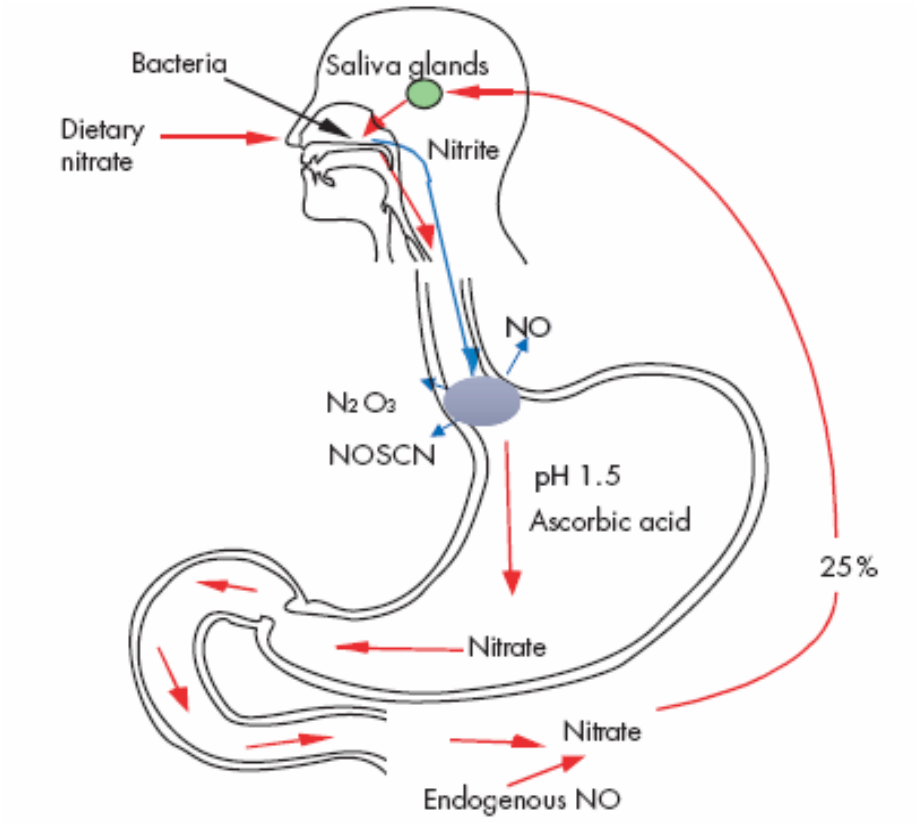


Figure 1.5: Dietary nitrate and entero-salivary recirculation, 25% of dietary nitrate is re-circulated, taken up by salivary glands and reduced to nitrite by the reducing bacteria on the dorsum of the tongue. The nitrite in the swallowed saliva is immediately converted to nitrous acid and nitrosating species such as N_2O_3, NO^+ when encounter acidic gastric juice. Further reaction with ascorbic acid takes place to produce nitric oxide and the former is oxidised to dehydroascorbic acid. The gastric cardia is where the nitrite in saliva first meets gastric acid, however, in patients with GORD the reactant site is the distal oesophagus [87].

Previous studies have reported that the active luminal nitrite chemistry takes place where the reactants first meet (i.e. when the nitrite in saliva encounters acidic gastric juice) [48, 87]. In the healthy acid-secreting stomach, the gastric cardia is the site where nitrite in saliva first meets gastric acid. However, in the patient with gastro-oesophageal reflux disease, the reaction site is moved proximally. Thus, the distal oesophagus instead of the cardia region has the highest concentration of nitrosating species and nitric oxide where the refluxing gastric acid meets the saliva [48, 55, 87, 103]. In addition, at these anatomical locations there is maximum local consumption of antioxidants in gastric juice, particularly ascorbic acid, as a result of active luminal chemistry [53].

It is also important to highlight the chemical environment of proximal gastric cardia and GOJ which may be relevant to high incidence of mutagenesis and neoplasia. Firstly at these locations, the nitrite to ascorbic acid ratio is high. Secondly, there is an adequate concentration of thiocyanate, which is an important catalytic agent for the nitrite conversion reaction, consequently causing the more rapid consumption of ascorbic acid. Thirdly, oxygen tension, swallowed food is the main source of oxygen delivered into the stomach; in addition to the epithelial cells, which are contains oxygen as well. In the presence of oxygen nitric oxide re-forms nitrosating species and this recycling pathway will continue until either the oxygen or ascorbic acid is completely consumed. Finally, gastric juice acidity is an important factor for the conversion reaction of nitrite to nitrosating species and it increases the rate of the recycling pathway [48, 53, 55, 87, 103, 104].

This suggests that in the healthy acid secreting stomach, the gastric cardia of the stomach is most fulfilling these criteria, while in patients with GORD the distal oesophagus is the optimal site. Indeed this might contribute to the high incidence of metaplasia and neoplasia in the proximal region of stomach and GOJ.

Therefore, an investigation of the influence of ascorbic acid, thiocyanate, oxygen tension, and different pH values on nitrite chemistry at the human GOJ was important.

1.5 Inhibition of nitrosative stress by vitamin C

Vitamin C is one of the most essential vitamins for good human health, as it plays an important role in numerous biological systems and also has a protective effect against carcinogenesis processes in many cells [105]. It acts as co-factors for many enzymes, which are involved in the synthesis of hormones, neurotransmitters, collagen, and other substances [106]. In addition, several studies have confirmed that it is one of the most important antioxidants in the aqueous fluid of most living tissues, as it neutralises the reactive oxygen and nitrogen species [99, 107, 108].

Vitamin C has two major forms: ascorbic acid (AA) and dehydroascorbic acid (DHA), and both of these forms have vitamin C activity and are inter-convertible by redox chemistry [109] . Under fasting conditions vitamin C is present in gastric juice mainly in the reduced form, ascorbic acid[54, 110] and is a potent antioxidant known to prevent gastric carcinogenesis through it ability to quench free radicals and inhibit nitrosamine formation[101, 111, 112].

However, little is known about the regulation of ascorbic acid secretion by gastric mucosa, other than that it is concentrated in the gastric mucosa and then actively secreted into gastric juice reaching a concentration of 20-300μM [52, 54, 113, 114]. Many factors affect the ascorbic acid concentration in gastric juice, for example, elevated pH of the gastric juice; chronic gastritis and infection with *H. pylori* significantly reduce the ascorbic acid concentration in gastric juice [54, 115, 116].

The main protective effect of ascorbic acid is not only that it neutralises the harmful DNA damaging effect caused by free radicals generated by *H. pylori* infection or smoking [117, 118]. Ascorbic acid reduces *N*-nitroso compounds formation by actively competing with secondary amines and amides for reaction with nitrosating species [54, 98-100, 112]. Ascorbic acid has a protective effect against gastric carcinogenesis through its ability to quench nitrosating species as well as inhibiting *N*-nitroso compounds formation in gastric juice.

However, it has been recognised that the luminal nitric oxide can react with oxygen and re-form nitrosating species, which can react with any remaining ascorbic acid [61, 104, 119, 120]. The rate of the reaction between nitric oxide and oxygen is increased by increasing the nitric oxide concentration, as it is related to the concentration of oxygen and the square of the nitric oxide concentration [61]. The recycling of nitric oxide in the presence of oxygen to re-form the nitrosating species continues until the ascorbic acid is depleted and this induces more nitrosative stress [48, 50, 104]. Moreover, the nitrosating species such as N_2O_3 are potentially carcinogenic compound directly through their ability to deaminate DNA bases or indirectly by forming of *N*- nitroso compounds [58-60]. Furthermore, nitric oxide in high concentration is shown to be mutagenic via the inhibition of a number of DNA repair enzymes [62].

However, recent studies have demonstrated that ascorbic acid may promote the nitrosation reaction within the epithelial lipid compartment despite inhibiting nitrosation within the luminal compartment [53, 121]. This due to that the nitric oxide generated by the reaction between salivary nitrite and ascorbic acid diffusing into adjacent epithelial cells and lipid compartments and reacting there with oxygen to generate N_2O_3 [56, 57, 61]. Indeed, the rate of reaction between the nitric oxide and oxygen is more rapid within the lipid than the aqueous compartment because both are more soluble in lipid than aqueous solutions [60, 122]. Thus, the presence of the lipid transforms the effect of ascorbic acid from a protecting against to promoting nitrosation [121]. The presence of lipid significantly alters acid

catalysed nitrosative chemistry (Figure 1.6). The lipid converts the effect of ascorbic acid from a inhibiting to promoting *N*-nitroso compounds formation. The ascorbic acid completely inhibits *N*-nitrosamines formation in the aqueous phase but not in the presence of the lipid [121].

Thus, other dietary antioxidants such as dietary phenolics have attracted considerable attention in recent years, as a consequence of ascorbic acid poor lipo-solubility and it is limitation in the adjacent lipid compartment.

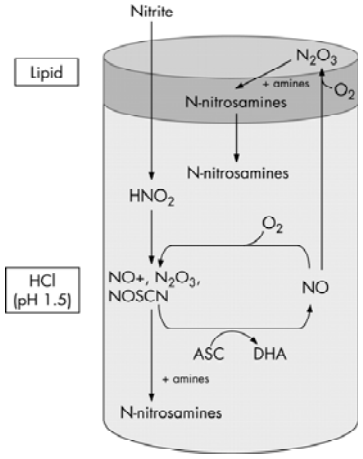


Figure 1.6: Proposed mechanism of *N*-nitrosamine formation in a dual-phase system on the presence of AA in the aqueous phase [121].

1.6 Dietary antioxidants

1.6.1 Phenolics in the diet

Dietary antioxidants have received considerable attention and interest in recent years. This arises from evidence that they may have beneficial effects against reactive oxygen species (ROS) and reactive nitrogen species (RNS). These ROS and RNS have been implicated in

the aetiology of many diseases such as cancer of the oesophagus and gastric cardia [123-125]. One the most important natural dietary antioxidants are dietary phenolics [126-129]. These water-soluble phenolic antioxidants are naturally present in the human diet, in a variety of fruits and vegetables as well as most beverages [130, 131]. It has been suggested that dietary phenolics play a beneficial role against nitrosation and nitrating species, however, their exact mechanism of action is still unclear [127, 132, 133].

Phenolic compounds are one of the major groups of polyphenol compounds, which consist of flavonoids, stilbenes, and lignans [130]. These polyphenol compounds are classified into different groups according to their phenol rings and the elements that bind these rings [130, 131]. In this study we examined a range of phenolics: caffeic acid (CA), ferulic acid (FA), chlorogenic acid (CGA), and gallic acid (GA). Gallic acid, belonging to the hydroxybenzoates, is mainly found in tea (tea leaves contain up to 4.5g/kg) and fruits while caffeic acid, ferulic acid, and chlorogenic acid belonging to the hydroxycinnamates, and are found in coffee and a variety of fruits such as apples, pears, peaches, plums [130, 131]. Vegetables (stems and leaves) are also an important source of dietary phenolics [130, 131].

Dietary phenolics are believed to have an important beneficial effect on human health. Many studies have evaluated the antioxidant activity of phenolic compounds in relation to atherosclerosis [134-136] . However less attention has been paid to the antioxidant activities of phenolic compounds in relation to gastric cancer. Therefore, an investigation of the antioxidant activity of dietary phenolics is important.

1.6.2 Antioxidant capacities

The stomach, particularly the cardia is subjected to many oxidative stresses (ROS and RNS) [48, 50, 53]. The production of nitrosating species, which are derived from the acidification of nitrite, can cause injury to gastric mucosa and play a significant role in the

aetiology of gastric adenocarcinoma [28, 49, 78]. Several studies have shown that an increased intake of dietary antioxidants may be associated with a lower risk of gastric cancer [123-125, 137]. The exact mechanism by which these dietary antioxidants may reduce the risk of gastric cancer has not been completely elucidated, although some studies suggested these antioxidants may provide protection against cancer by scavenging the nitrosating species and inhibiting the formation of *N*-nitroso compounds [127, 132, 133]. Several studies have reported that caffeic acid and related compounds provides protection against the *N*-nitrosamine compounds (reaction of nitrite and secondary amines in gastric juice) by the inhibition of the *N*-nitrosation reactions [138-140]. Furthermore, chlorogenic acid and related polyphenols competitively inhibit the nitrosation reaction of DNA by the nitrosating agent produced by nitrite under acidic conditions [141] and this may contribute to the protective effects of dietary phenolics against gastric cancer.

Moreover, recently many studies have demonstrated that the consumption of green tea inhibits nitrosation by competing with secondary amines for the nitrosating species [142, 143].

1.7 Aim

The aim of this *in vitro* study was to model the chemical reactions occurring at the human gastro-oesophageal junction (GOJ) after ingestion of nitrite; a mechanism which may be relevant to upper GI malignancies. The study aimed to elucidate the role of ascorbic acid (AA), sodium thiocyanate (NaSCN), different pH values (1.5, 2.5 and 3.0) and oxygen (O_2) on the nitrite chemistry in the simulated gastric juice.

This study also investigated the influence of a range of water- soluble dietary phenolics on the nitrite chemistry at simulated GOJ environment to compare their effect with that of ascorbic acid.

Chapter 2 – Materials and Methods

2.1 Chemicals and reagents

All chemicals (solvents and reagents) used in the studies were obtained from Sigma Aldrich (Poole, UK), except the ethylenediaminetetraacetic acid (EDTA) which was obtained from BDH Ltd., (Liverpool, UK).

2.2 The bench-top model

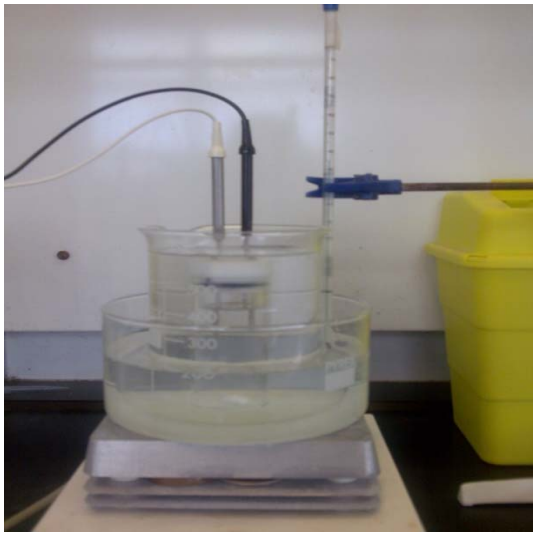
All studies were performed in a purpose-designed closed bench-top model, representing the human gastro-oesophageal junction, to study the chemical reaction occurring when nitrite encounters the simulated gastric juice. These studies focused on the measurement of nitric oxide formation and oxygen consumption by electrochemical detection.

The model consisted of a glass cylinder (75 mm tall and 25 mm internal diameter) equipped with a tight-fitting cap with two ports enabling insertion of the electrochemical nitric oxide and oxygen probes, as well as a third sampling port (Figure 2.1). The nitric oxide and dissolved oxygen meters were interfaced to a computer, converting the electrical signal (volts) to nitric oxide concentration in μM and oxygen level in %, respectively.

The capped glass cylinder was filled with 36ml of simulated gastric juice. The simulated gastric juice (HCl, 0.1M, pH 1.5) contained 1mM EDTA, 1mM sodium thiocyanate (NaSCN), and ascorbic acid or phenolic acid. Ascorbic acid (AA) was tested in final concentrations of 100μM, 500μM or 2000μM in parallel with negative controls. Dietary phenolics including ferulic acid, caffeic acid, gallic acid, and chlorogenic acid were tested in final concentrations of 250μM and 2000μM.

The system was immersed in degassed water and the experiments carried out in a 37°C water bath (Figure 2.1). The total incubation time was 70 minutes and the assay was initiated by adding $NaNO_2$ after ten minutes from incubation.

(A)



(B)

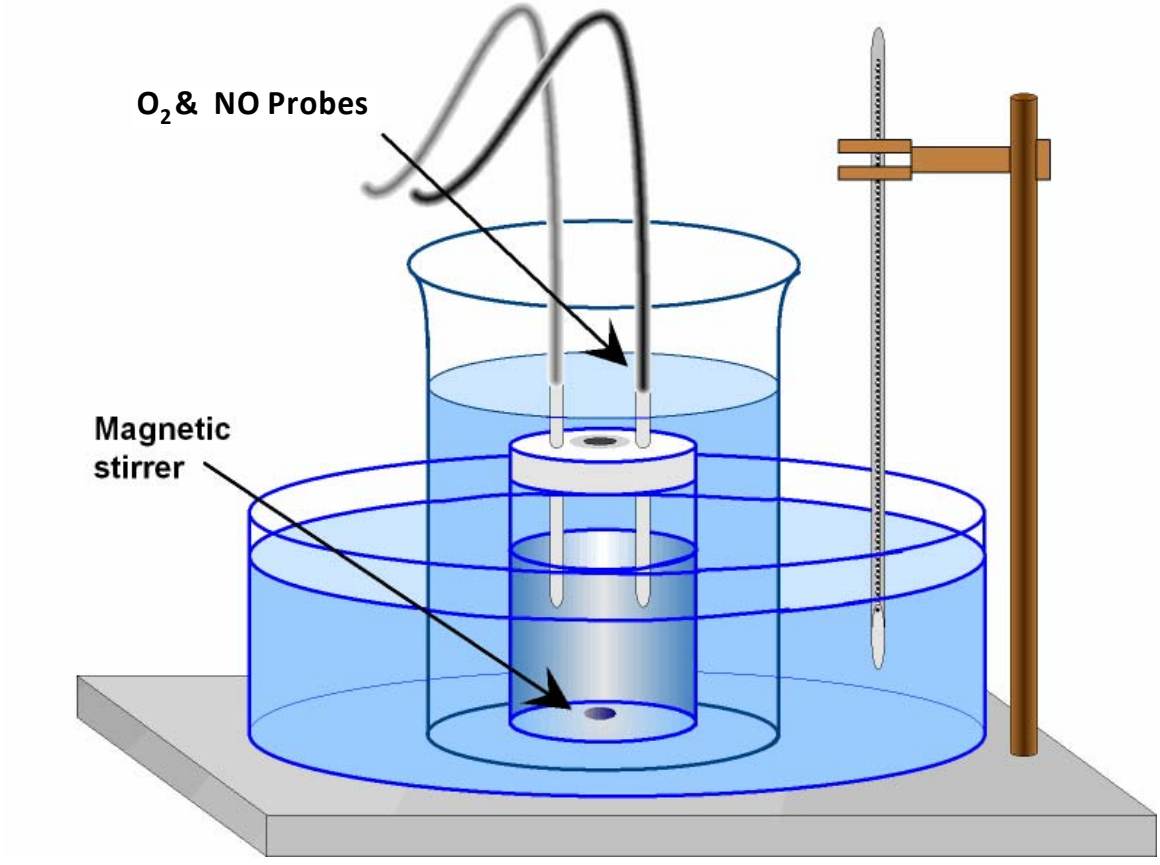


Figure 2.1 The bench-top model, (A) photograph and (B) diagram, representing the human GOJ, to study the chemical reactions occurring when nitrite encounters the simulated gastric juice

Elimination of oxygen from the system

One of the most important shortcomings of the early experimental set-up under anaerobic condition was the entry of oxygen into the system. To overcome this problem, a tight fitting cap was especially designed. The system was then immersed under deoxygenated water to reduce any entry of oxygen into the system. Indeed, it was found in subsequent experiments that this set-up resulted in the maximum reduction of oxygen entering the system. Furthermore, to achieve anaerobic conditions, the head space of the capped cylinder was filled with argon gas.

2.3 Assay set-up

To study the influence of AA, NaSCN, O_2 and different pH values (1.5, 2.5 and 3.0) on the nitrite chemistry was carried out in a bench-top model; described under section 2.2.

To investigate the influence of ascorbic acid in nitrite chemistry, the simulated gastric juice (HCl, 0.1M, pH 1.5) contained 1mM EDTA, 1mM NaSCN, in the absence or presence of ascorbic acid (100μM, 500μM or 2000μM).

Under aerobic conditions, the contents of the cylinder were aerated for two minutes before the incubation, whereas for anaerobic conditions the dissolved oxygen was removed from the solutions by purging helium through it for 5- 10 minutes, followed by a flow of argon to fill the headspace of the capped cylinder.

The contents of the cylinder were constantly mixed with a magnetic stirrer and the system was immersed under water, which was also purged with helium and placed in water bath at 37°C (Figure 2.1).

The assay was initiated by the addition of sodium nitrite (100 μM) at t=10 minutes and the incubated time was 60 minutes.

Furthermore, to investigate the influence of other parameters on the nitrite chemistry, each of the experiment was performed with and without NaSCN (1mM), at different pH values (1.5, 2.5 or 3.0) under both aerobic and anaerobic conditions.

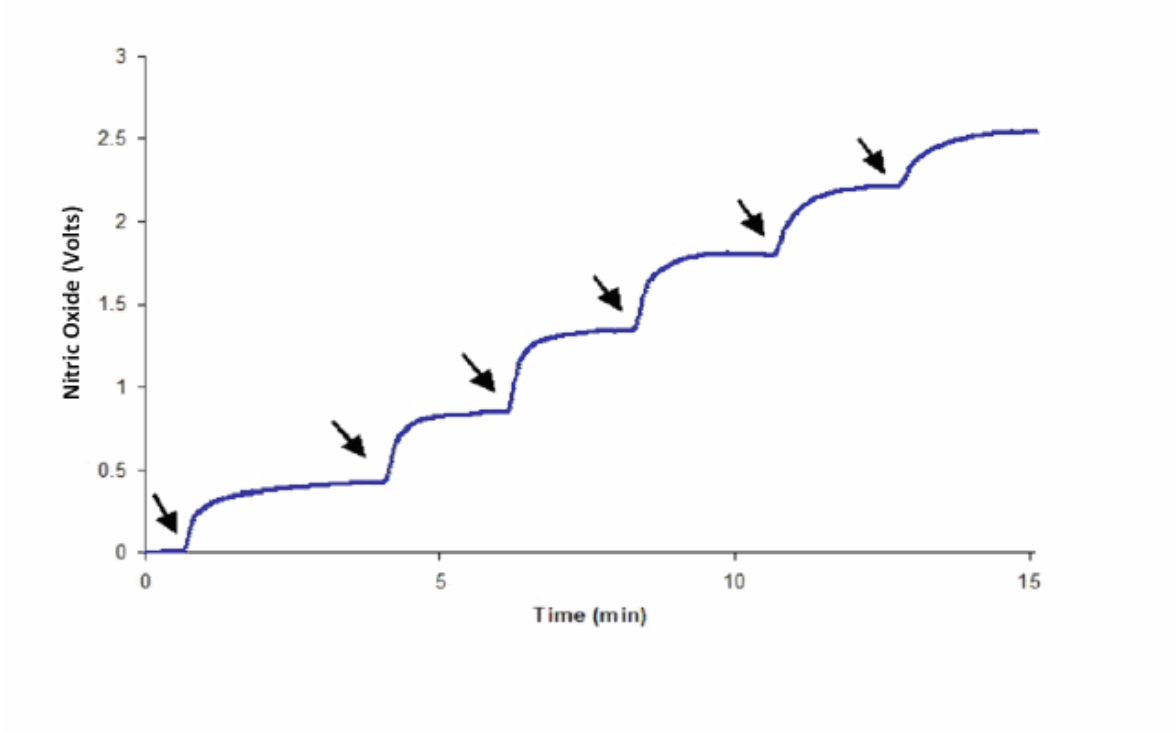
Samples were taken from the cylinder to measure the AA and DHA at the start and end (50µl or 500µl) respectively, of the incubation period. All experiments were carried out in duplicate.

To assess the influence of a range of dietary phenolics on nitrite chemistry in comparison with ascorbic acid, the studies were carried out in the bench-top model described under section 2.2. The simulated gastric juice (HCl, 0.1M, pH 1.5) contained 1mM EDTA, 1mM NaSCN, in the presence of either ascorbic acid or dietary phenolics. The dietary phenolics, including ferulic acid, caffeic acid, gallic acid and chlorogenic acid were tested in final concentrations of 250µM and 2000µM. The studies were performed under both aerobic and anaerobic conditions. All experiments were carried out in duplicate.

2.3.1 Nitric oxide measurement

Nitric oxide levels were monitored with an isolated dissolved nitric oxide electrode and meter (ISO – NO Mark II; World Precision Instruments, Sarasota, Florida, U.S.A). The nitric oxide probe was calibrated by adding successive bolus of 50µl $NaNO_2$ (0.7µM) to sulphuric acid (0.1 M) containing potassium iodide (0.1M) in a starting volume of 36 ml. Before adding the bolus of $NaNO_2$ to the solution, dissolved oxygen was removed by purging the system with helium for five minutes. Under these conditions, nitric oxide concentration in the solution was equal to nitrite concentration, i.e. one mole of nitrite produces one mole of nitric oxide. The NO meter was interfaced to a computer, converting the electrical signal (volt) to nitric oxide concentration in µM; the electrode response was linear to the range of concentrations tested (Figure 2.2A and B).

(A)



(B)

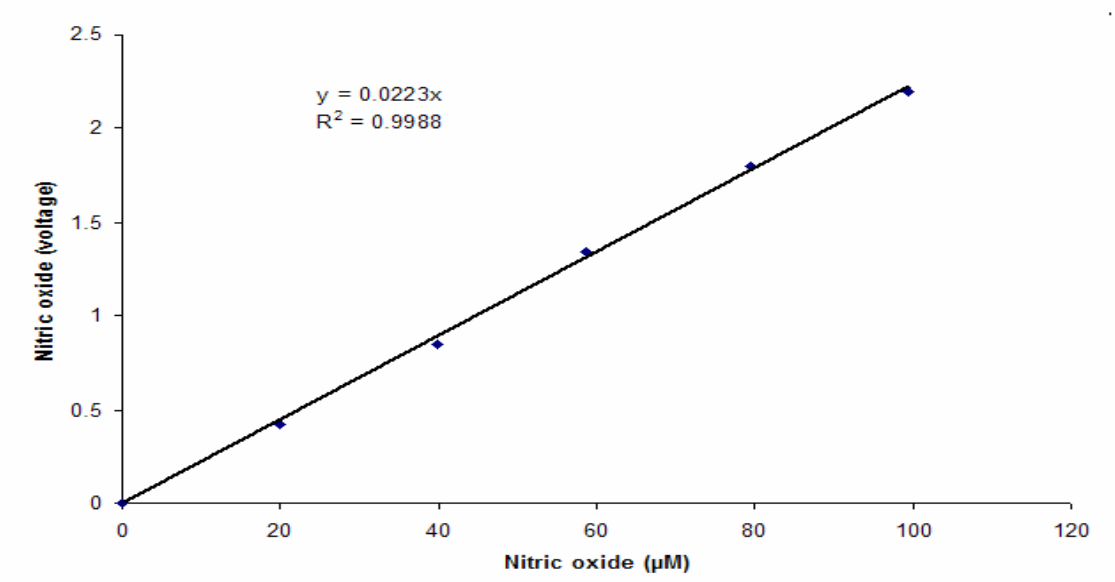
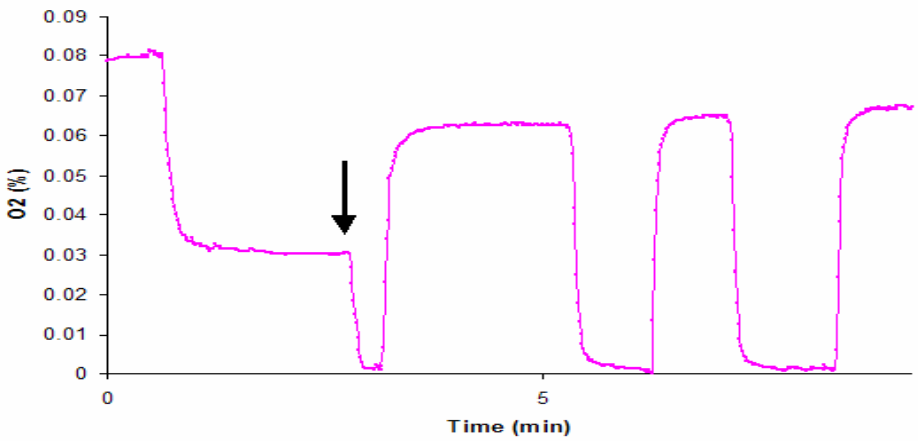


Figure 2.2 Calibration of nitric oxide probe, (A) by adding successive bolus dose of 50µl NaNO2 to sulphuric acid (0.1M) containing potassium iodide (0.1M), and (B) obtaining a linear response between electrical signal of NO (volts) and NO concentration (µM).).

2.3.2 Oxygen Measurement

The dissolved oxygen in the solution was monitored using an isolated dissolved oxygen meter and electrode (Mark II, World Precision Instrument Inc., Sarasota, U.S.A). The oxygen probe was calibrated at 37ºC by repeatedly transferring the probe between two beakers filled with aerated HCl (20.4% oxygen) and degassed HCl (bubbling helium through) under constant stirring. The NaSCN (1mM), AA (2000µM) and $NaNO_2$ (100µM) were added to degassed HCl in order for the meter to display zero% of oxygen (Figure 2.3A and B).

(A)



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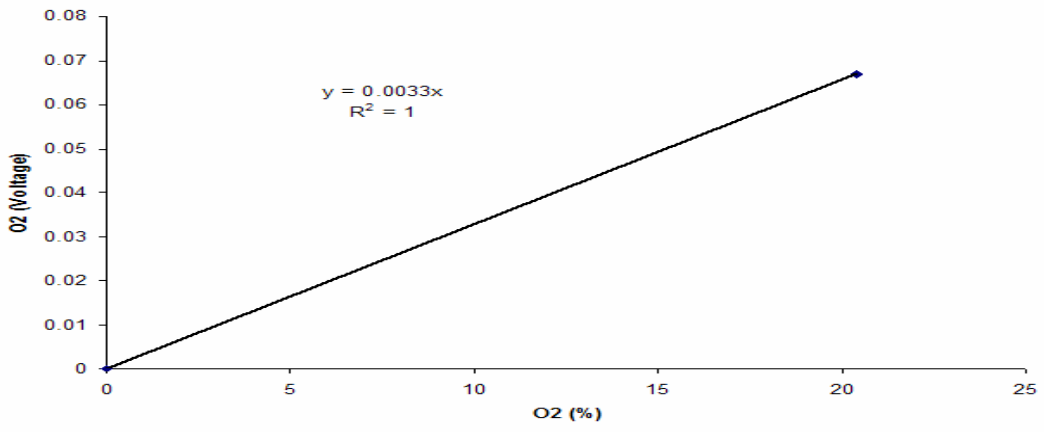


Figure 2.3 Calibration of oxygen probe, (A) the arrow is indicates the time of the addition of NaSCN, AA, and $NaNO_2$ and (B) obtaining a linear response between electrical signals of O2 (volts) andO_2 levels (%).

One of the major difficulties of these experiments was the accurate calibration of the oxygen probe. The difficulties were mainly due to the extremely sensitive nature of the probe to achieve a right calibration. Any inaccuracy in the calibration process can result in over- or under-estimation of the value of the dissolved oxygen in the solution. This, in turn, would undermine the measurement processes. Therefore, great care was taken during the calibration of the oxygen probe. Indeed, it was common practice to calibrate the oxygen probe daily before starting the experiment. It was found that with this practice the experiments were highly reproducible.

2.4 Sampling procedure

Before starting the reaction, two 50µl samples were taken from the cylinder to be used to measure the baseline values of ascorbic acid (AA) and dehydroascorbic acid (DHA). After sixty minutes from the addition of nitrite, a further two samples (500µl) were taken to assess final AA/ DHA levels.

The samples for AA measurement (50µl or 500µl) were added to Eppendorf tubes containing an equal volume of 2% metaphosphoric acid and 0.5% sulfamic acid. The samples for DHA measurement (50µl or 500µl) were added to Eppendorf tubes containing an equal volume of 6 mg/ml dithiothreitol (DTT) and 2% metaphosphoric acid.

The purpose of the DTT was to generate AA from any DHA in the sample, thus allowing quantification of the total vitamin C (TVC) levels, allowing the amount of DHA present in the sample to be estimated by subtracting AA from TVC. To remove any remaining nitrite, sulfamic acid was added to the sample. All samples were kept at - 20°C until analysis by high performance liquid chromatograph (HPLC), based upon the method of Sanderson and Schorah [144, 145]

2.4.1 Ascorbic acid analysis

Before the analysis, samples were thawed and centrifuged at 1000g ALC (Multi-Speed Refrigerated Centrifuge) (CPK 131R) from Thermo Life Sciences. Remove 0.3ml of the samples supernatant were further centrifuged in a minifuge at 9000g (Mini-spine, Eppendorf). AA and TVC levels were measured by HPLC. The instrumentation was comprised of a Shimadzu L-ECD-6A Electrochemical Detector, Shimadzu LC-10AT VP pump, and Shimadzu model SIL-10AD VP with 50µl loop injector. AA was measured using reverse- phase on a Phenomenex 5µm C 18 Luna analytical column 150 x 4.6 mm and protected by an Anachem guard column 20 x 3 mm; Packing Material, Lichroprep RP-18 (25-40µm). The acetate buffer solution consisted of 0.1M sodium acetate and 0.1M octylamine adjusted to pH 4.3 using 50% acetic acid. The mobile phase pumped through HPLC consisted of 85% acetate buffer and 15% acetonitrile. The flow rate was 0.8ml/min, generating a column pressure of approximately 1000psi. The retention time of AA was 2.4 minutes. The aqueous stock standard solution was prepared by adding 5 mg AA to 5.0ml of DDT (3.5mg/ml) in MPA/SA 50/50 volumes. In working standards of 5, 10, 20, 30, and 40µg/ml of AA were prepared by taking 50, 100, 200, 300 and 400 ml of 1 mg/ml AA stock standard solution (1mg/ml), making the volume up to 10 ml in MPA/SA 50/50 volumes. The auto-sampler was programmed to inject 25µl aliquots of standards and samples. To remove the oxidizing sites on the column 25µl of the DDT solution was injected prior to the standards and samples. The number of injections through the HLPC was restricted to 55 due to the decomposition of AA, these include the standards before and after two sets of 20 samples [145, 146].

2.5 Data analysis

All the data is presented as mean values of two experiments. The nitric oxide and oxygen values were highly reproducible and thus only two experiments performed per study and

presented as mean value. The Pearson correlation coefficient (CC) was calculated for typical experiments in which there were changes in nitric oxide values with time (experiment 3.1.2) between the two duplicates and this gave values of CC=0.75, 0.97 and 0.91 for 100μM, 500 μM and 2000 μM, respectively. Similarly, for experiment 3.1.3 where oxygen varied with time and again the changes in the mean values of the two duplicates of the each experiment were correlated tightly, the Pearson correlation coefficient were 0.79, 0.91 and 0.63 for 100μM, 500μM and 2000 μM, respectively. The statistical analysis of data of ascorbic acid results was performed either by ANOVA or by Student t- test. Statistically significance of the differences was depicted with P value of less than 0.01 and 0.05.

Chapter 3 – Results and Discussions

OVERVIEW

In this chapter experimental results are presented using the simulated gastric juice system described in Chapter 2.2, to study the role of various factors affecting nitrite chemistry at the simulated GOJ environment.

Also presented are the effects of a range of dietary phenolics (water-soluble antioxidant) on the nitrite chemistry and a comparison of their effect with ascorbic acid.

The study focused on the measurement of NO and O_2 levels by electrochemical detection. The result of these sections is followed by brief discussion.

3.1 Nitrite chemistry in the simulated gastric juice: effect of ascorbic acid

3.1.1 Nitric oxide and dissolved oxygen levels following nitrite addition in the absence of ascorbic acid

The addition of nitrite (100µM) to the simulated gastric juice (with NaSCN, pH 1.5, under aerobic condition) in the absence of ascorbic acid only led to a limited amount of nitric oxide being formed (0.34µM) (Figure 3.1). Meanwhile, dissolved oxygen levels decreased slightly after nitrite addition (from 20.7% to18.7%) before returning to baseline levels at t= 70 minutes (Figure 3.1).

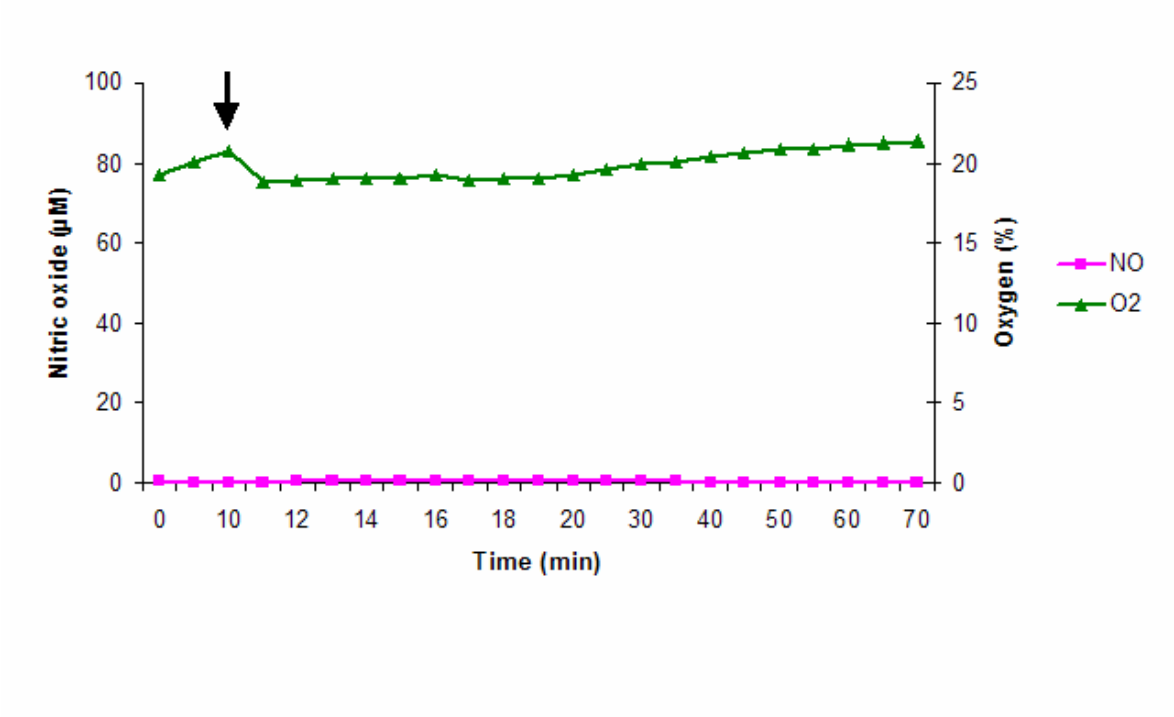


Figure 3.1: Nitric oxide levels (µM) (squares) and dissolved oxygen levels (%) (triangles) in the simulated gastric juice (NaSCN, pH 1.5) following nitrite addition (100µM) at t=10 min, in absence of ascorbic acid under aerobic condition. Values are presented as mean of two experiments.

3.1.2 Nitric oxide levels following nitrite addition in presence of different concentrations of ascorbic acid

When ascorbic acid was present in the system described under 3.1.1, it increased the production of nitric oxide and produced a more marked fall in oxygen concentration (Figure 3.2 and 3.3). With ascorbic acid 100µM, the nitric oxide concentration reached a peak value of 17.45µM one minute after the addition of nitrite. The nitric oxide concentration then slowly decreased to 2.2µM by the end of the incubation time period. With ascorbic acid 500µM, the nitric oxide concentration peaked at 68µM approximately five minutes after the addition of nitrite and then fell to 3µM after thirty minutes. With ascorbic acid 2000µM, the nitric oxide rose in a similar manner to that observed following ascorbic acid 500µM, reaching a value of 63.1µM at ten minutes. However, unlike the two lower concentrations of ascorbic acid, the nitric oxide concentration following ascorbic acid 2000µM remained elevated throughout the duration of the experiment (Figure 3.2).

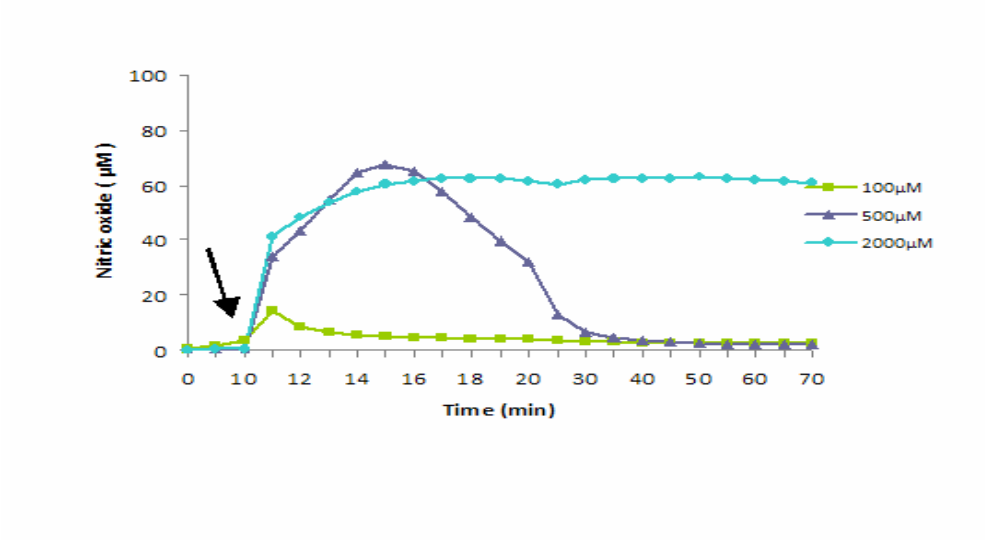


Figure 3.2: Nitric oxide levels (µM) in the simulated gastric juice (NaSCN, pH 1.5), following nitrite addition (100µM) at t=10 min, in presence of ascorbic acid 100µM (squares), 500µM (triangles) or 2000µM (circles) under aerobic condition. Values are presented as mean of two experiments.
The CC between the two duplicates was 0.75, 0.97 and 0.91 for 100µM, 500µM and 2000µM, respectively.

3.1.3 Dissolved oxygen levels following nitrite addition in presence of different concentrations of ascorbic acid

In the presence of ascorbic acid 100µM, the rise in nitric oxide concentration was mirrored by a fall in oxygen concentration from a baseline value of 20% to 16% one minute after the addition of nitrite. Thereafter, the oxygen concentration remained steady or slightly increased back towards baseline level. With ascorbic acid 500µM, the more marked rise in nitric oxide was mirrored by a more marked fall in dissolved oxygen concentration with the nadir of oxygen concentration occurring at five minutes after the addition of nitrite being only 1% and then slowly increasing and returning to 15% by the end of the experiment. With ascorbic acid 2000µM, the oxygen concentration decreased to become undetectable within the first three minutes and remained undetectable throughout the duration of the experiment (Figure 3.3).

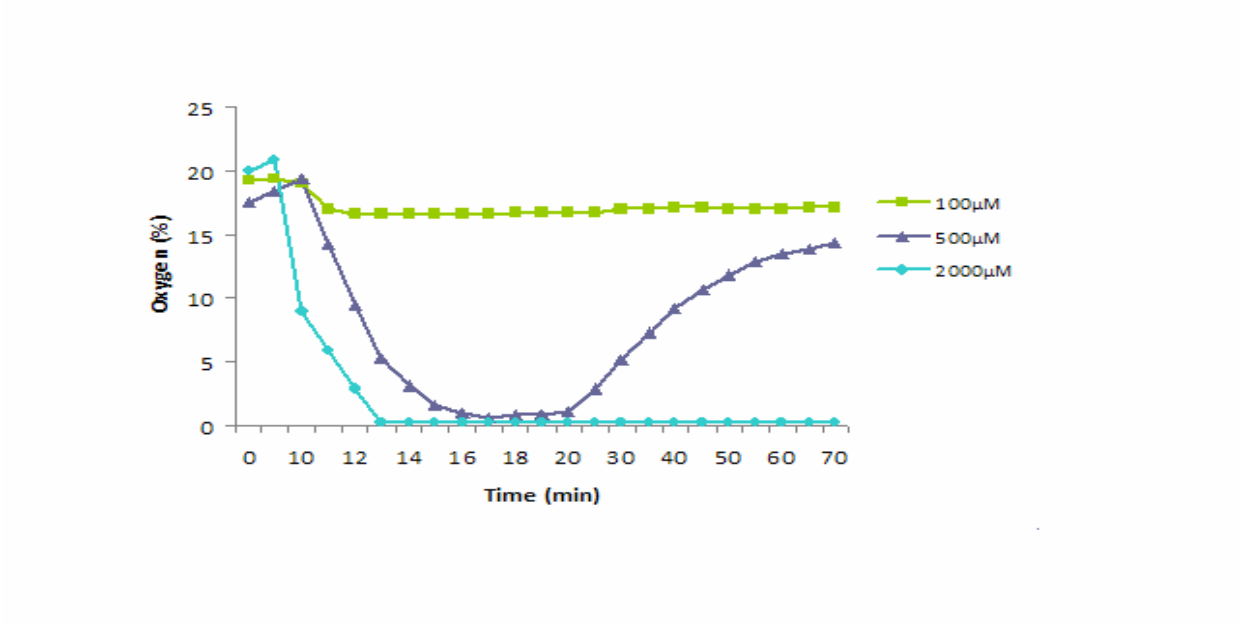


Figure 3.3: Dissolved oxygen levels (%) in the simulated gastric juice (NaSCN, pH1.5), following nitrite addition (100µM) at t=10 min, in presence of ascorbic acid 100µM (squares), 500µM (triangles) or 2000µM (circles). Values are presented as mean of two experiments.
The CC between the two duplicates was 0.79, 0.91 and 0.63 for 100µM, 500µM and 2000µM, respectively.

3.1.4 Ascorbic acid measurements

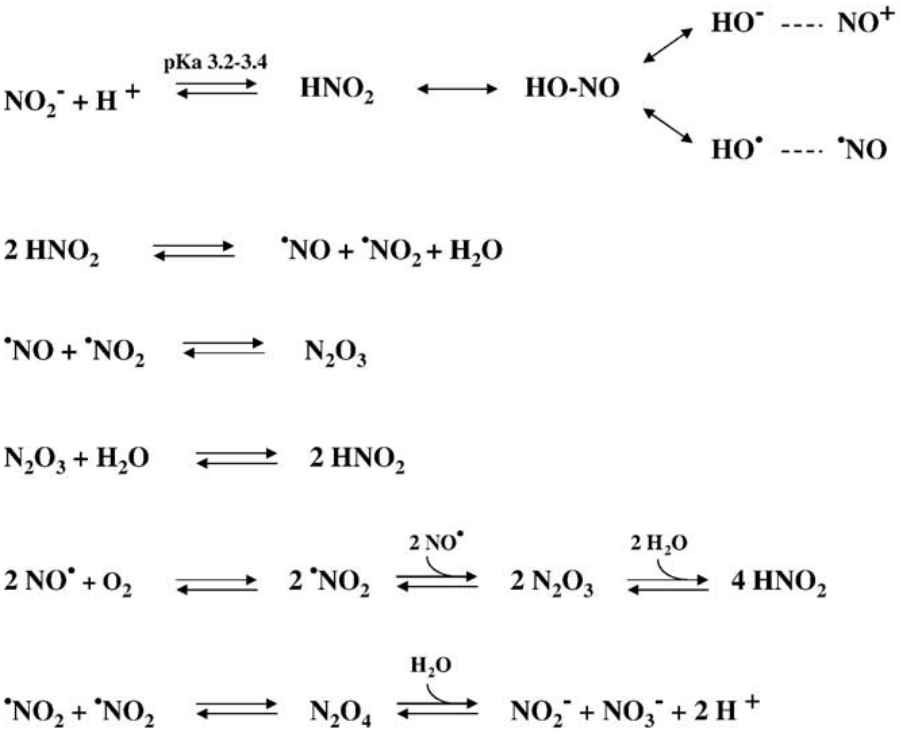
In the experiment with 100μM ascorbic acid, the ascorbic acid was entirely consumed by the end of the experiment. In the experiment with 500μM ascorbic acid, 91.5% of the ascorbic acid was consumed. With 2000μM ascorbic acid significant amounts of ascorbic acid remained at the end of the experiment with only 32% being consumed (Table 3.1).

Table 3.1: Ascorbic acid consumed (%) in the simulated gastric juice (NaSCN, pH 1.5), following nitrite addition (100μM) in the presence of ascorbic acid 100μM, 500μM or 2000μM over 60 minutes incubation, under aerobic conditions. Values are presented as mean of two experiments and their statistical difference.

[AA] μM (at t= 0)	AA consumed (%) (at t= 70 min)	P value
100	99.18	$P < 0.05$
500	91.47	$P < 0.05$
2000	32.05	$P < 0.01$

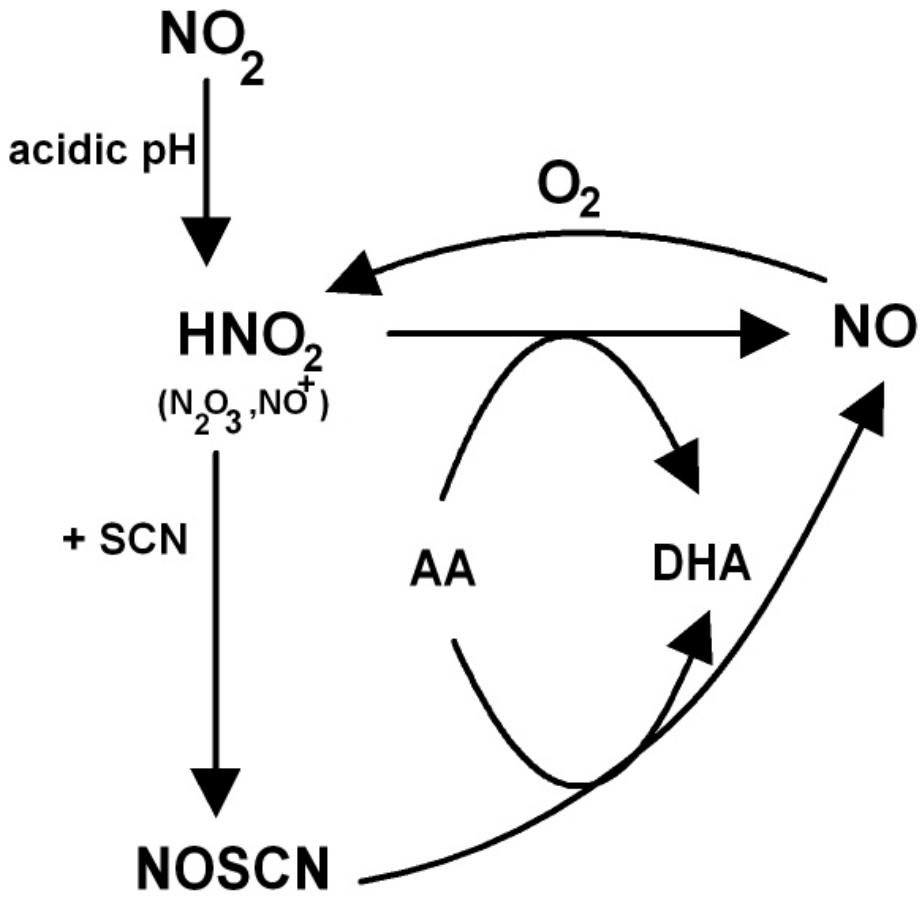
3.1.5 Discussion

The addition of nitrite 100μM to the HCl pH1.5 containing sodium thiocyanate 1mM produced only a very small increase in the nitric oxide concentration reaching a peak of 0.34μM. The pKa of nitrite is 3.5 and at the experimental pH of 1.5, the added nitrite is converted to nitrous acid, nitrosating species including N_2O_3, NO^+ and NOSCN, and the small concentration of nitric oxide [28, 49, 78]. When the experiment was repeated in the presence of 100μM ascorbic acid, the rise in nitric oxide was much more marked reaching a peak of 17.45μM. This can be explained by the ascorbic acid reacting with the nitrosating species with the latter being reduced to nitric oxide and the former being oxidised to dehydroascorbic acid [52-54, 101].



Scheme 3.1:. Nitrite chemistry in acidic aerobic solutions [147].

In the presence of ascorbic acid 100μM, the addition of nitrite also resulted in a fall in the dissolved oxygen concentration from 20% to 16% and this fall mirrored the rise in nitric oxide. This fall in oxygen can be explained by the nitric oxide formed by the reaction between the ascorbic acid and the nitrosating species reacting with the dissolved oxygen to form NO_2, N_2O_3, nitrous acid and nitrosating species scheme 3.2 [61, 104, 119, 120].



Scheme 3.2: Chemical reaction occurring when nitrite encounter the simulated gastric juice containing thiocyanate and ascorbic acid under aerobic condition.

The rate of the reaction between nitric oxide and oxygen is directly related to the concentration of oxygen and to the square of the nitric oxide concentration [61, 119]. The reaction between the nitric oxide and dissolved oxygen to re-form nitrosating species results in recycling of the nitrosating species and thus consumption of the ascorbic acid.

This can explain why only 17.45μM nitric oxide was formed when equimolar amounts of ascorbic acid and nitrite were present. As the nitric oxide was being produced, it was also being consumed by reacting with the oxygen. This recycling also meant that the ascorbic acid would be fully consumed; preventing any further formation of nitric oxide and thus the nitric oxide concentration fell. In the experiments with ascorbic acid 100μM, there was no ascorbic acid detectible at the end of the experiment.

The experiment with the ascorbic acid 500μM showed a much more marked rise in nitric oxide, a more marked fall in oxygen, and again the great majority of ascorbic acid was consumed by the end of the experiment. The changes noted with ascorbic acid 500μM can again be explained by the mechanism discussed above.

When the experiment was performed with ascorbic acid 2000μM present, clear differences were seen from those observed with the lower doses. The nitric oxide concentration reached a peak of 63μM and remained at this level throughout the study period. In addition, the oxygen concentration fell to undetectable levels and remained undetectable throughout the experiment. Furthermore, the experiment differed from the earlier experiments in that all the added ascorbic acid was not consumed during the experiment. The higher concentration of ascorbic acid in this experiment would mean that recycling of nitrosating species could continue until the oxygen was completely depleted. In contrast, in the earlier experiments the rate controlling factor in the recycling process was the ascorbic acid which became completely depleted rather than the oxygen. The complete depletion of the oxygen and the persistence of ascorbic acid can explain why the nitric oxide concentration remained elevated throughout the experiment without showing any evidence of a fall. The main reason for the nitric oxide falling in the earlier experiments was that it was reacting with oxygen to re-form nitrosating species and nitrous acid [61, 104, 119, 120]. In the absence of oxygen, this reaction cannot take place. In addition, if any small amounts of

oxygen were to enter the system and react with nitric oxide to re-form nitrosating species then the remaining ascorbic acid would rapidly react with these nitrosating species to reproduce nitric oxide.

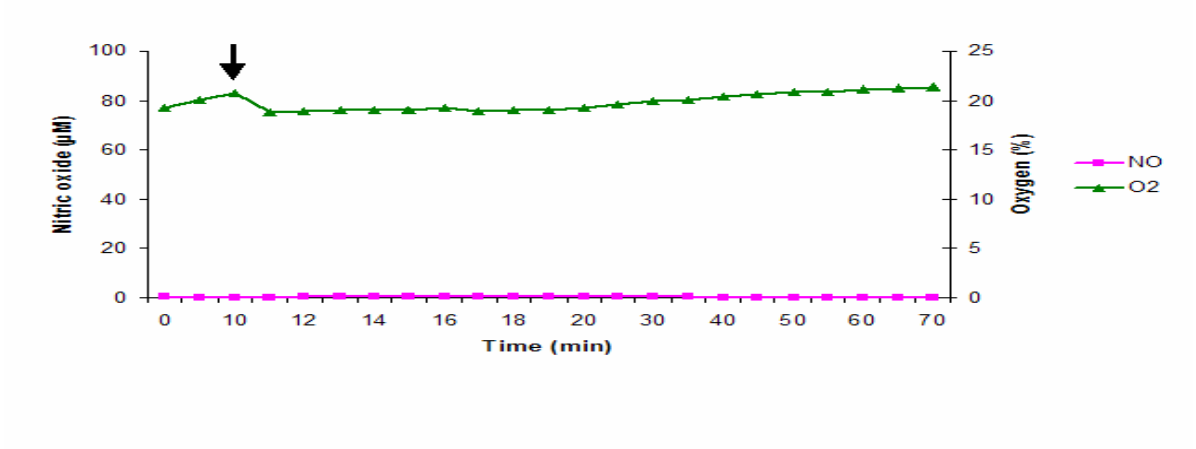
Though we added nitrite 100μM we did not produce nitric oxide100μM. There are several explanations for this. The first is that some of the nitric oxide may have been lost from the system. The second is that during the recycling process some of the products end up as nitrate and in this way can no longer be recycled [147].

3.2 Nitrite chemistry in the simulated gastric juice: effect of thiocyanate

3.2.1 Nitric oxide and dissolved oxygen levels following nitrite addition in the absence of ascorbic acid

In the absence of ascorbic acid, the change in nitric oxide and oxygen on adding nitrite to hydrochloric acid pH1.5 was identical in the presence or absence of sodium thiocyanate 1mM (Figures 3.4A and B).

(A)



(B)

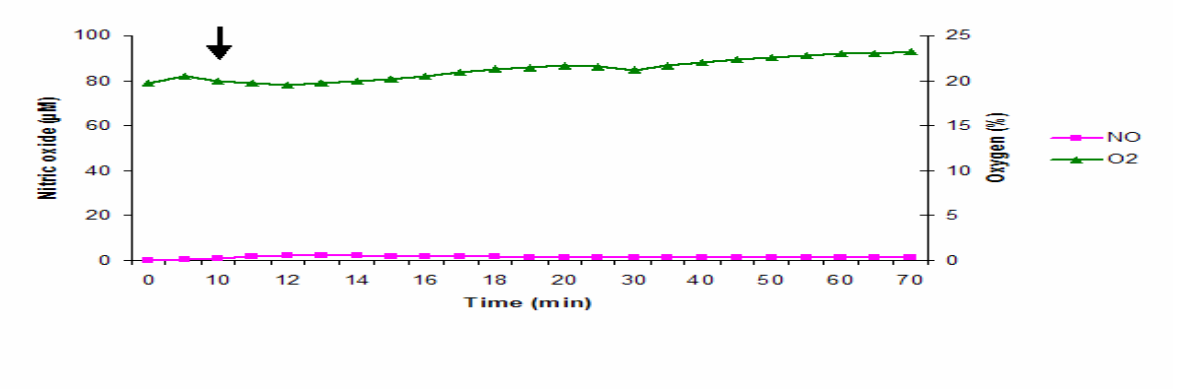


Figure 3.4: Effect of thiocyanate, presence (A) and absence (B) on nitric oxide levels (µM) (squares) and dissolved oxygen (%) (triangles) in the simulated gastric juice, following nitrite addition (100µM) at t=10 min, in absence of ascorbic acid. Values are presented as mean of two experiments.

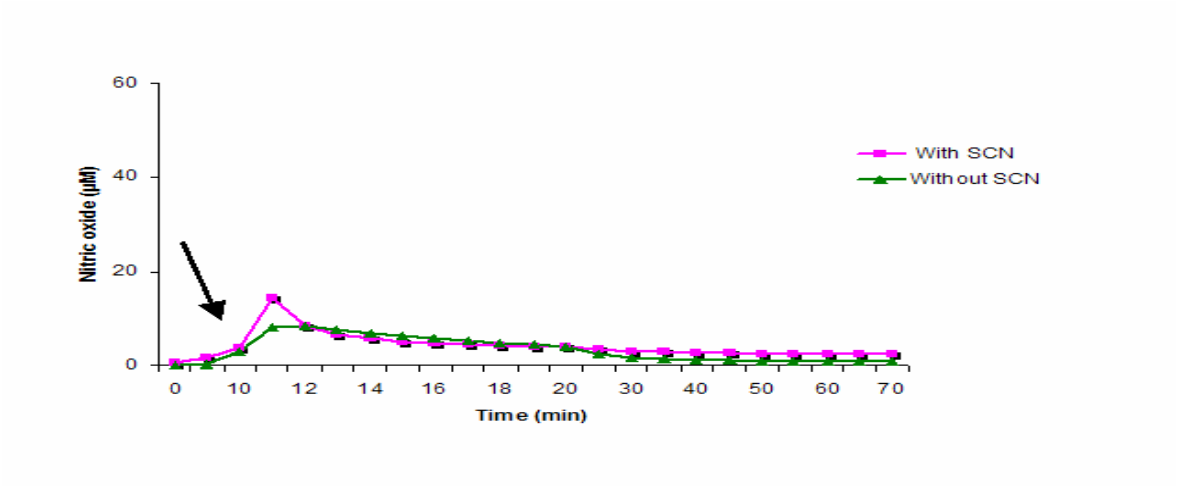
3.2.2 Nitric oxide levels following nitrite addition in the presence of different concentrations of ascorbic acid

When nitrite 100μM was added to the HCl pH1.5 containing ascorbic acid 100μM in the presence of sodium thiocyanate it produced a higher peak nitric oxide concentration of 17.45μM compared with 8.6μM in the absence of thiocyanate. In the presence of thiocyanate the peak nitric oxide concentration occurred one minute after the addition of nitrite and then slowly fell. The rise in nitric oxide in the absence of thiocyanate appeared to be slower and not occurring until two minutes after the addition of nitrite (Figure 3.5A).

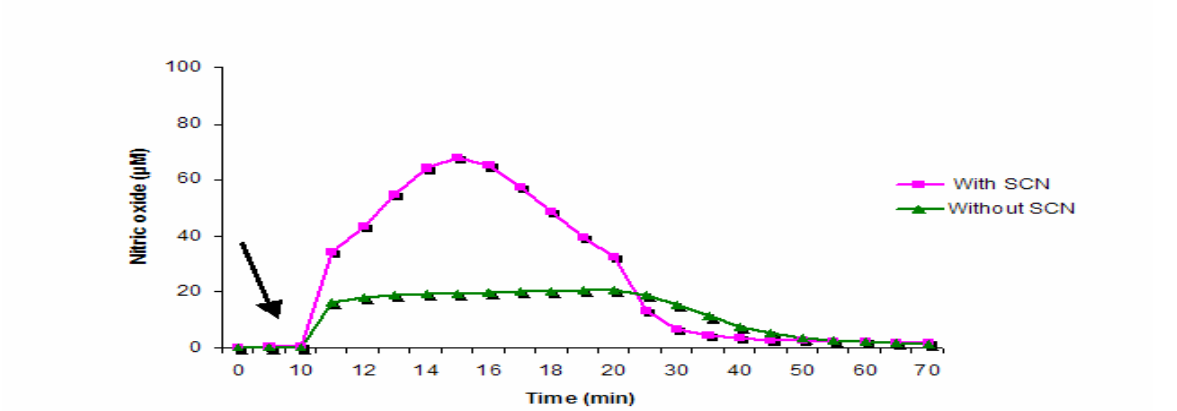
With ascorbic acid 500μM there was a marked difference in the peak nitric oxide concentration reached. In the presence of thiocyanate a peak nitric oxide concentration of 68μM was reached five minutes after adding nitrite. In contrast, the peak nitric oxide concentration in the absence of thiocyanate was 20.7μM and occurred ten minutes after the addition of nitrite. The profile of the rise in nitric oxide was also different in the presence and absence of thiocyanate. In the presence of thiocyanate there was a clear rise and then clear fall in the nitric oxide concentration. In contrast, in the absence of thiocyanate the nitric oxide spheres to plateau at a level of approximately 20μM and remained at that level between two and ten minutes after adding the nitrite and then it slowly fell (Figure 3.5B).

With ascorbic acid 2000μM there was a marked rise in nitric oxide both in the presence and absence of thiocyanate and in both cases the nitric oxide remained elevated throughout the duration of the experiment. However, there did appear to be some differences in the profile of the nitric oxide response in the presence versus absence of thiocyanate.

(A)



(B)



(C)

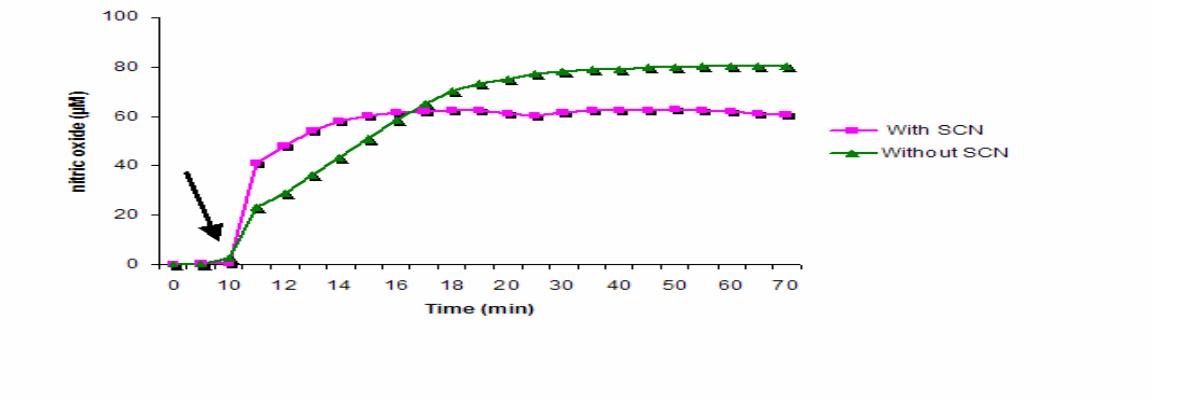


Figure 3.5: Nitric oxide levels (µM) in the simulated gastric juice at pH 1.5, on absence of thiocyanate (triangles) and presence (squares), following nitrite addition (100µM) at t=10min, in the presence of ascorbic acid 100µM (A), 500µM (B) or 2000µM (C) under aerobic condition. Values are presented as mean of two experiments.

In the presence of thiocyanate, the initial rise in nitric oxide was more rapid than in the absence of thiocyanate and in the presence of thiocyanate the level of the plateau of nitric oxide was approximately 15μM lower than that obtained in the absence of the thiocyanate (Figure 3.5C).

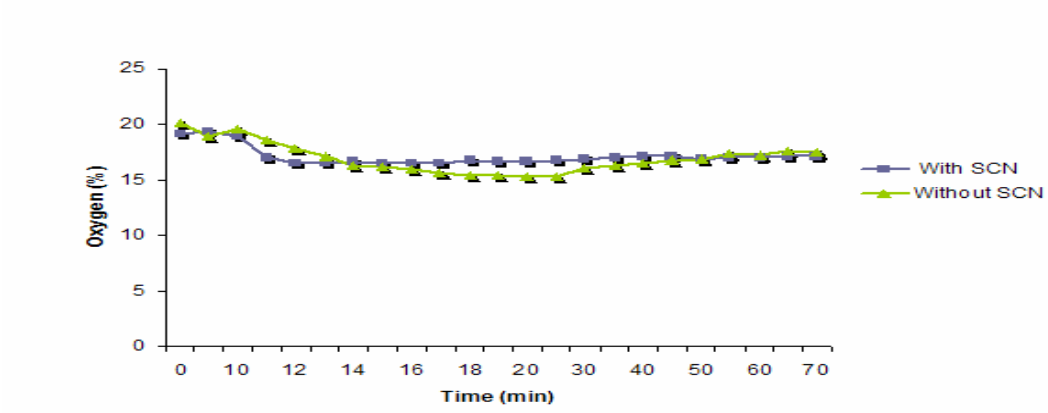
3.2.3 Dissolved oxygen levels following nitrite addition in presence of different concentrations of ascorbic acid

With the ascorbic acid 100μM, the change in oxygen concentration reflected the changes in nitric oxide with a more rapid fall in oxygen in the presence of thiocyanate (Figure 3.6A).

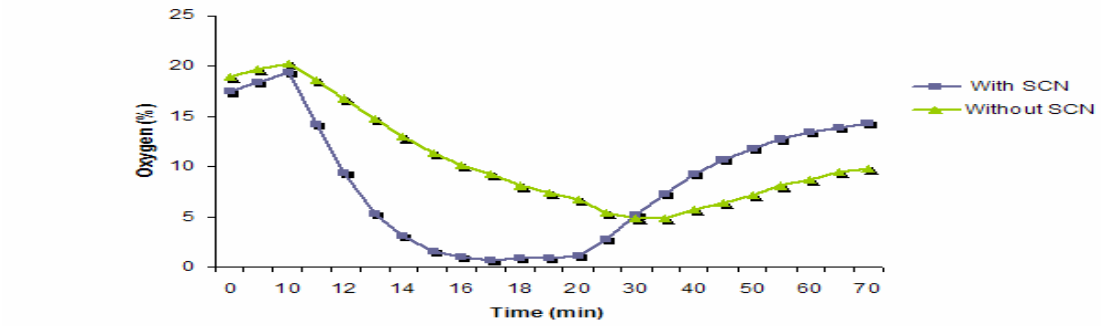
With the ascorbic acid 500μM, the changes in oxygen also reflected the changes in nitric oxide. In the presence of thiocyanate the oxygen level fell more rapidly and was almost undetectable for five minutes and then slowly increased again. In contrast, in the absence of thiocyanate the fall in oxygen was slower, reaching a nadir of 5% at twenty minutes and then showing a slow rise (Figure 3.6B).

With the ascorbic acid 2000μM, the oxygen was completely depleted in the presence of thiocyanate within the first three minutes and remained undetectable throughout the experiment. In contrast the fall in oxygen was less rapid in the absence of thiocyanate, reaching a nadir of 1.5% at six minutes and then slowly rising (Figure 3.6C).

(A)



(B)



(C)

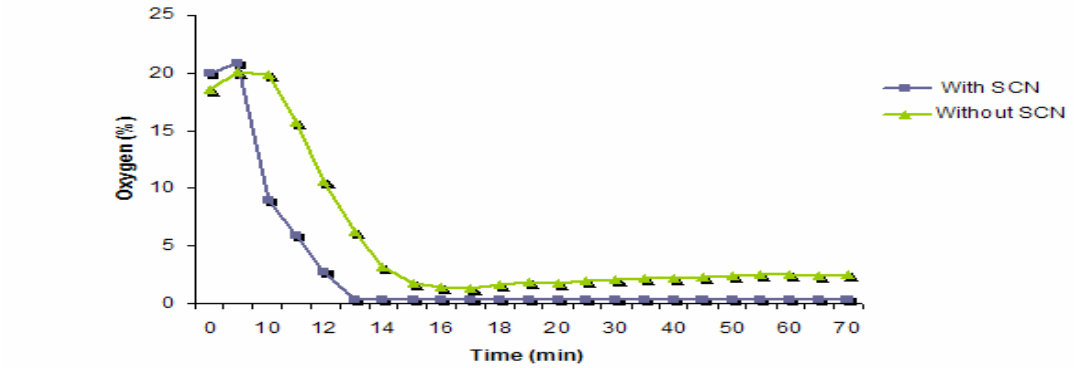


Figure 3.6: Dissolved oxygen levels (%) in the simulated gastric juice on absence of thiocyanate (triangles) and presence (squares), following nitrite addition (100µM) at t=10 min, in the presence of ascorbic acid 100µM (A), 500µM (B) and 2000µM (C). Values are presented as mean of two experiments.

3.2.4 Ascorbic acid measurements

In the ascorbic acid 100μM, all of the ascorbic acid was consumed in the presence of thiocyanate whereas only approximately 50% was consumed in the absence of thiocyanate. In addition with ascorbic acid 500μM, 91% was consumed in the presence of thiocyanate compared with only 80% in the absence of thiocyanate. With the ascorbic acid 2000μM substantial amounts remained present in both the presence and absence of thiocyanate (Table 3.2).

Table 3.2 Thiocyanate effect on ascorbic acid consumption (%) in the simulated gastric juice pH 1.5 under aerobic condition, following nitrite addition (100μM) in presence of ascorbic acid 100μM, 500μM or 2000μM over 60 minutes incubation. Values are presented as mean of two experiments.

[AA]μM at t=0	AA consumed (%) with NaSCN at t= 70 min	AA consumed (%) without NaSCN at t= 70 min	P Value
100	99.18	44.43	$P < 0.05$
500	91.47	80.72	$P < 0.05$
2000	32.05	29.24	

3.2.5 Discussion

In the presence of thiocyanate, the rise in nitric oxide concentration was more rapid and at the two lower concentrations of ascorbic acid, produced a higher peak concentration. In the presence of thiocyanate, at acidic pH, the NO^+ reacts with thiocyanate to form the nitrosating species NOSCN [49, 78, 90-92]. This is the dominant species which is thought to react with ascorbic acid to form nitric oxide [49, 78, 90-92]. In contrast, in the absence of thiocyanate the nitrosating species are N_2O_3 and NO^+. The more rapid rise in nitric oxide concentration can be explained by the raised affinity of NOSCN following ascorbic acid compared to these other nitrosating species at the pH of this experiment.

In the experiment with ascorbic acid 500μM, the presence of thiocyanate produced a clear peak nitric oxide concentration and then clear fall, whereas in the absence of thiocyanate there was a lower peak concentration but more of a plateau of nitric oxide with a less rapid fall. The more rapid fall in nitric oxide in the presence of thiocyanate may be explained by the more rapid consumption of ascorbic acid and the fall in nitric oxide concentration occurring when the ascorbic acid has been depleted[104]. The consumption of ascorbic acid occurs due to the recycling of nitric oxide and its reactive oxygen to reform nitrosating species. In the thiocyanate the fall in oxygen was more rapid. This can be explained by the higher concentration of nitric oxide produced in the presence of thiocyanate and the reaction between nitric oxide and oxygen being related to the square of nitric oxide concentration [61, 119, 120]. The changes in the ascorbic acid concentration would also be consistent with the ascorbic acid being more rapidly consumed in the presence of thiocyanate as the concentrations of ascorbic acid at the end of the experiment were lower in the presence versus absence of thiocyanate. The slower consumption of ascorbic acid in the absence of thiocyanate would mean that when nitric oxide reacted with oxygen to reform nitrosating species, the latter could still react with ascorbic acid and reform nitric

oxide. One would therefore have a steady state with the consumption of nitric oxide by its reaction with oxygen equal to the rate of its regeneration by the reaction of nitrosating species with ascorbic acid and therefore producing the plateau level of nitric oxide.

In the presence of thiocyanate the oxygen concentration was higher at the end of the experiment than in the absence of thiocyanate. The rise in oxygen in the presence of thiocyanate can be explained by oxygen diffusing into the system. Though this would also happen in the absence of thiocyanate, the persisting nitric oxide in the absence of thiocyanate would be able to react with the oxygen converting it into nitrous acid.

In the experiment with ascorbic acid 2000μM, the rise in nitric oxide was more rapid in the presence of thiocyanate but the plateau level achieved was slightly lower. The more rapid rise in nitric oxide can again be explained by the higher affinity of NOSCN for ascorbic acid than that of other nitrosating species. The lower level of the plateau in the presence of thiocyanate is likely to be explained by greater recycling of nitric oxide in the presence of thiocyanate leading to a greater proportion ending up as nitrate [104]. The greater rate of production of nitric oxide in the presence of thiocyanate will lead to a greater rate of recycling and thus a higher proportion of the nitric oxide ending up as nitrate[104, 147]. Further evidence of this greater rate of recycling was seen in the fact that the oxygen concentration in the presence of thiocyanate fell more rapidly in the presence of thiocyanate than in its absence [120].

In the absence of thiocyanate, a small amount of oxygen was still detectible throughout the experiment with ascorbic acid 2000μM present. In contrast, no oxygen was detectible after three minutes in the presence of thiocyanate. The reason for this is unclear. In both experiments nitric oxide was maintained at a high level throughout the experiment and indeed was slightly higher towards the end of the experiment in the absence of thiocyanate. Reasons for this are unclear.

3.3 Nitrite chemistry in the simulated gastric juice: effect of different pH values (1.5, 2.5 and 3.0)

3.3.1 Nitric oxide and dissolved oxygen levels following nitrite addition in the presence of different concentrations of ascorbic acid

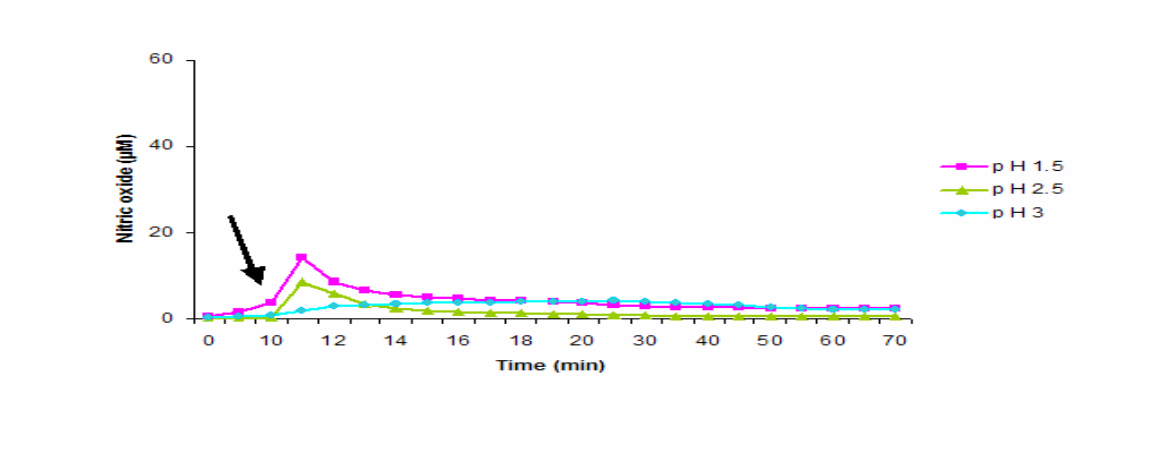
With ascorbic acid 100μM present, the rise in nitric oxide concentration on adding nitrite 100μM to HCl containing thiocyanate was related to the pH of the HCl. The rise in nitric oxide was greatest at the lowest pH of 1.5 and least at pH 3. In addition, the rate of rise in nitric oxide was more rapid at pH1.5 and much slower at pH 3 (Figure 3.7A). These changes in nitric oxide were reflected in similar changes in degree of fall in the oxygen concentration (Figure 3.8A).

With ascorbic acid 500μM the rise in nitric oxide was more marked than with the ascorbic acid 100μM and again the degree of rise was influenced by the pH. The increase in nitric oxide was greatest at pH 1.5 reaching a peak of 68μM at five minutes and then falling to 6.5μM at twenty minutes. At pH 2.5, the peak nitric oxide was 30.7μM reached at 5 minutes and again slowly falling. At pH 3, the rise in nitric oxide concentration was very slow but continuous, reaching a peak of 11.9μM at the end of the experiment (Figure 3.7B). The changes in oxygen concentration with the ascorbic acid 500μM again reflected the changes in nitric oxide at the different pH with the most marked and rapid fall in oxygen being seen at pH 1.5. Interestingly, the rise in oxygen concentration in the latter part of the experiment was most marked also with the pH 1.5 solutions (Figure 3.8B).

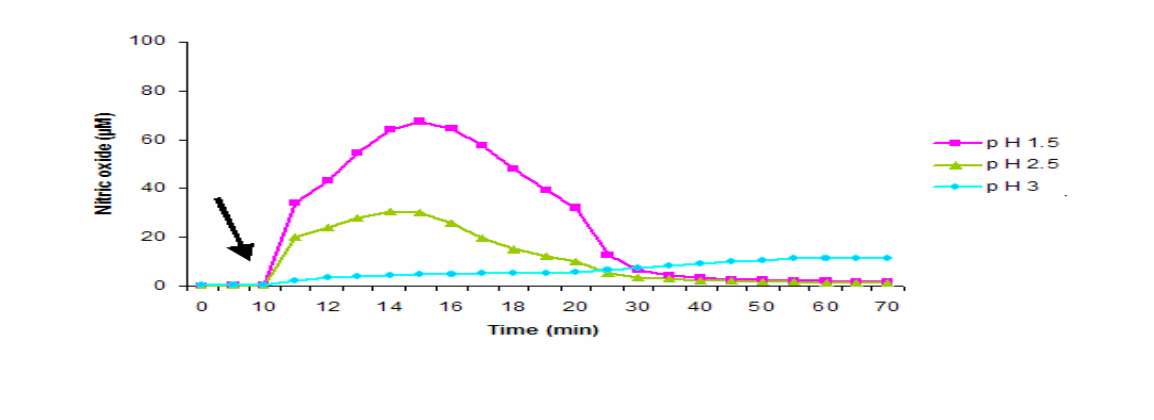
With ascorbic acid 2000μM the rate of rise in nitric oxide was closely related to the pH being most rapid at pH 1.5 and slowest at pH 3. At each pH, the final concentration obtained was approximately 70μM, though the plateau did seem to be slightly lower in the

lowest pH solutions (Figure 3.7C). The change in oxygen concentration again reflected the rise in nitric oxide being most rapid at pH 1.5 and slowest at pH 3. In addition, at pH 3 there seemed to be some residual oxygen present (Figure3.8C).

(A)



(B)



(C)

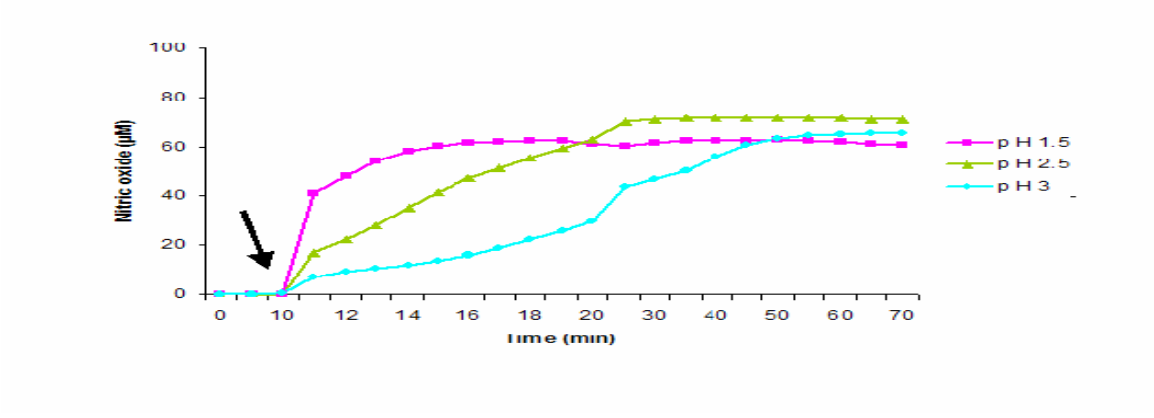
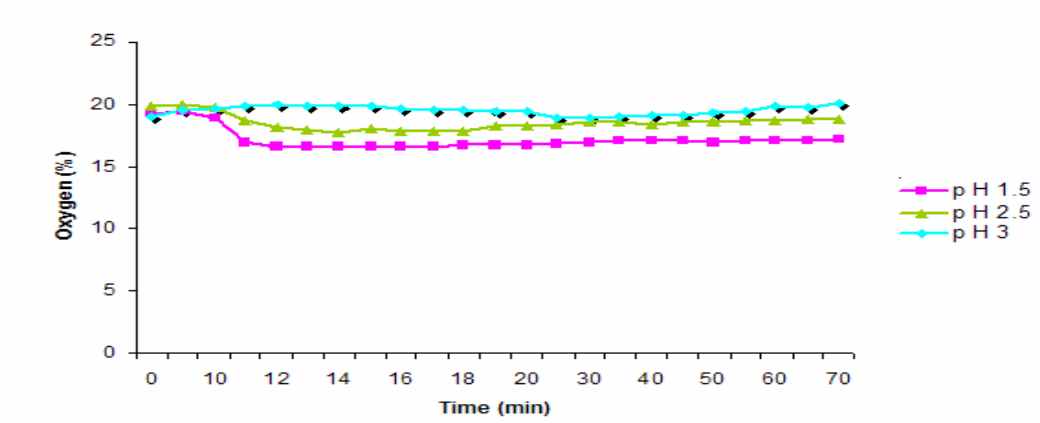
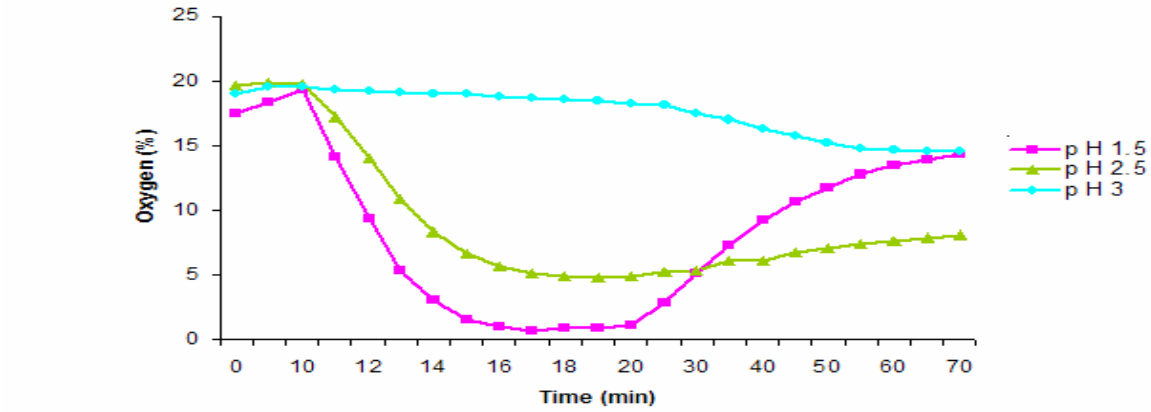


Figure 3.7: Nitric oxide levels (µM) in the simulated gastric juice (NaSCN, aerobic condition) of different pH values pH 1.5 (squares), 2.5 (triangles) and 3.0 (circles), following nitrite addition (100µM) at t=10 min, in the presence of ascorbic acid 100µM (A), 500µM (B) or 2000µM (C). Values are presented as mean of two experiments.

(A)



(B)



(C)

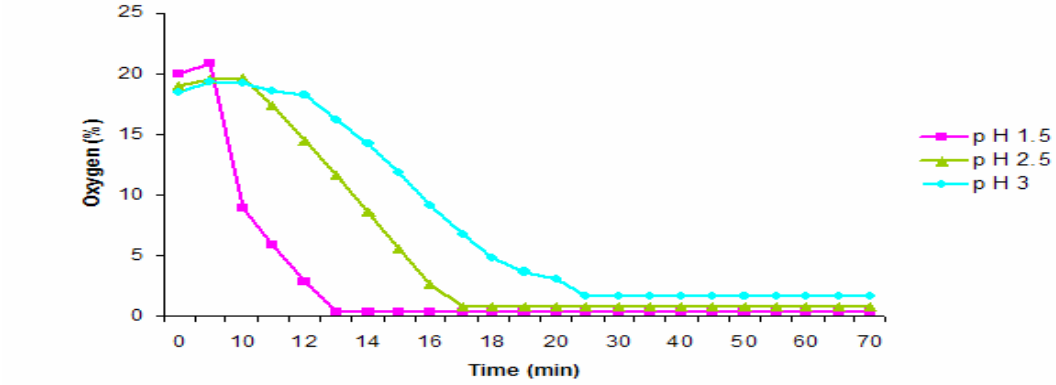


Figure 3.8: Dissolved oxygen levels (%) in the simulated gastric juice (NaSCN, under aerobic condition) of different pH values pH 1.5 (squares), 2.5 (triangles) and 3.0 (circles) on, following nitrite addition (100µM) at t=10 min, in the presence of ascorbic acid 100µM (A), 500µM (B) or 2000µM (C). Values are presented as mean of two experiments.

3.3.2 Discussion

The effect of pH on nitric oxide and oxygen were similar at the ascorbic acid 100μM and ascorbic acid 500μM experiments. However, the changes are clearer in the ascorbic acid 500μM experiment and therefore discussion will focus on that experiment. With the ascorbic acid 500μM the rise in nitric oxide was most rapid and greatest at pH 1.5 and slowest at pH 3. This can be explained by the effect of the pH on the proportion of nitrosating species present with a high affinity for reacting with ascorbic acid. The concentration of NO^+ in the solution of nitrite is inversely related to the pH value being greatest at lowest pH [50, 104]. The reactive nitrosating species in the presence of thiocyanate is thought to be NOSCN, which is formed by a reaction between NO^+ and SCN^-. Consequently, lowering the pH will increase the concentration of NOSCN and thus increase the rate of reaction of ascorbic acid and the rate of formation of nitric oxide. The initial fall in the oxygen concentration was also greatest at lowest pH, again being consistent with the fall in oxygen being directly related to the concentration of nitric oxide which will then react with the oxygen to form nitrosating species again [50, 104, 120].

In the ascorbic acid 500μM experiment the fall in nitric oxide concentration following its peak was more marked at pH 1.5 than at pH 2.5 and this was reflected in a more rapid rise in oxygen following its nadir at pH 1.5 than at pH 2.5. This is likely to be related to the fact that at pH 1.5 the more rapid recycling will result in the complete depletion of ascorbic acid whereas at pH 2.5 the slower chemistry results in a slower rate of recycling and thus some ascorbic acid will remain [104]. Though ascorbic acid was not measured in this particular experiment, the results from earlier experiments indicate that at pH 1.5 in the presence of thiocyanate, ascorbic acid would be fully depleted.

In the 500μM experiment at pH 3 the rise in nitric oxide concentration was very slow but at the end of the experiment it was higher than at the lower pHs where nitric oxide concentration had fallen back to almost zero. In this experiment the slow rate of production of nitric oxide results in very slow recycling and thus ascorbic acid remains. In the presence of adequate ascorbic acid equilibrium will therefore be produced between the production of nitric oxide by the reaction in nitrosating species and ascorbic acid and the loss of nitric oxide due to the reaction of nitric oxide with the oxygen. The latter reaction will be very slow at this low concentration of nitric oxide.

With the ascorbic acid 2000μM, the rise in nitric oxide was most rapid with the pH 1.5 solution and slowest with the pH 3 solution. In each case, the final concentration achieved at the end of the experiment was fairly similar at approximately 70μM and it seemed to be slightly lower with the lowest pH. The fall in oxygen concentration again reflected the rise in nitric oxide. In this experiment the ascorbic acid being in gross excess will not be rate-controlling and therefore one can see the clear effects of the pH on the rate of production of nitric oxide. Again the slightly lower plateau with nitric oxide at pH 1.5 may be explained by the more rapid recycling at this pH and thus greater conversion of the nitrosating species to nitrate which can no longer be converted to nitric oxide.

3.4 Nitrite chemistry in the simulated gastric juice: effect of oxygen

3.4.1 Nitric oxide levels following nitrite addition in theabsence of ascorbic acid

The addition of nitrite (100μM) to the simulated gastric juice (with NaSCN, pH 1.5) in absence of ascorbic acid under both aerobic and anaerobic conditions produced very limited amount of nitric oxide were, 0.34μM and 2.25μM, respectively.

3.4.2 Nitric oxide and dissolved oxygen levels following nitrite addition in presence different concentration of ascorbic acid

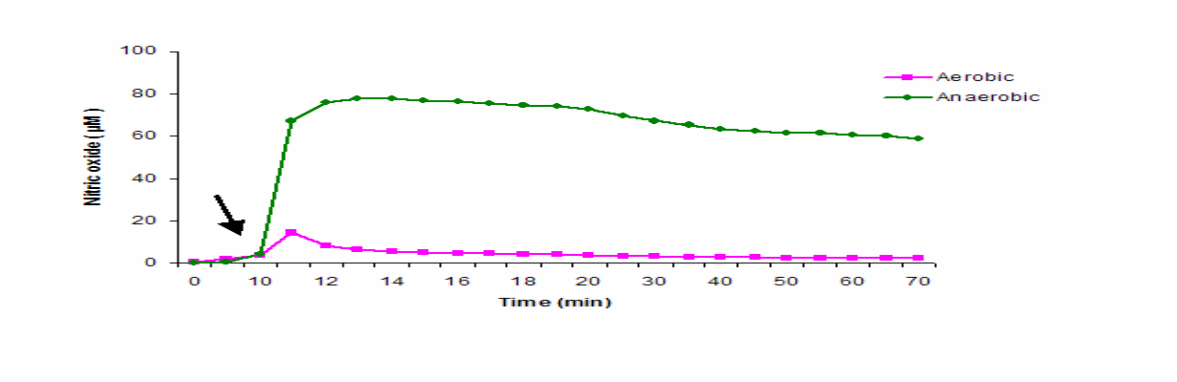
The presence of oxygen had a profound effect on the rise in nitric oxide when nitrite 100μM was added to HCl pH 1.5 containing thiocyanate and this was most marked at the lowest ascorbic acid concentration.

With ascorbic acid 100μM in the presence of oxygen, the peak nitric oxide concentration was 17.4μM occurring one minute after the addition of nitrite and then it slowly fell during the experiment. In contrast, in the absence of oxygen a peak nitric concentration of 78μM occurred one-two minutes after adding nitrite and this only showed a slight fall throughout the remainder of the experiment (Figure 3.9A).

With the ascorbic acid 500μM and ascorbic acid 2000μM, the rises in the nitric oxide level under the anaerobic conditions were similar to that seen with ascorbic acid100μM. However, under aerobic conditions the rise in nitric oxide concentration was greater with the ascorbic acid 500μM reaching a peak of 68μM at five minutes and then falling to undetectable levels at the end of the experiment (Figure 3.9B). With the ascorbic acid

2000µM, the rise in nitric oxide under the aerobic conditions reached a peak of 63µM at ten minutes and remained elevated throughout the experiment (Figure 3.9C).

(A)



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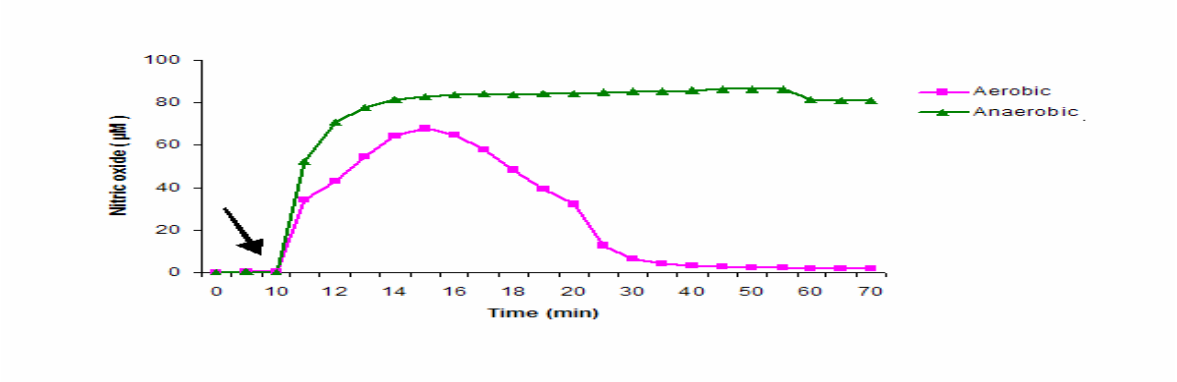

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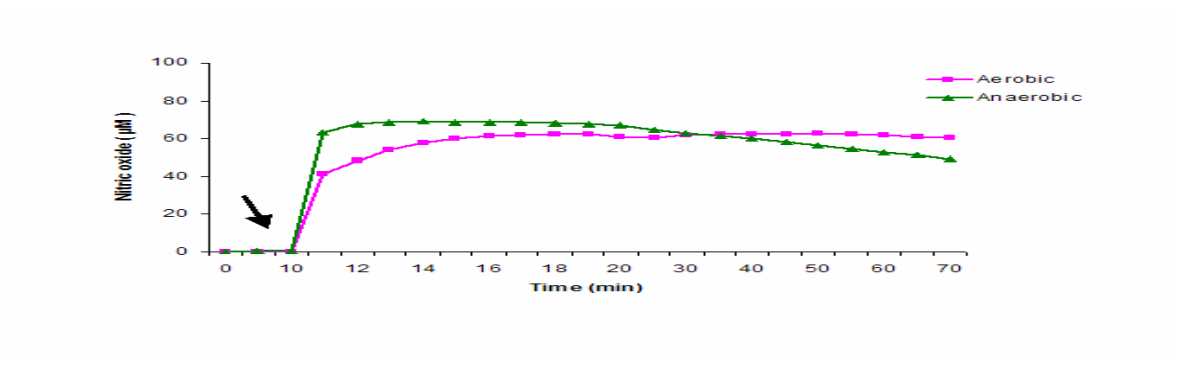


Figure 3.9: Nitric oxide levels (µM) in the simulated gastric juice (NaSCN, pH 1.5) under aerobic (squares) and anaerobic (triangles), conditions, following nitrite addition (100µM) at t=10min, in the presence of ascorbic acid 100µM (A), 500µM (B) or 2000µM (C). Values are presented as mean of two experiments.

3.4.3 Ascorbic acid measurements

The presence of oxygen had a marked effect on the amount of ascorbic acid consumed during the experiment. In the absence of oxygen ascorbic acid remained present at the end of each experiment even when only ascorbic acid 100μM had been added. In contrast, in the presence of oxygen all the ascorbic acid was consumed during the experiment at 100μM and more than 90% with the ascorbic acid 500μM but only 32% with the ascorbic acid 2000μM (Table 3.3).

Table 3.3: Ascorbic acid consumed (%) in the simulated gastric juice (NaSCN, pH 1.5), following nitrite addition (100μM) in the presence of ascorbic acid 100μM, 500μM or 2000μM over 60 minutes incubation, under both aerobic and anaerobic conditions. Values are presented as mean of two experiments.

[AA]μM at t=0	AA consumed (%) with oxygen at t= 70 min	AA consumed (%) without oxygen at t= 70 min	P Value
100	99.18	72.13	$P < 0.05$
500	91.47	48.89	
2000	32.05	10.12	

3.4.4 Discussion

These experiments show the dramatic effect that the presence of oxygen has on this chemistry. With ascorbic acid 100μM in the presence of oxygen, the peak nitric oxide concentration was only 17.4μM and then rapidly fell whereas in the absence of oxygen the nitric oxide concentration reached a peak value of 78μM and remained elevated throughout the experiment. Consequently, the area under the nitric oxide time curve was of the order of 20 times greater in the absence than in the presence of oxygen. As shown in earlier experiments, the key reaction in producing the nitric oxide in the acidified nitrite solution is the presence of ascorbic acid. The much lower nitric oxide concentration in the ascorbic acid 100μM experiment in the presence of oxygen can be explained by the rapid consumption and depletion of ascorbic acid [50, 104]. This is consistent with the fact that no ascorbic acid was detectible at the end of the experiment. Stoichiometrically, one molecule of ascorbic acid should convert one molecule of nitrite in acidic conditions to one molecule of nitric oxide. This chemistry was apparent in the absence of oxygen where nitrite 100μM produced 80μM nitric oxide. However, in the presence of oxygen the nitric oxide formed in this way rapidly reacted with the oxygen to reform nitrosating species which can further react with the ascorbic acid and they converted that to nitric oxide. However, these recycling results in the rapid consumption of ascorbic acid and consequently the nitrosating species formed cannot be converted back into nitric oxide. The rapid rate of this recycling appears as 1.5 in the presence of thiocyanate and is demonstrated by the fact that ascorbic acid must be consumed within a minute of adding the nitrite, as the nitric oxide concentration was markedly lower at this time point which represents its concentration in the presence of oxygen.

In the anaerobic experiments increasing the ascorbic acid concentration to 500μM or 2000μM had no significant effect on the nitric oxide response. This can be explained by

the fact that adequate ascorbic acid was available even at its lowest concentration and therefore increasing the concentration had little effect on the nitric oxide profile. In contrast, in the presence of oxygen increasing the ascorbic acid concentration markedly changed the nitric oxide profile and at the ascorbic acid 2000μM concentration the nitric oxide profile became very similar to that in the absence of oxygen. The latter can be explained by the fact that at high concentrations of ascorbic acid the recycling of the nitrite results in depletion of oxygen (see earlier experiment 3.1.2) and consequently the experiment then becomes an anaerobic experiment. In the absence of oxygen, the nitric oxide formed remains as nitric oxide as it cannot react with oxygen to return to nitrosating species and nitrite.

3.5 Nitrite chemistry in the simulated gastric juice: effect of different concentrations of dietary phenolics in the presence of oxygen

3.5.1 At low concentration (250μM)

3.5.1.1 Nitric oxide and dissolved oxygen levels following nitrite addition in the presence of caffeic acid (CA), ferulic acid (FA), chlorogenic acid (CGA) and gallic acid (GA) individually in comparison with ascorbic acid

In the presence of oxygen the addition of nitrite 100μM to the simulated gastric juice containing NaSCN (1mM) and AA or a range of dietary phenolics (FA, CA, GA and CGA, 250μM) individually at pH 1.5, resulted in nitric oxide production and a fall in oxygen concentrations (Figure 3.10 and 3.11). With ferulic and caffeic acid (250μM), the nitric oxide concentration reached a peak value of 3.5μM and 5.6μM respectively two minutes after addition of nitrite. These nitric oxide concentrations then slowly decreased to undetectable level by the end of the experiment. With gallic acid (250μM) the nitric oxide reached a plateau at 7.8μM and remained at that level between two and ten minutes after adding the nitrite. The nitric oxide concentration then slowly fell to 4μM by the end of the incubation time period. With chlorogenic acid (250μM) the nitric oxide rose in a similar manner to that observed following gallic acid, reaching a plateau at 6.5μM, although, it remained elevated throughout the duration of the experiment. With ascorbic acid (250μM) the nitric oxide concentration reached a peak at 23.5μM, occurring at one minute from the addition of nitrite. This rise in the nitric oxide was different from that seen after other phenolics acids were added. There was a clear rise and then clear fall in the nitric oxide concentration (Figure 3.10).

With ferulic acid or caffeic acid (250μM) the change in nitric oxide concentrations were reflected in similar changes in the degree of fall in the oxygen concentration. The oxygen

concentrations were decreased from a baseline value 20% to 19% and 18%, respectively. Thereafter the oxygen concentrations remained steady or slightly increased back towards the baseline level. The change in oxygen concentration with gallic acid and chlorogenic acid (250µM) again reflected the change in nitric oxide with the most marked and rapid fall in oxygen being seen with gallic acid. With ascorbic acid (250µM), the marked rise in nitric oxide was reflected by a marked fall in the dissolved oxygen concentration with the lowest point of oxygen concentration occurring two minutes after the addition of nitrite being 14.5% and then slowly increasing and returning to 19% at the end of the experiment (Figure 3.11).

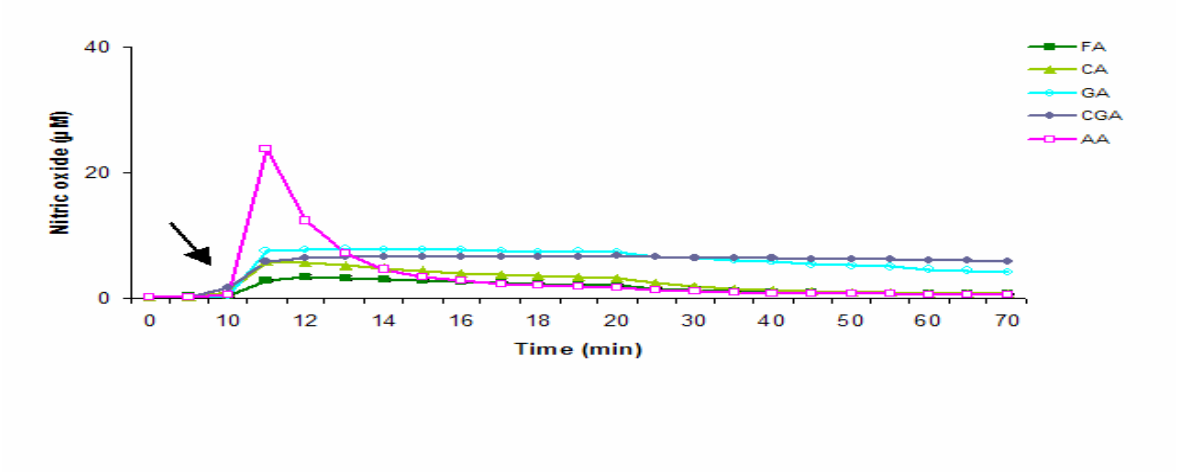


Figure 3.10: Effect of presence of 250µM ferulic acid (squares), caffeic acid (triangles), gallic acid (open circles), chlorogenic acid (circles) or ascorbic acid (open squares) on nitric oxide levels (µM) in the simulated gastric juice, following nitrite addition (100µM) at t=10 min under aerobic conditions. Values are presented as mean of two experiments.

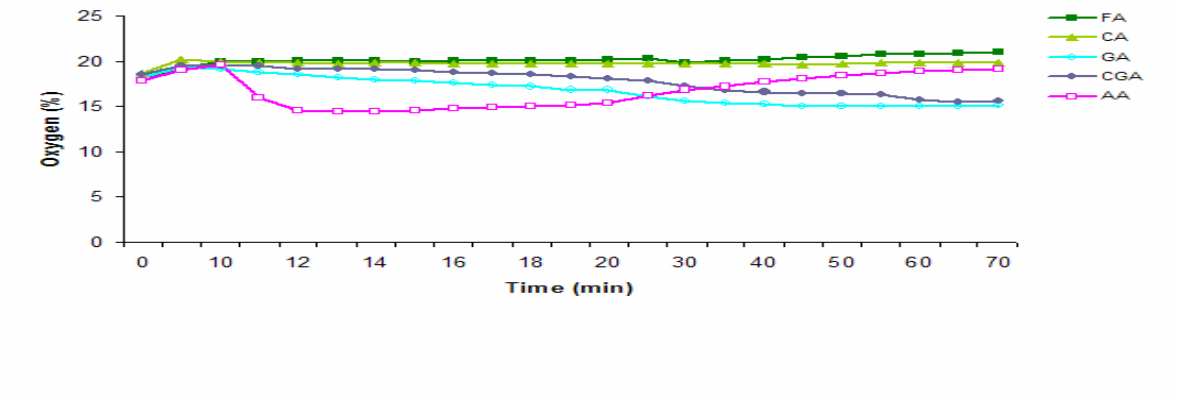


Figure 3.11: Effect of presence of 250µM ferulic acid (squares), caffeic acid (triangles), gallic acid (open circles), chlorogenic acid (circles) or ascorbic acid (open squares) on dissolved oxygen levels (%) in the simulated gastric juice, following nitrite addition (100µM) at t=10 min. Values are presented as mean of two experiments.

3.5.2 At high concentration (2000μM)

3.5.2.1 Nitric oxide and dissolved oxygen levels following nitrite addition in the presence of caffeic acid (CA), ferulic acid (FA), chlorogenic acid (CGA) and gallic acid (GA) individually in parallel with ascorbic acid

When ferulic acid (2000μM), was present in the system described under 3.5.1.1, it produced nitric oxide at a peak value of 5.2μM within minute of the addition of nitrite. With caffeic acid (2000μM), the nitric oxide rose in a similar manner to that observed following ferulic acid, reaching a value 7.5μM minute after the addition of nitrite. Under both conditions the nitric oxide concentrations then slowly decreased to become undetectable by the end of incubation time period. With gallic acid (2000μM), the nitric oxide concentration peaked at 20μM ten minutes after the addition of nitrite, and then slowly decreased to 6μM by the end of the experiment. With chlorogenic acid (2000μM) the rise in the nitric oxide concentration gradually reached a peak of 39.5μM at the end of the experiment. In the presence of ascorbic acid (2000μM), there was a marked rise in nitric oxide, which reached a value 63.1μM approximately ten minutes after the addition of nitrite. The nitric oxide concentration remained elevated throughout the duration of the experiment (Figure 3.12).

With ferulic acid (2000μM) the rise in nitric oxide concentration was mirrored by a fall in the oxygen concentration from 20% to 19% within the first two minutes, and then it slowly decreased to 17% by the end of the experiment. With caffeic acid (2000μM), the dissolved oxygen concentration fell from a baseline value of 20% to 19% within the first two minutes after the addition of nitrite. Thereafter the oxygen concentration remained steady and slightly increased back towards baseline level. With gallic acid (2000μM), the rise in nitric oxide was mirrored by the fall in dissolved oxygen concentration with the nadir of 8% oxygen concentration at fifteen minutes after the addition of nitrite. This then slowly

increased and returned to12% at the end of the experiment. With chlorogenic acid (2000µM), the oxygen concentration gradually decreased to become undetectable at 20 minutes and then slowly increasing to 2% at the end of the experiment. With ascorbic acid (2000µM), the oxygen concentration rapidly decreased to become undetectable at first three minutes and remained undetectable for the duration of the experiment (Figure 3.13).

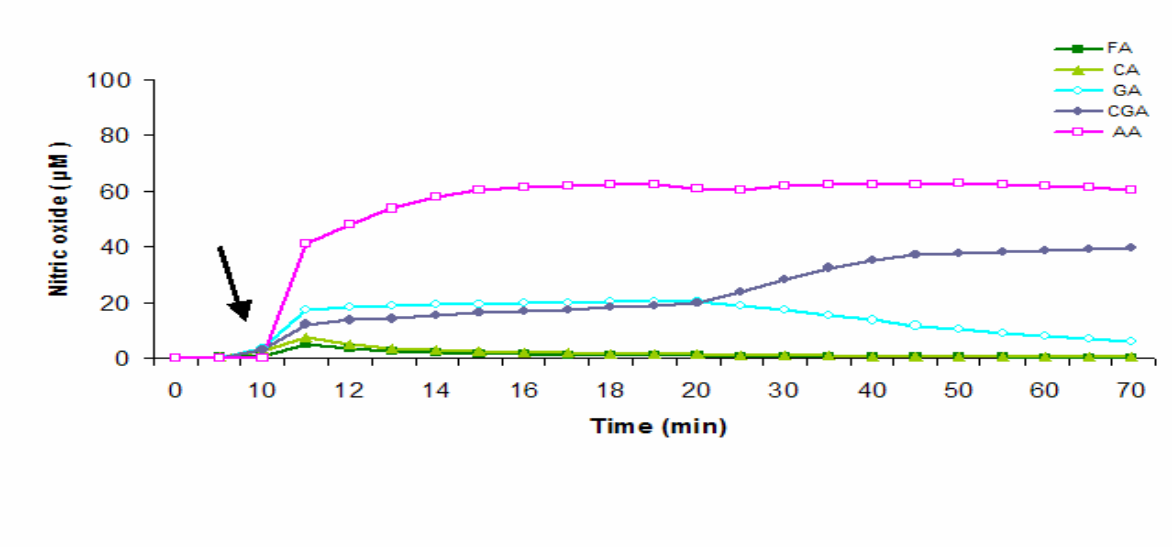


Figure 3.12: Effect of presence of 2000µM ferulic acid (squares), caffeic acid (triangles), gallic acid (open circles), chlorogenic acid (circles) or ascorbic acid (open squares) on nitric oxide levels (µM) in the simulated gastric juice, following nitrite addition (100µM) at t=10 min under aerobic conditions. Values are presented as mean of two experiments.

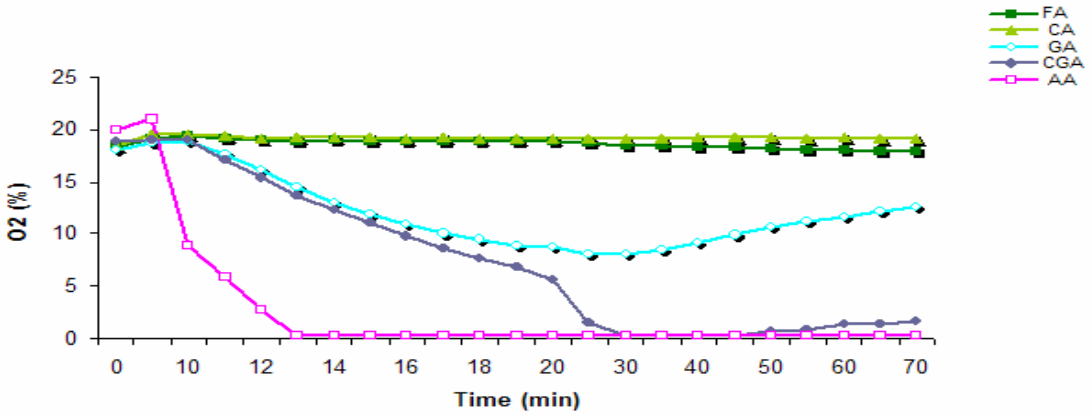


Figure 3.13: Effect of presence of 2000µM ferulic acid (squares), caffeic acid (triangles), gallic acid (open circles), chlorogenic acid (circles) or ascorbic acid (open squares) on dissolved oxygen levels (%) in the simulated gastric juice, following nitrite addition (100µM) at t=10 min. Values are presented as mean of two experiments.

3.6 Nitrite chemistry in the simulated gastric juice: effect of different concentrations of dietary phenolics in the absence of oxygen

3.6.1 Nitric oxide levels following nitrite addition in the presence of caffeic acid (CA), ferulic acid (FA), chlorogenic acid (CGA) and gallic acid (GA) individually, in compare with ascorbic acid

3.6.1.1 At low concentration (250μM)

In the absence of oxygen the addition of nitrite 100μM to simulated gastric juice (with NaSCN, pH 1.5) containing ferulic acid (250μM) produced nitric oxide reaching a peak value of 10μM at three minutes after the addition of nitrite. The nitric oxide concentration then slowly decreased to 2μM by the end of the incubation time period. With caffeic acid (250μM) the nitric oxide concentration peaked at 18.4μM two minutes after the addition of nitrite and then slowly decreased to 2.5μM by the end of the experiment. With the chlorogenic acid (250μM) the rise in nitric oxide concentration was gradual but continuous, reaching a peak of 36.4μM by the end of the experiment. With gallic acid (250μM) there was a marked rise in nitric oxide reaching a peak of 50μM at twenty five minutes and then slowly fall to 40.7μM by the end of the experiment. With ascorbic acid (250μM) there was a clear rise in the nitric oxide concentration reaching a peak value of 89.5μM minute after the addition of nitrite. The nitric oxide then slowly decreased to 58μM by the end of the incubation time (Figure 3.14).

3.6.1.2 At high concentration (2000μM)

In the absence of oxygen, the rise in the nitric oxide profile after adding nitrite (100μM) to simulated gastric juice (with NaSCN, pH 1.5) was similar in the presence of ferulic or caffeic acid (2000μM). With ferulic acid (2000μM) the nitric oxide concentration reached

a peak value of 17.5µM minute after the addition of nitrite and then slowly decreased to 2.2µM at seventy minutes. With caffeic acid (2000µM) the nitric oxide rose in a similar manner to that observed following ferulic acid, reaching a value approximately 15µM higher than that obtained with ferulic acid (2000µM). With chlorogenic (2000µM) the rise in nitric oxide concentration gradually reached a plateau at a level of 54µM occurring at ten minutes from adding nitrite. The nitric oxide concentration remained elevated throughout the duration of the experiment. With gallic acid or ascorbic acid (2000µM), the addition of nitrite it produced high peak nitric oxide concentration occurred approximately ten minutes after the addition of nitrite. With gallic acid, the nitric oxide peak value was 80µM and then this gradually decreased to 46µM by the end of the incubation time period. With ascorbic acid, the nitric oxide peak was approximately 15µM lower than that observed with gallic acid and by the end of experiment it was 49µM (Figure 3.15).

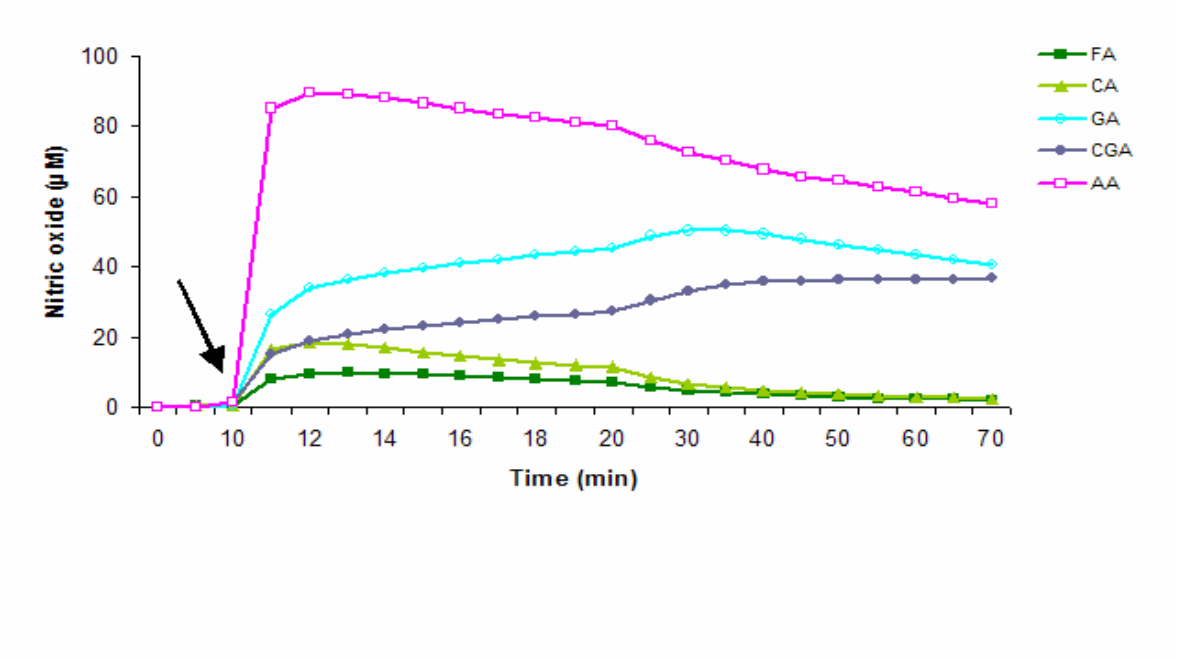


Figure 3.14: Nitric oxide levels (µM) in the simulated gastric juice, following nitrite addition (100µM) at t=10 min, in presence 250µM of ferulic acid (squares), caffeic acid (triangles), gallic acid (open circles), chlorogenic acid (circles) or ascorbic acid (open squares) under anaerobic conditions. Values are presented as mean of two experiments.

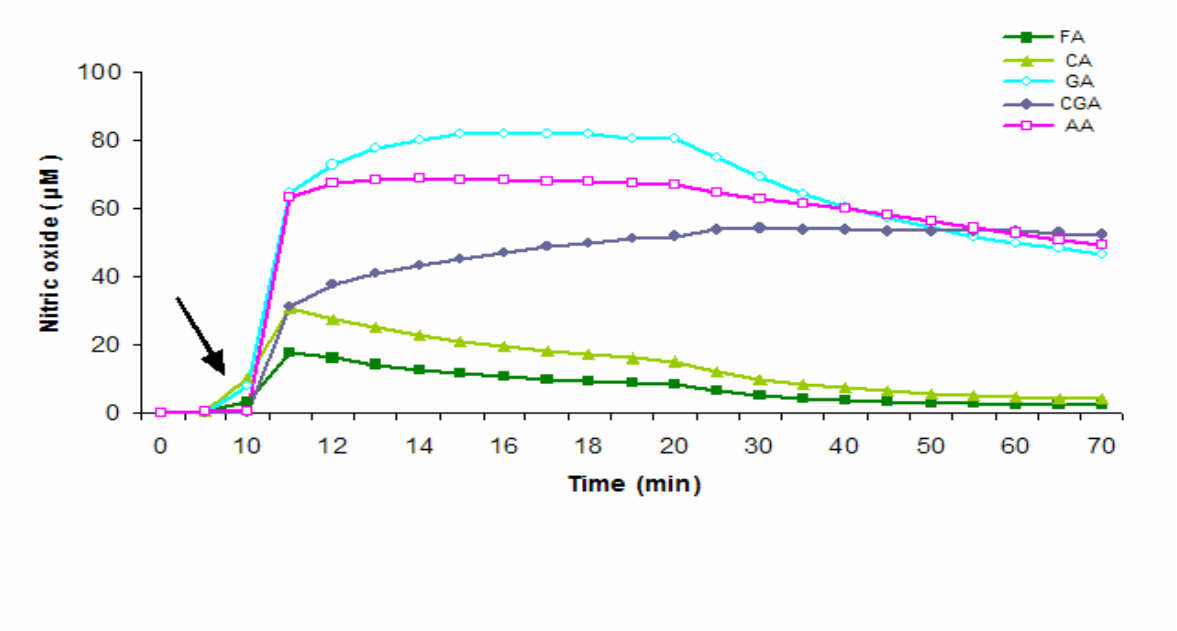


Figure 3.15: Nitric oxide levels (µM) in the simulated gastric juice, following nitrite addition (100µM) at t=10 min, in presence 2000µM of ferulic acid (squares), caffeic acid (triangles), gallic acid (open circles), (circles) or ascorbic acid (open squares) under anaerobic condition. Values are presented as mean of two experiments.

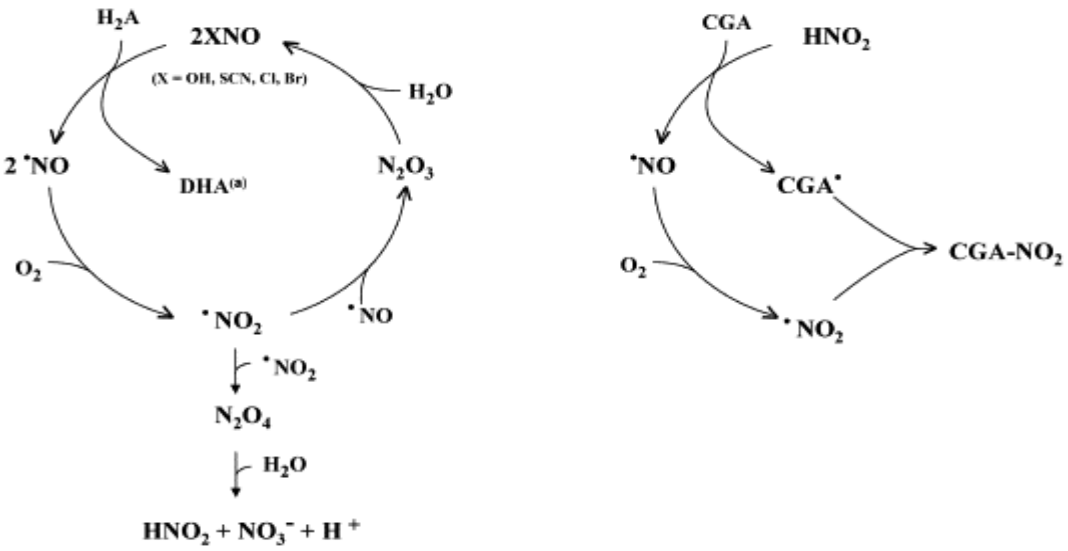
3.6.2 Discussion

The second aim of this study was to compare the ability of common dietary antioxidants, belonging to the phenolics, with that of ascorbic acid at reducing nitrite to nitric oxide under conditions simulating those occurring in the upper gastrointestinal tract. Four phenolic acids were tested alongside ascorbic acid in two different concentrations: caffeic acid, ferulic acid, chlorogenic acid, and gallic acid. Phenolic acids occur in most fruits, vegetables, and dicotylenous plants either in free or conjugated form. Caffeic and ferulic acids are hydroxycinnamates occurring in fruits and vegetables; chlorogenic acid, an ester of caffeic acid and quinic acid, is abundant in coffee and apples; meanwhile, gallic acid is a major building block in the synthesis of tannins, which are abundant and confer their astringent properties to tea and red wines [130, 131]. Ascorbic acid, which is actively secreted in the stomach, is a major inhibitor of acid catalysed nitrosation in the lumen [148-150]. Meanwhile, the potential chemo-protective effect of phenolic compounds against nitrosative stress has been highlighted in the past [151-153]. Previous studies investigated the nitrosation inhibiting potential of dietary phenolics in single-phase systems, based on their nitric oxide promoting abilities [154, 155]. However, recent studies have demonstrated that high nitric oxide production in the presence of lipids is the main cause for the transfer of the nitrosative stress from the aqueous to the lipid compartment, an issue relevant to transfer of nitrosation to the gastric epithelium [156].

The present experiments compared the nitric oxide generating abilities of the four phenolic acids with ascorbic acid. The addition of nitrite (100μM) to the simulated gastric juice containing 1mM NaSCN and low concentration ascorbic acid (250μM) caused a rapid rise in nitric oxide to 23μM followed by a fall in nitric oxide accompanied by an initial fall in oxygen. At high concentration ascorbic acid (2000μM) increased nitric oxide to 63μM which was maintained and accompanied by the complete depletion of oxygen. When the

low dose ascorbic acid experiment was repeated under anaerobic conditions, the nitric oxide reached 89.5μM and was maintained at this level. The rapid rise and then fall of nitric oxide with low dose ascorbic acid in the presence of oxygen can be explained by the rapid depletion of ascorbic acid due to the nitric oxide reacting with oxygen to re-form nitrosating species, which can react again with the remaining ascorbic acid as shown in Scheme 3.3. This recycling has been shown to lead to the rapid depletion of ascorbic acid under aerobic conditions [157, 158]. The reaction between nitric oxide and oxygen is second order with respect to nitric oxide and the rapid depletion of ascorbic acid can be explained by the high concentrations of nitric oxide initially produced by the ascorbic acid [159, 160].

When the experiment was performed in the presence of low concentration gallic acid and chlorogenic acid, it produced a slower and less marked initial rise in nitric oxide than ascorbic acid (i.e. longer lasting than a burst); however, the nitric oxide level was maintained and there was a less marked early depletion of oxygen. When the low dose gallic acid and chlorogenic acid experiment was repeated under anaerobic conditions, the final concentration obtained was lower in both cases than with the ascorbic acid. The less rapid initial rise in nitric oxide suggests that these phenolic acids have a lower affinity for the nitrosating species than ascorbic acid under similar experimental conditions. Previous studies proposed that the nitric oxide-promoting ability of chlorogenic acid is related to the presence of the catechol group, enabling formation of nitric oxide upon encounter with nitrous acid, and leading to the formation of an intermediate *o*-semiquinone radical able to then react with nitrogen dioxide to generate a nitrated polyphenol end-product, this depleting the system in nitrosating species and limiting further nitric oxide formation scheme 3.3 [155].



Scheme 3.3: proposed mechanism of nitric oxide release from acidified nitrite in presence of ascorbic acid *(left)* and chlorogenic acid *(right)*.[147].

Ferulic acid and caffeic acid produced a different response with respect to both nitric oxide and oxygen than the other antioxidants tested. At both low and high concentrations they produced a rapid but limited rise in nitric oxide to 5μM at low concentration and 7.5μM at high concentration, followed by a rapid fall and in both cases with little discernable change in oxygen concentration. In the absence of oxygen the initial rise in nitric oxide was higher (35μM) but was again followed by a rapid fall with similar pattern with both low and high concentrations. The nitric oxide response observed with caffeic acid and ferulic acid suggests that the nitrite are reduced to nitric oxide but that a subsequent reaction then occurs leading to a fall in nitric oxide. It has been demonstrated that both ferulic and caffeic acids can form nitroso-derivatives upon encounter with acidified nitrite, via preferential reaction between their side chain and the nitrosating species, with limited or no nitric oxide production [161-164]. This mechanism is key in ensuring the efficient, stable scavenging of nitrosating species, without generating nitric oxide. According to Peri *et al.*(2005*)* the limited nitric oxide production by ferulic acid is linked to the absence of a catechol group, preventing the formation of an *o*-semiquinone radical to react with

nitrogen dioxide [155]. However, this research indicates, however, that the nitric oxide promoting ability of caffeic acid was similar to that of ferulic acid, despite the fact that caffeic acid possesses a catechol group. It is therefore likely that the nitric oxide promoting abilities of phenolic acids may not be entirely based on the presence or lack of a catechol group.

Chapter 4 - Conclusion

General conclusion

Over the last fifty years there has been concern about luminal nitrite as a risk factor for upper gastro-intestinal malignancies [49, 78]. This has arisen from evidence that salivary nitrite generates nitrosating species and nitric oxide when encountering acidic gastric juice [49, 78, 100].

Therefore it was important to study the influence of ascorbic acid, sodium thiocyanate, oxygen, and pH on nitrite chemistry in the simulated GOJ environment. It was also relevant to investigate the effect of other dietary antioxidants such as ferulic acid, caffeic acid, gallic acid, and chlorogenic acid on the nitrite chemistry under conditions simulating the lumen at the GOJ.

Previous studies both in the simulated bench-top system and *in situ* have examined the fate of salivary nitrite when it encounters acidic gastric juice [48, 103, 104]. This study, in accordance with the literature, has demonstrated that nitrite in the simulated GOJ environment is rapidly converted to nitric oxide in the presence of ascorbic acid [50, 53, 104].This is due to fact that only a very limited amount of nitric oxide is produced in the absence of ascorbic acid. Thus ascorbic acid is effectively reducing the nitrosating species to nitric oxide and in the process is oxidized to dehydroascorbic acid. However, the presence of oxygen reduces the efficacy of ascorbic acid because nitric oxide can react with dissolved oxygen to reform nitrosating species. The recycling of nitric oxide means more ascorbic acid is consumed and this was clearly observed in this study. We also observed that ascorbic acid at low or medium concentrations is an important controlling factor for the recycling processes, while with higher concentrations of ascorbic acid dissolved oxygen in the system was the main factor controlling the recycling pathway. These findings further support previous observations that nitric oxide recycling is maintained until either the ascorbic acid or oxygen is depleted [104].

The present study also demonstrated that the pH of the simulated gastric juice had a clear effect on the rate of nitric oxide production in the presence of ascorbic acid. The nitric oxide concentration in a solution of nitrite containing ascorbic acid is inversely related to the pH, being greatest at the lowest pH of 1.5. The presence of sodium thiocyanate in the simulated gastric juice also enhances the conversion of nitrite to nitric oxide and increases the ascorbic acid consumption. Moreover, the absence of oxygen in the system markedly increases nitric oxide concentration and also resulted in ascorbic acid being present throughout the experiment.

This study has demonstrated a different pattern of nitric oxide production from acidified nitrite in the presence of a range of dietary phenolics concentrations compared with ascorbic acid under conditions simulating the GOJ. Ferulic and caffeic acids produced only a small increase in nitric oxide, which was not sustained under both aerobic and anaerobic conditions. However, unlike ferulic and caffeic acids, gallic and chlorogenic acids produced a much more marked rise in nitric oxide which remained elevated under both aerobic and anaerobic conditions. In contrast, ascorbic acid produced a strong increase in nitric oxide production, which was followed by a clear fall under aerobic conditions. However, with high concentration of ascorbic acid and under anaerobic conditions, nitric oxide was sustained throughout the duration of experiment.

In summary, nitric oxide has numerous potential effects in the mediation of physiological and pathological mechanisms. It remains to be seen whether the generation of nitric oxide at the GOJ and the factors we have demonstrated to influence this are involved in the high incidence of disturbed physiology and pathology occurring at this anatomical site.

Further studies are required to investigate the biological significance of the nitric oxide generated at the GOJ. In addition, it may be important to study the effect of the various dietary anti-oxidants in modifying any biological effects.

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