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Pharmacology and Therapeutic Role of Inorganic Nitrate and Nitrite in Vasodilatation

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Abstract

Nitrite has emerged as an important bioactive molecule that can be biotransformed to nitric oxide (NO) related metabolites in normoxia and reduced to NO under hypoxic and acidic conditions to exert vasodilatory effects and confer a variety of other benefits to the cardiovascular system. Abundant research is currently underway to understand the mechanisms involved and define the role of nitrite in health and disease. In this review we discuss the impact of nitrite and dietary nitrate on vascular function and the potential therapeutic role of nitrite in acute heart failure.

Keywords:

Nitrate; Nitrite; Nitric oxide; Vasodilatation; Heart failure.

Abbreviations:

AHF, acute heart failure; ALDH2, mitochondrial aldehyde dehydrogenase; BP, blood pressure; cGMP, cyclic guanosine monophosphate; CHF, congestive heart failure; CVD, cardiovascular disease; DBP, diastolic blood pressure; deoxyHb, deoxyhemoglobin; eNOS, endothelial nitric oxide synthase; FBF, forearm blood flow; FBF-R, forearm blood flow ratio; FMD, flow mediated dilatation; GTN, glyceryl trinitrate; Hb, hemoglobin; iNOS, inducible nitric oxide synthase; MAP, mean arterial pressure; Mb, myoglobin; NO, nitric oxide; nNOS, neuronal nitric oxide synthase; NOS, nitric oxide synthase; O_2^-, superoxide; $ONOO^-$, peroxynitrite; oxyHb, oxyhemoglobin; RBC, red blood cell; ROS, reactive oxygen species; SAP, systemic arterial pressure; SBP, systolic blood pressure; sGC, soluble guanylate cyclase; UVA, ultra violet; XO, xanthine oxidase; XOR, xanthine oxidoreductase.

Table of contents:

1. Introduction

Cardiovascular disease (CVD) is the leading cause of death worldwide accounting for approximately 30% of all global deaths (Global status report on non-communicable diseases 2010. Geneva, World Health Organization, 2011). The number of people who will die from CVD, mainly from heart disease and stroke, are projected to increase and reach approximately 23.3 million by 2030 (Mathers & Loncar, 2006). Despite major advances in the treatment of patients with CVD, the morbidity and mortality associated with CVD is high, and there remains significant space for improvement in new therapeutic interventions. With more potentially promising candidate therapeutics on the horizon, it is particularly important to test these new treatments in a clinical setting in order to improve the outcome for CVD patients through application of more effective therapies.

The vascular endothelium is involved in many aspects of cardiovascular health, including regulating vascular tone, hemostasis, thrombosis, permeability and cell adhesion (Hirase & Node, 2012; Padilla, et al., 2013). The endothelium releases vasodilatory substances including nitric oxide (NO), prostacyclin, C-type natriuretic peptide and endothelium-derived hyperpolarizing factor, as well as vasoconstrictors including endothelin-1, angiotensin II and thromboxane A_2 (Danser, et al., 1994; Moncada & Vane, 1981; Needleman, et al., 1976; Vanhoutte & Katusic, 1988; Yanagisawa, et al., 1988). In the healthy endothelium, a balanced production of these factors plays an important preventative role against vascular disease. However endothelial dysfunction disturbs this balance and is associated with an increased risk of development of CVD, such as atherogenesis, increased arterial stiffness (arteriosclerosis) and associated hypertension.

2. Role of nitric oxide in vascular function

The discovery in the 1980's that NO could be produced endogenously in the vasculature and exert vasodilatator effects led to a plethora of studies which demonstrated its pleotropic effects including control of blood pressure (BP) and vascular tone (Furchgott & Zawadski, 1980; Ignarro, 1999; Ignarro, et al., 1987; Loscalzo & Welch, 1995; Moncada, et al., 1988; Murad, et al., 1978). NO is produced by a family of enzymes known as NO synthases (NOS) (Michel & Feron, 1997) utilizing the substrate L-arginine, molecular oxygen and nicotinamide adenine dinucleotide phosphate (Palmer, et al., 1988). Three different isoforms of NOS have been identified. Two of these are constitutively expressed in cells and synthesise NO in response to increased intracellular calcium concentrations (Mayer, et al., 1989; Moncada & Palmer, 1990; Mulsch, et al., 1989). The constitutive enzymes are known as neuronal NOS (nNOS or NOS I) and endothelial NOS (eNOS or NOS III) (Michel & Feron, 1997). The third isoform, termed inducible NOS (iNOS or NOS II), is expressed in response to cytokines and other inflammatory factors in a variety of cells including macrophages, neutrophils, cardiac myocytes and endothelial cells (Szabo, et al., 1994) and produces NO independently of changes in intracellular calcium concentration. More recently it has become clear, however, that the production of either constitutive NOS isoform can also be induced under certain conditions, and that some tissues express low levels of iNOS already constitutively. Under normal physiological conditions low levels of NO produced by eNOS function as an important regulatory messenger and maintain vessel tone (Amezcua, et al., 1989; Loscalzo & Welch, 1995). NO-induced vasodilatation is mediated by activation of soluble guanylate cyclase (sGC), which converts guanosine triphosphate into cyclic guanosine monophosphate (cGMP), and subsequently relaxes vascular smooth muscle (Ignarro, et al., 1981).

When NO is produced at elevated levels, such as from iNOS during septic shock, NO can also have cytotoxic effects (Szabo, et al., 1993). While lower levels of constitutively produced NO are involved in the regulation of mitochondrial activity by competing with oxygen at the level of complex IV of the respiratory chain (Erusalimsky & Moncada, 2007), higher concentrations of NO have been shown to persistently inhibit mitochondrial function by nitrosylation of electron chain complexes, in particular complex I (Clementi, et al., 1998), and to cause DNA damage (Delaney, et al., 1993; deRojas-Walker, et al., 1995). Endothelial dysfunction is apparent in conditions such as hypertension, heart failure, coronary artery disease, and atherosclerosis (Cai & Harrison, 2000; de Berrazueta, et al., 2010; Vanhoutte, 2009). During these pathophysiological conditions, eNOS-dependent conversion of L-arginine to NO is impaired and thus NO bioavailability is reduced, concurrent with decrease in NO-mediated vasorelaxation (Harrison, 1997; Pou, et al., 1992; Shimokawa, et al., 1991; Wilcox, et al., 1997). Enhanced degradation of NO through scavenging by reactive oxygen species (ROS), together with a reduction in production *via* eNOS, has also been shown to be involved in reducing NO bioavailability (Harrison, 1997).

NO scavenging by ROS (in particular superoxide, O_2^-) is normally controlled through a well-balanced production of NO and ROS, (Cai & Harrison, 2000). However during pathological conditions when the production of NO decreases, the balance is perturbed (Griendling & Fitzgerald, 2003). Superoxide reacts with NO to form peroxynitrite ($ONOO^-$) (Burney, et al., 1999). Unlike NO, $ONOO^-$ is a potent pro-oxidant which has been demonstrated to play a role in initiating lipid peroxidation in both membranes and lipoproteins (Radi, et al., 1991; Rubbo, et al., 1994; Thomas, et al., 1998). Consequently, $ONOO^-$-modified low density lipoprotein has been shown to be involved in the accumulation of cholesteryl esters in the fatty streaks characteristic of atherosclerosis (Darley-Usmar, et al., 1992; Guy, et al., 2001).

Endothelial eNOS function may also become compromised as a result of increased oxidative stress. In atherosclerosis, for example, the flow of electrons from the reductase domain of eNOS to the oxidase domain (where L-arginine is oxidized) may become uncoupled from NO production. Under these conditions, the enzyme produces O_2^- or $ONOO^-$ in place of NO, a process referred to as "eNOS uncoupling". Uncoupling with a concomitant reduction in NO production occurs primarily when eNOS becomes monomeric (normally functional eNOS is a dimer) (Forstermann & Munzel, 2006; Zou, et al., 2004) and dissociation and/or oxidation of the eNOS cofactor tetrahydrobiopterin has been suggested to play a key role in uncoupling (Bendall, et al., 2005). Tetrahydrobiopterin availability is thought to be an important regulator in activity and uncoupling of eNOS (Bendall, et al., 2005). A recent study in diabetes-induced mice suggests that tetrahydrobiopterin oxidation leads to eNOS uncoupling and dysfunction, with exogenous administration of tetrahydrobiopterin promoting eNOS dimerization and normalisation of eNOS function (Abudukadier, et al., 2013). Uncoupling of eNOS has been demonstrated in human arteries and veins concomitant with an increase in O_2^- production (Margaritis, et al., 2013). Increases in O_2^- production with consecutive endothelial dysfunction have been attributed to eNOS uncoupling (Vasquez-Vivar, et al., 1998), enhanced activity of NADPH oxidase (an enzyme whose primary function is to produce O_2^-) and xanthine oxidase (Jacobson, et al., 2007).

Independent of oxidative stress and in addition to situations where the availability of the NOS substrate L-arginine may be compromised, a number of methylated arginine derivatives such as asymmetric dimethylarginine act as endogenous inhibitors of NOS and thus of NO generation (Böger, 2003). Asymmetric dimethylarginine concentrations in plasma and tissues tend to increase in particular in patients with heart and renal failure, contributing to risk of adverse outcomes in such individuals (Visser, et al., 2010).

Thus, endothelial dysfunction can be a result of several different abnormalities affecting either production or availability of NO, and under many pathophysiological conditions it is possibly a multi-factorial process. Therefore, in most cases no causative treatment option will be available. An attractive alternative to rescuing compromised endothelial function by correcting the true cause of the malfunction is administration of an alternative source of NO. Current treatment options for conditions such as angina, myocardial infarction, and heart failure include administration of organic nitrates, for example glyceryl trinitrate (GTN) as a source of NO, causing coronary vasodilatation (Marsh & Marsh, 2000). However, undesirable side effects including a throbbing headache and orthostatic problems as well as tolerance development (limiting drug efficacy when administered for a prolonged period of time) have led to an intense search for superior treatment options. As judged by the outcome of several animal experimental studies, inorganic nitrite (NO_2^-) administration may hold promise for the treatment of conditions associated with endothelial dysfunction by improving vascular function following biotransformation to NO; if found to be of value in human trials as well, it may become an attractive treatment alternative to organic nitrates in the future.

In this review, we discuss nitrite as a potential NO substitute, the mechanisms involved in nitrate bioactivity and the role of nitrite in cardiovascular health and function.

3. Nitrite as a potential nitric oxide substitute

The nitrate-nitrite-NO pathway has been proposed as an alternative pathway for NO generation (Lundberg et al., 2008). NO produced through this pathway has been proposed to represent a NOS-independent alternative to the classical pathway in which NO is produced by oxidation of L-arginine in a reaction catalyzed by NOS (Lundberg et al., 2008). Under normal physiological pH and oxygen tension, nitrite is an endogenous substance produced *via* the oxidation of NO (in cells by cytochrome C oxidase (Torres et al, 2000), in blood principally by ceruloplasmin; Shiva et al., 2006), and for many years nitrite was simply considered a relatively inert metabolic end-product of NO. However in the past decade, it has become apparent that under certain conditions nitrite exerts potent biological effects, and several research groups have identified that – particularly at low pH and oxygen tension - nitrite is reduced by various nitrite reductases to NO (Aamand, et al., 2009; Cosby, et al., 2003; Feelisch, et al., 2008; Rassaf, et al., 2007; Shiva, et al., 2007a; Totzeck, et al., 2012a; Webb, et al., 2004; Webb, et al., 2008a). Table 1 summarises various animal studies that have evaluated the efficacy of nitrite as a vasodilator and/or BP lowering agent. *In vivo*, nitrite is readily oxidised to nitrate (NO_3^-) by cellular and acellular processes, and the latter can be reduced back to nitrite *via* mechanisms involving the commensal bacterial flora of the oral cavity and the gut as well as reduction by xanthine oxidoreductase (XOR) in the host tissues (Lundberg, et al., 2009). This had led to the conceptualisation that a 'nitrogen oxide cycle' exists by which dietary sources of nitrite and nitrate mix in with nitrite and nitrate produced by the oxidation of endogenous NO, suggesting a common circulating pool contributes to bodily NO production.

An important physiological role for the nitrate-nitrite-NO pathway is increasingly becoming apparent in the literature as its activity under various conditions is being uncovered. It has been shown by a number of research groups that nitrite-derived bioactivity is cardioprotective by minimising cell death by apoptosis (Dezfulian, et al., 2007; Duranski, et al., 2005; Shiva, et al., 2007a; Shiva, et al., 2007b; Tripatara, et al., 2007; Webb, et al., 2004), exerts anti-aggregatory effects (Corti, et al., 2013; Park, et al., 2013; Srihirun, et al., 2012; Velmurugan, et al., 2013), inhibits hypoxic and inflammatory pulmonary arterial hypertension (Bueno, et al., 2013; Sparacino-Watkins, et al., 2012; Baliga, et al., 2012), and that it increases forearm blood flow (FBF) and decreases BP by acting as a vasodilator (Cosby, et al., 2003; Dejam, et al., 2007; Maher, et al., 2008). In addition, nitrate has been shown to improve exercise performance by reducing the oxygen cost of exercise in skeletal muscle (Larsen, et al., 2011). Since this profile of action resembles that of NO (and the latter can be measured as a reaction product under specific reaction conditions) it is widely believed that NO is the active principle that underpins most if not all of the actions of nitrite (and nitrate). However, to the best of our knowledge, this has not been unequivocally demonstrated. In this review, we shall focus on the vasodilator effects of nitrite and dietary nitrate, together with the therapeutic potential of nitrite in acute heart failure (AHF).

4. Dietary sources of nitrite and nitrate

4.1. Nitrate and conversion to nitrite

Nitrate (NO_3^-) is a ubiquitous constituent of our environment and plays an essential role in the global nitrogen cycle. Symbiotic Rhizobia bacteria located in the root nodules of leguminous plants can fix atmospheric nitrogen and hydrogen to produce ammonia which can be transported into the soil through plant roots, or by decomposition (Gilchrist, et al., 2010). The ammonia can be converted into nitrite by denitrifying *Nitrosomonas* bacteria (and ammonia oxidizing Archaea) in the soil and further to nitrate by *Nitrobacter*. Nitrate can be taken up from the soil through transporter channels in plant root cells, providing a source of nitrogen for amino acids, proteins and nucleotides essential for growth and development (Wang, et al., 2012). The genetic make-up of the plant and thus the degree of nitrate influx and efflux *via* transporter channels can affect the nitrate content of the plant (Wang, et al., 2012). In addition, there are several environmental impacts affecting the degree of nitrogen fixation, including temperature, precipitation, soil type and the extent of agricultural fertilizer use, and the intensity of exposure to sunlight all of which can have an impact on the nitrate content of plants (Seljasen, et al., 2012).

4.2. Dietary sources of nitrate

Green leafy vegetables are the major source of dietary nitrate. The highest reported nitrate content is found in rocket (arugula; range 963-4305 mg nitrate/kg) (Santamaria, et al., 1999), followed by radish (range 1117-2993 mg nitrate/kg) (Santamaria, et al., 1999), spinach (range 961 – 2453 mg nitrate/kg) (Koh, et al., 2012), beetroot (range 644-1800 mg nitrate/kg) (Tamme, et al., 2006) and lettuce (range 428-1766 mg nitrate/kg depending on type) (Santamaria, et al., 1999). Cured meats are also a source of dietary nitrates and nitrites through the use of potassium and sodium nitrate and nitrite as curing/preserving agents in these products (Binkerd & Kolari, 1975; Kim & Conca, 1990). Drinking water is another source of nitrate (Knobeloch, et al., 2013; Nemčić-Jurec, et al., 2013); the concentration of nitrate in drinking water will vary according to geographical location, regional rules regarding safe levels of nitrate in tap water, or the consumption of bottled water (Espejo-Herrera, et al., 2013).

4.3. Route of dietary nitrate after consumption

Tracer studies with the stable nitrogen isotope ^{15}N revealed that about 60% of an oral ^{15}N-nitrate dose ingested is excreted *via* the kidneys within the following 48h; the fate of the remainder is unclear and assumed to be subject to metabolic transformation to other nitrogen-containing species (Wagner, et al., 1984). About 25% of the circulating pool of nitrate is actively taken up from blood *via* an anion exchange channel called sialin (Qin, et al., 2012) and secreted by the salivary glands into saliva (Lundberg, et al., 2008). The salivary nitrate is reduced to nitrite by commensal bacteria (*Actinomyces* and *Veillonella* spp.) residing on the surface of the tongue (Doel, et al., 2005; Pannala, et al., 2003; Tannenbaum, et al., 1976). Nitrite is then swallowed into the stomach; in this strongly acidic environment nitrite is protonated to form nitrous acid (HNO_2; pK_a 3.15; the pK_a is the pH at which 50% of the acid is dissociated), and can spontaneously give rise to the generation of NO through the following sequence of reactions: $2HNO_2 \rightarrow H_2O + N_2O_3$ and $N_2O_3 \leftrightarrow NO + NO_2$ (Butler and Feelisch, 2008), or re-enters the circulation as nitrite (Benjamin, et al., 1994; Lundberg, et al., 1994). This cycle is known as the 'enterosalivary recirculation pathway' of nitrate.

Several studies have highlighted the importance of oral bacteria flora in the reduction of nitrate to nitrite. In earlier studies by Lundberg and Govoni (2004), the authors showed that avoiding swallowing after nitrate ingestion can abrogate increases in plasma nitrite (Lundberg & Govoni, 2004). In 2008 Govoni and colleagues expanded this work by studying the effects of commercially available antibacterial mouthwash on salivary and plasma levels of nitrate and nitrite following an oral intake of dietary nitrate (sodium nitrate) in healthy subjects. The authors reported that rinsing the mouth with antibacterial mouthwash prior to ingestion of nitrate reduces the conversion to nitrite in the saliva and attenuates the rise in plasma nitrite (Govoni, et al., 2008). To corroborate these findings further, Petersson and colleagues (2009) explored the role of oral commensal bacteria in bioactivation of dietary nitrate to nitrite and NO. Rats were treated twice daily with antiseptic mouthwash while they were given nitrate-supplemented drinking water (10 mmoles/L sodium nitrate) (Petersson, et al., 2009). The authors reported a reduction of nitrate-reducing oral bacteria with a consecutive attenuation of circulating nitrite levels and the gastroprotective effects of nitrate. Moreover, nitrite-dependent BP lowering effects of nitrate were abolished, suggesting oral bacteria play an essential role in the regulation of gastrointestinal and cardiovascular function *via* the bioactivation of salivary nitrate (Petersson, et al., 2009). Furthermore, Kapil and colleagues have shown in healthy volunteers that seven days treatment with an antiseptic mouthwash reduces oral nitrite production by up to 90%, with a concomitant decrease in plasma nitrite levels to 25% of control values (Kapil et al., 2013).

5. Circulating nitrate and nitrite

5.1. Relationship between plasma nitrate and nitrite

The basal level of nitrite in the plasma of healthy individuals has been measured, with considerable variation between subjects and methods used, with values ranging from a range of 50-150 nmoles/L to almost 1000 nmoles/L (Gladwin et al., 2000; Lauer et al., 2001; Rassaf et al., 2003; Kleinbongard, et al., 2003; Govoni et al., 2008). The reason for this variability is not exactly clear but is likely to involve methodological issues such as ongoing uptake by blood cells during the centrifugation process. The majority of basal nitrite originates from the oxidation of NO (Kleinbongard, et al., 2003; Moncada & Higgs, 1993; Rhodes, et al., 1995), with the remainder stemming from the metabolic conversion of dietary nitrate. Ingestion of nitrate causes a rapid increase in circulating plasma nitrate within 30 minutes of consumption; the level of plasma nitrate peaks at 3 hours with levels remaining elevated for up to 24 hours (Kapil, et al., 2010a; McKnight, et al., 1997). Elevations in plasma nitrate are followed by a delayed increase in plasma nitrite secondary to bioconversion; nitrite levels rise over 1 to 2 hours and form a plateau between 2 to 6 hours after which concentrations decline (Kapil, et al., 2010a; McKnight, et al., 1997). Further research has demonstrated maximal reduction in BP in humans at 2 to 3 hours after consumption of nitrate, corresponding with the point of peak plasma nitrite concentration (Petersson, et al., 2009; Webb, et al., 2008b). It has also been suggested that BP reduction by nitrates acts in a dose-dependent manner (Kapil, et al., 2010a), although it is appreciated now that physiologically, the basal level of nitrite is an important contributor to blood flow regulation.

5.2. The effect of dietary nitrate on circulatory nitrate and nitrite levels

The Mediterranean diet is noted for its high content of vegetables, fruit and fish and has been linked to a lower incidence of CVD (Appel, et al., 1997; Grosso, et al., 2014; Joshipura, et al., 2001; Lundberg, et al., 2006) and diabetes (Salas-Salvado, et al., 2014). The volume of vegetable consumption is notably higher in the Mediterranean diet than the Western diet; for example in the UK the average vegetable consumption per day is estimated at 160 g (Meah, et al., 1994), whilst the Mediterranean diet contains approximately 550 g of vegetables per day (Trichopoulou, et al., 2003). In terms of vegetable-provided dietary nitrate, the Mediterranean diet is estimated to contain 400 mg nitrate per day, which is over four times the amount in a typical Western diet (estimated at 77 mg per day) (Raat, et al., 2009). The Dietary Approaches to Stop Hypertension (DASH) diet has also been suggested to aid in reduction of CVD, and a review of current dietary recommendations based on the levels of beneficial dietary nitrate and nitrite has recently been suggested (Hord, 2011).

Numerous studies have demonstrated that intake of nitrate-rich vegetables can increase the levels of circulating plasma nitrite (Kapil, et al., 2010b; Lidder & Webb, 2012; Lundberg, et al., 2006; Machha & Schechter, 2012). An acute application of sodium (or potassium) nitrate in water or fruit juice or supplementation of the diet with nitrate has been shown to increase the levels of plasma nitrite (Kapil, et al., 2010a; Larsen, et al., 2006; Lundberg & Govoni, 2004). Beetroot juice has a relatively high nitrate content and is frequently used in human studies as a convenient source of nitrate, not least because it is classified as 'food', simplifying the administrative effort required to carry out human studies in the UK and other countries. Beetroot juice consumption in healthy volunteers has been shown to be associated with an increase in the levels of plasma nitrate (by 16 fold) and nitrite (by 2 fold) (Webb, et al., 2008b). Cermak and colleagues reported a similar trend with regard to plasma nitrite levels in trained male cyclists. Control subjects who consumed 140 ml nitrate-depleted beetroot juice had plasma nitrite levels of 271 nmoles/L, whereas subjects consuming the same volume of beetroot juice (containing 8.7 mmoles nitrate) showed a two-fold higher plasma nitrite level of 532 nmoles/L (Cermak, et al., 2012). It has also recently been demonstrated that nitrite/nitrate handling in humans shows sexual dimorphism: increases in plasma nitrate and nitrite levels as well as inhibition of platelet function after consumption of beetroot juice revealed a clear gender difference (Velmurugan, et al., 2013).

The consumption of dietary nitrate from beetroot juice in healthy volunteers has been shown by Wylie and colleagues to increase plasma nitrate and nitrite levels in a dose-dependent manner (Wylie, et al., 2013). Volunteers were given beetroot juice containing ~4.2, 8.4 or 16.8 mmoles nitrate; the level of circulating nitrate peaked at values of 160±43 µmoles/L, 269±92 µmoles/L and 581±209 µmoles/L, respectively (all P <0.05). These corresponded with increases in plasma nitrite to 220±104 nmoles/L, 374±173 nmoles/L and 653±356 nmoles/L (all P <0.05) respective to increasing dose (Wylie, et al., 2013). Furthermore, Vanhatalo et al demonstrated that continued beetroot juice consumption over a period of 15 days resulted in sustained elevation of plasma nitrite levels (Vanhatalo, et al., 2010). The authors found that consumption of beetroot juice containing 5.2 mmoles nitrate/day significantly elevated plasma nitrite levels by 35% 2.5 hours after ingestion, 25% at 5 days and 46% at 15 days, as discussed further in section 6.1.

6. Effects of nitrate and nitrite on cardiovascular function

6.1. Effect of dietary nitrates

As discussed in section 5.2 and depicted in table 2, several research groups have demonstrated that ingestion of dietary nitrate (beetroot juice) in healthy subjects results in increased plasma nitrite concentration *via* bioconversion *in vivo* (Cermak, et al., 2012; Kapil, et al., 2010a; Velmurugan, et al., 2013; Webb, et al., 2008b; Wylie, et al., 2013) and substantial research efforts have focused to investigate the role of this bioactive nitrite on cardiovascular function (Bondonno, et al., 2012; Lansley, et al., 2011; Vanhatalo, et al., 2010; Webb, et al., 2008b; Wylie, et al., 2013). For instance, Webb and colleagues reported a substantial reduction in systolic blood pressure (SBP) of 10.4 mmHg 3 hours after ingestion of beetroot juice, and this effect inversely correlated with peak increases in plasma nitrite levels (Webb, et al., 2008b). In order to ascertain that nitrite was indeed the carrier of bioactivity that accounts for the BP change, a complementary spitting study was conducted. The results clearly demonstrated that spitting caused interruption of the enterosalivary recirculation of dietary nitrate in as much as it abolished both the rise in plasma nitrite and the decrease in BP (Webb, et al., 2008b).

The consumption of nitrate rich spinach has been shown to decrease SBP, by 2.7 mmHg, and increase flow mediated dilatation (FMD) of the brachial artery, by 0.5%, in healthy volunteers (Bondonno et al., 2012). The response of healthy volunteers to dietary nitrate *via* beetroot juice consumption has been demonstrated to show a dose-response relationship between nitrate load and BP. Ingestion of beetroot juice decreased SBP and mean arterial pressure in relation to nitrate dose (Wylie, et al., 2013). Decreases in diastolic blood pressure (DBP) were only found with the higher doses of nitrate in this study. These changes in BP correlated with changes in plasma nitrate and nitrite. The same study also demonstrated that dietary nitrate can reduce steady-state oxygen uptake during moderate intensity exercise and increases time to task failure (Wylie, et al., 2013). In agreement with the lack of tolerance development to the vasodilator effects of nitrite alluded to earlier, Vanhatalo and co workers have shown that elevated plasma nitrite levels during subchronic consumption of beetroot juice over a period of 2 weeks is associated with a sustained decrease in SBP and DBP and reduced O_2 cost of submaximal exercise (Vanhatalo, et al., 2010). Similarly, Lansley and colleagues showed that elevated plasma nitrate and nitrite in healthy competitive male cyclists improved performance in time trial cycling without alteration of VO_2 max by improving exercise efficiency (Lansley, et al., 2011). In contrast to these results in fit but untrained or moderately trained athletes, dietary nitrate supplementation has not been demonstrated to have beneficial effects on exercise performance or endurance in elite athletes (Christensen, et al., 2013; Peacock, et al., 2012).

Overall, the results from these studies suggest that oral nitrate administration, particularly *via* beetroot juice, increases plasma nitrite levels and produces a moderate vasodilator response in healthy volunteers. The effects of dietary nitrate have also been investigated in the setting of chronic environmental hypoxia and under pathophysiological conditions (Martin, et al., 2013; Ghosh, et al., 2013). A double-blind placebo-controlled study of the effects of beetroot juice on various bodily functions was carried out in 28 healthy human volunteers, first at sea level and then during 5 days at high altitude (4,559m) (Martin, et al., 2013). The purpose of this study was to investigate whether the beneficial effects of nitrate on mitochondrial efficiency observed in some of the studies described above are maintained or might even be enhanced under conditions of reduced oxygen availability; if the latter was true this could be of benefit for critically ill patients suffering from hypoxemia. Publication of results from this study are eagerly awaited.

In another investigation in patients with grade 1 hypertension it has been shown that the consumption of beetroot juice can elevate plasma nitrite levels by 1.5 fold, with the increase being associated with a decrease in both SBP and DBP (Ghosh, et al., 2013). Grade 1 hypertensives were classed as those with SBP between 140-159 mmHg or DBP 90-99 mmHg. Dietary nitrate was consumed by drinking 250 ml of beetroot juice with nitrate concentration of 13.2 mmoles/L. The SBP in these patients was shown to decrease, with peak mean fall in pressure occurring between 3 and 6 hours after consumption of beetroot juice at 11.2 mmHg compared to 0.7 mmHg in controls (Ghosh, et al., 2013). DBP was also reduced in hypertensive patients who consumed beetroot juice, with a peak mean fall in pressure of 9.6 mmHg. Similar to healthy volunteers, in hypertensive patients the decrease in SBP was inversely correlated with plasma nitrite but not nitrate levels. It is interesting that compared to healthy volunteers, a lower dose of dietary nitrate is required to produce a comparable drop in SBP and DBP in hypertensive patients, perhaps because of these patients' higher BP at baseline (Ghosh, et al., 2013).

Another recent investigation is of interest in the context of discussions about the effects of nitrite and nitrate on BP. In this study, the skin of 24 healthy human volunteers was exposed for 20 min to ultraviolet (UVA) radiation from tanning lamps. During and up to half an hour after UVA exposure DBP was significantly lowered by approximately 5 mmHg, and these hemodynamic changes were associated with opposite changes in circulating nitrate and nitrite concentrations (Liu, et al., 2014). These light-induced BP changes were independent of changes in vitamin D levels and suggested translocation of NO bioactivity from a preformed storage pool in the skin to the circulation, resulting in an elevation of plasma nitrite at the expense of nitrate. Surprisingly, an acute 10-fold elevation of circulating nitrate levels (from basal concentrations of 10.7 µmoles/L on a low nitrate diet to 108 µmoles/L 1 hour after acute oral ingestion of sodium nitrate) did not alter the hemodynamic effects of UVA light. Thus, in addition to the bioactivation of nitrate to nitrite *via* oral commensal bacteria, another endogenous pool of nitrate (or a closely related NO species that can give rise to nitrite) appears to exist in human skin that may contribute to BP regulation in response to exposure of the body to UVA/sunlight.

These studies built on earlier investigations on the effects of light on vascular tone of isolated rabbit aortic strips in organ baths (Furchgott et al., 1961), a phenomenon known as 'photorelaxation'. In those studies, addition of sodium nitrite to the organ bath potentiated light-induced vasorelaxation in an endothelium-independent manner (Matsunaga & Furchgott, 1989). This effect of nitrite was potentiated by the presence of superoxide dismutase or other O_2^- scavengers (Matsunaga & Furchgott, 1989; Matsunaga and Furchgott, 1991), demonstrating the importance of the balance between NO and O_2^-. Later studies in rat aortic rings showed that the release of NO from vascular storage forms comprising S-nitrosothiols and nitrite account for the phenomenon of photorelaxation (Rodriguez et al, 2003).

6.3. Nitrite-mediated vasodilatation in physiological and pathophysiological conditions

6.3.1. Animal studies

The use of nitrite as a BP lowering agent dates back to the beginning of the last century (Butler & Feelisch, 2008). In 1953, the ability of nitrite to act as a vasodilator was first demonstrated by Furchgott and Bhadrakom who showed that administration of sodium nitrite to pre-contracted rabbit aortic strips *in vitro* induced vasorelaxation (Furchgott & Bhadrakom, 1953). In subsequent studies 200 µmoles/L nitrite was shown to relax rat aorta *in vitro* under normoxic conditions, with a reduction to 40 µmoles/L required under hypoxia (Modin, et al., 2001). Whilst in a canine model under normoxic conditions aortic relaxation has been shown to occur with administration of nitrite at levels between 100 to 1000 µmoles/L (Arai, 2005). In more recent work, high micromolar to millimolar pharmacological concentrations of exogenously administered nitrite have been demonstrated to relax pre-constricted isolated blood vessels (Maher, et al., 2008; Ormerod, et al., 2011). Thus, when studied using isolated vascular preparations nitrite is far less potent a vasodilator than NO itself. In very early work by Reichert and Mitchell (1880), potassium nitrite was found to exert a dose-dependent effect on pulse (at the time these experiments were conducted routine BP measurement was not yet part of clinical practice and only possible using somewhat cumbersome invasive methods (Booth, 1977); thus, pulse rate was used as a proxy for systemic effects on the circulation). In man, 2 grains of potassium nitrite showed little effect on pulse (1 grain is a unit of mass equal to approximately 65 mg; 2 grains translate into a dose of approximately 2mg/kg). When the dose was increased to 6 grains, the pulse (measured 40 minutes after nitrite administration) was increased by approximately 30 beats per minute; this was associated with throbbing vessels and warmth to the face. Ten grains of potassium nitrite increased pulse to an even greater extent within 25 minutes, concurrent with flushed face and hands and a throbbing headache. These observations are consistent with systemic and peripheral vasodilatation. The same authors also reported the effects of potassium nitrite on arterial BP in animals. In experimental rabbits, cats and dogs, large doses of potassium nitrite (0.2 g in rabbits and cats and 0.5-1.0 g in dogs) caused an immediate and continual decrease in BP to zero. However, a smaller dose (0.08 g) increased BP within 30 seconds after administration, which was followed by a decline in pressure; further administration of nitrite caused a similar pattern of transient increase followed by a large, sustained fall in blood pressure (Reichert & Mitchell 1880). A similar dose-dependent effect of nitrite on BP has been reported more recently by Feelisch and colleagues (Bryan et al, 2005). Intraperitoneally administered low doses (0.1 mg/kg) of sodium nitrite in rats caused a small, non-significant increase in mean arterial blood pressure (MAP) while higher doses (1.0 and 10 mg/kg) decreased BP by a maximum of 5% and 27% of controls, respectively. Since no direct vasoconstrictor effects to nitrite have been observed *in vitro*, these studies suggest that *in vivo* nitrite has a dual effect on the vasculature, acting as a vasodilator at higher doses while eliciting a transient pressor effect at lower to intermediate doses; the latter may be secondary to functional interaction with other vasoactive factors.

In an *in vivo* study in Wistar rats, it has been demonstrated that administration of either infused sodium nitrite, or potassium nitrite supplemented in the drinking water is associated with a decrease in MAP in both anaesthetized and freely moving rats (Vleeming, et al., 1997). In a mouse model of ischemic (hypoxic) hind-limb, hypertensive mice showed a significantly decreased blood flow compared to sham operated mice, and blood flow could be recovered in both hypertensive and normotensive mice when treated with sodium nitrite compared to non-treated mice. Inhibition of XOR prevented this recovery, suggesting a role for XOR-mediated reduction of nitrite to NO in blood flow under these conditions, as suggested in *in vitro* studies (Amin, et al., 2012; Li, et al., 2003). Hypertensive mice also demonstrated a decrease in cGMP levels compared to control mice in the hind limb (Amin, et al., 2012). However, treatment with sodium nitrite was found to significantly enhance cGMP levels; thus in this study, it was suggested that sodium nitrite could be used as a therapy for full recovery of blood flow. Table 1 summarises studies to date investigating the role of nitrite on vasodilatation and BP in animal models.

6.3.2. Translational studies in man

The vasodilatory effects of nitrite infusion were later translated to healthy human volunteers. In 2003, Cosby and colleagues showed that sodium nitrite vasodilates the forearm vasculature when infused into the brachial artery (Cosby, et al., 2003). Initially, a high dose of 36 µmoles/min (2.4 mg/min), resulting in an approximate intravascular nitrite concentration of 200 µmoles/L, was tested and found to significantly increase FBF both with and without NOS inhibition by *N*-methylarginine (n = 10, P <0.01). FBF increased further on forearm exercise during continued nitrite infusion, despite relative reduction in nitrite concentration due to increased blood flow. Cosby and colleagues went on to test a "near-physiological" dose of 400 nmoles/min (27.6 µg/min), which significantly increased FBF from 3.5 ± 0.2 to 4.5 ± 0.3 ml/min/100 ml tissue (n = 10, P <0.006) at rest, and to a greater extent on exercise (Cosby, et al., 2003). The authors concluded that basal levels of nitrite are capable of influencing resting vascular tone and subserving hypoxic vasodilatation. In subsequent studies, the same group extended their previous observations with systemic nitrite. A lower dose of sodium nitrite, resulting in a plasma concentration of just 350 nmoles/L, was found to cause a significant drop in BP in healthy volunteers. In addition, the authors showed a dose-dependent increase in sodium nitrite infusion (0 to 110 µg/kg/min) increased FBF from 2.8 to 12.3 ml/min/100 ml tissue (Dejam, et al., 2007).

In support of an enhanced role of nitrite during hypoxia, the effects of sodium nitrite infusion during normoxia and hypoxia, respectively in healthy volunteers were investigated by our group (Maher, et al., 2008). Sodium nitrite was infused into the forearm brachial artery at doses from 40 nmoles/min to 7.84 µmoles/min (Maher, et al., 2008). Under normoxic conditions, large decreases in forearm venous tone were found at doses between 784 nmoles/min and 7.84 µmoles/min, with peak venodilatation of 35.8% (±7.5% P <0.005) occurring at the highest infused dose of 7.84 µmoles/min. The forearm blood flow ratio (FBF-R: FBF corrected for control arm) was increased during the two highest doses of nitrite infusion at 3.14 µmoles/min and 7.84 µmoles/min, increasing from a baseline of 1.0 to 1.8 and 1.6 respectively (Maher, et al., 2008). Under hypoxic conditions, FBF-R was enhanced following infusion of 7.84 µmoles/min and FBF-R significantly increased compared to the same dose under normoxic conditions (P <0.05). We concluded that under normoxic conditions, nitrite was a potent vasodilator of capacitance vessels but only a modest dilator of resistance vessels (compared with other vasodilating agents), whilst under hypoxic conditions administration of exogenous sodium nitrite has a substantial relaxation effect on resistance vessels. This may be due to the relatively low PO_2 in the capacitance bed compared to resistance vessels under physiological conditions; thus the effect of nitrite on capacitance vessels will be more pronounced in normoxia, whereas in hypoxia nitrite demonstrates a greater effect on the resistance vessels due to the decrease in PO_2 approaching levels (previously seen but not markedly enhanced) in the capacitance vasculature. This study thus demonstrated that oxygen tension plays an essential role in determining the vasodilatory response to nitrite (Maher, et al., 2008), although it does not exclude the involvement of other contributing factors. Table 2 summarises the recent studies that have investigated the efficacy and potential mechanisms of nitrite-mediated vasorelaxation and BP reduction in healthy and pathophysiological conditions, such as patients with hypertension or heart failure.

There are noticeable discrepancies between studies regarding to the dose of nitrite and increased blood flow in normoxia (Cosby, et al., 2003; Lauer, et al., 2001; Maher, et al., 2008). It has been proposed that in normoxia, both acetylcholine (Larrousse, et al., 2006) and bradykinin (Wotherspoon, et al., 2005) are more effective vasodilators, inducing increase in blood flow by three to four fold, but that in hypoxia it appears that nitrite plays a dominant role. This would suggest that different pathways exist to confer vasodilatation during normoxia and hypoxia and the differing conversion of nitrite to NO under varying oxygen tensions is thus believed to be an important feature integral to hypoxic signalling.

The results on nitrite on human FBF under normoxic conditions (Cosby, et al., 2003) are in stark contrast to earlier findings by Lauer et al (Lauer, et al., 2001), claiming that nitrite had no direct vasodilator effect when intra-arterially infused, for one minute, at a rate identical to that used in the Cosby study. An explanation for this discrepancy is discussed in section 7 (mechanisms of nitrite-mediated vasodilatation).

With regard to nitrite administration in pathophysiological conditions, a study comparing the intraarterial effects of nitrite administration in healthy volunteers with congestive heart failure (CHF) patients highlighted differing effects of nitrite administration between the two groups (Maher, et al., 2013), with evidence of hyperresponsiveness in forearm resistance vessels in the latter (Maher, et al., 2013). In contrast there was reduced venodilation in the heart failure patients, however at any given nitrite infusion dose the venous levels of plasma nitrite were lower in the heart failure patients *vs* controls indicating increased clearance across the forearm vascular bed and possibly explaining the apparent venous hyporesponsiveness (Maher, et al., 2013). The results from these studies in patients with CVD highlights complications which may arise when translating results from both animal models and healthy human models into clinical settings, and more research in the presence of particular disease states may be required to fully understand how nitrite, and indeed other forms of therapy, can be applied for maximum patient benefit and improved clinical outcome.

6.4. Role of nitrite at different oxygen tensions

Studies have shown that the level of nitrite in plasma appears with an apparent arteriovenous gradient, showing greater vasodilatation activity in the capacitance vessels under normoxic conditions and in the resistance vessels under hypoxic conditions (Maher, et al., 2013; Maher, et al., 2008). This effect has also been observed in studies of human FBF, most notably during exercise (Cosby, et al., 2003; Gladwin, et al., 2000). It has been suggested that this gradient may be due to consumption of nitrite and NO during transit along the vascular tree (Cosby, et al., 2003; Gladwin, et al., 2000); the nitrite reductase activity of deoxyhemoglobin (deoxyHb) in particular is suggested to play a role in nitrite-NO formation as described below.

One molecule which has garnered more attention than any other in this area is hemoglobin (Hb). Oxyhemoglobin (oxyHb) and deoxyHb are known as potent NO scavengers (Isbell et al., 2007; Joshi, et al., 2002), and have been shown to regulate the effects of NO (Griffith, et al., 1984). However, only oxyHb chemically reacts with NO to form nitrate, deoxyHb binds it to form nitrosylHb. The balance between the different states of oxygen saturation in Hb has been suggested to contribute to the gradient effect between arterial and venous systems, with deoxygenation of the heme moiety suggested to express reductase activity with maximal efficiency around the p50, i.e. the oxygen partial pressure at which 50% of the hemoglobin is oxygenated (Crawford, et al., 2006; Feelisch, et al., 2008; Gladwin & Kim-Shapiro, 2008; Huang, et al., 2005). It has been suggested that Hb acts as a nitrite reductase in the blood, with red blood cells (RBC) representing the principle source of Hb and a carrier of nitrite (Dejam, et al., 2005). Hb can interact with blood nitrite *via* a redox process determined through both the heme redox potential and oxygen saturation of Hb (Shiva, et al., 2011). A particular balance of oxyHb to deoxyHb is required for optimal reduction of nitrite to NO, peaking around the p50 value. The NO produced may then interact with mitochondrial cytochrome *c* oxidase which contains a binuclear centre to which oxygen ordinarily binds in the mitochondrial respiratory chain. However, upon cytochrome *c* oxidase-NO binding, oxygen binding becomes inhibited, and mitochondrial respiration is reduced (Shiva, et al., 2011). During hypoxia, NO-mediated inhibition of mitochondrial respiration is enhanced. This process may aid the extension of oxygen gradients in tissues ensuring oxygen delivery to a greater tissue area, regulating mitochondrial ROS generation and the action of Hypoxia Inducible Factor 1-α (Hagen, et al., 2003). Physiologically, the vasodilatory action of Hb reduction of nitrite to NO is suggested to mediate the cGMP-dependent pathway of vasodilatation in hypoxia (Cosby, et al., 2003; Crawford, et al., 2006; Huang, et al., 2005; Jeffers, et al., 2005).

The effect of oxygen tension on nitrite metabolism has also been investigated in animal experimental models. RBC homogenates showed the expected behaviour in as much as NO production from nitrite was found to be maximal around the p50 value of Hb (Feelisch, et al., 2008). In contrast, in rat tissue homogenates the *in vitro* reduction of nitrite to NO was shown to be limited under normoxic conditions, while nitrite to NO conversion progressively increased at lower oxygen concentrations in all organs, including vascular tissue (Feelisch, et al., 2008). This metabolic activity was associated with an increase in the formation of nitrosated and nitrosylated products *in vivo*. The formation of NO from nitrite was found to be largely enzymatic in nature as it was sensitive to heat inactivation and blockage of thiol groups (Feelisch, et al., 2008), supporting earlier studies implicating heme and thiol-containing reductases in this process (Bryan, et al., 2005). Data from a more recent *in vivo* study carried out in rats suggest that acute hypoxic vasodilatation is largely mediated by NO metabolites rather than by free NO from Hb-mediated nitrite reduction (Umbrello, et al., 2014).

The step-wise reduction of nitrate to nitrite and nitrite to NO has been demonstrated under anoxic conditions *in vitro* with a role for nitrate/nitrite reductase xanthine oxidase (XO) (Li, et al., 2003). The authors of this study also found that nitrite and NO production was pH-dependent with maximum observable NO production at pH 5.0, suggesting that XO reduces nitrate to nitrite and NO under acidic conditions, as associated with CVD (Li, et al., 2003).

7. Mechanisms of nitrite-mediated vasodilatation

Much of our current understanding about nitrite's mode of action as a vasodilator is based on animal experimental work and observed associations of changes in circulating plasma concentrations and blood flow and/or pressure at pharmacological doses; other pieces of information are derived from *in vitro* studies with isolated proteins or cultured cells. Considerably less information is available on the mechanism of vasodilatation by nitrite in human tissue and the role, if any, of endogenous nitrite for human physiology. The fact that nitrite can relax isolated segments of precontracted vascular tissue in organ baths does not necessarily mean that it is involved in the regulation of BP. Part of the paucity of mechanistic information is due to the fact that monitoring the rather low concentrations of endogenous nitrite requires specific analytical equipment such as HPLC or gas phase chemiluminescence, which is not available in every laboratory; another reason is that our current understanding of basic processes including nitrite/nitrate uptake, processing and excretion at the cellular and whole organism level is incomplete. Moreover, while biochemically interesting in principle some *in vitro* findings obtained with high nitrite concentrations under complete anoxia are likely to be of limited relevance to physiology. The lack of pharmacological tools such as specific 'nitrite scavengers' is also a limiting factor. The extrapolation of animal experimental data to human physiology requires particular care – not everything that looks promising in the animal experimental setting is ultimately going to work in humans.

It is important to acknowledge that no 'nitrite receptor' has yet been described; thus, there is no mechanistic basis for a direct coupling of a nitrite recognition site to a down-stream signalling event in smooth muscle to trigger vasodilatation. Therefore, the vasodilator effects of nitrite are not, at least at present, known to be a consequence of interaction with a specific receptor in vascular tissue; with no 'nitrite receptor' as target, there is likely no simple concentration-response relationship for its biological effect either. Although it cannot be excluded at present that nitrite may affect processes coupled to the transport of other anions in the vasculature (a process that could conceivably affect vascular tone), all evidence available to this date suggests that nitrite has to be metabolized to NO, or an NO-like species, in order to exert a biological effect. This process appears to require the presence of a sulfhydryl group and a heme moiety (Bryan, et al., 2005). There is no agreement in the literature as to whether nitrite is metabolised intracellularly or bioactivated in the extracellular space. Nitrite may require prior entry to the tissue first before it can act as a vasodilator. In this case, the plasma/tissue concentration gradient would seem to be important, but transport may well be *via* a carrier-facilitated uptake, with competition by other anions – little is known about any of this. Once inside the vascular tissue, nitrite may interact with one of the enzymes described below to become reduced to NO (or N_2O_3) and/or become biotransformed to nitroso and/or nitrosyl species before being able to interact with sGC to produce cGMP. However, nitrite transport into cells may not be required in all cases as it has recently been shown that nitrite reduction can also occur through red blood cell (or endothelial cell) nitrite reductases such as XOR (Ghosh et al., 2013) to mediate a vasodilatory response.

A surprisingly large number of different proteins have been identified that can reduce nitrite to NO *in vitro*, and detailed recent reviews on this topic are available (Kim-Shapiro & Gladwin, 2013; van Faassen, et al., 2009; Kapil, et al., 2010b; Lundberg and Weitzberg, 2010). However, there is neither agreement in the literature as to which of these potential nitrite bioactivation processes are the most relevant for vasodilatation (or any other biological process), nor do we understand the reason for this particularly high degree of redundancy. In principle, the processes involved are chemical/non-enzymatic or enzymatic, with different pH optima for nitrite reduction depending on the nature of the proteins involved. Many of the enzymatic pathways demand rather low concentrations of oxygen to reduce nitrite efficiently. In fact, oxygen appears to be a highly effective inhibitor of tissue 'nitrite reductase' activity (Feelisch, et al., 2008). Thus, hypoxia does not 'stimulate' nitrite reduction, it is rather that lower levels of oxygen result in less inhibition.

The simplest chemical pathway of NO generation from nitrite involves disproportionation of the corresponding acid, HNO_2. This pathway is probably not of much relevance for nitrite bioactivation in vascular tissue, unless it is rendered hypoxic for a prolonged period of time. Both Hb (Basu, et al., 2007) and carbonic anhydrase (Aamand, et al., 2009) have been reported to possess nitrite/nitrous acid anhydrase activity, a reaction in the course of which N_2O_3 is formed, eventually giving rise to NO and NO_2. While the former could theoretically provide a convenient way of exporting nitrite-derived NO from RBCs under hypoxic conditions, recent animal experimental results suggest that the majority of hypoxic vasodilatation is not mediated by Hb-mediated nitrite reduction (Umbrello, et al., 2014). The nitrite reductase activity of carbonic anhydrase appears to be linked to the coupling of cerebral blood flow and metabolic activity in response to visual stimulation (Aamand, et al., 2009; Aamand, et al., 1985).

It would perhaps be desirable to group proteins according to the mechanism involved in nitrite reduction, but this is not known for all proteins to date. Moreover, a single protein may employ several different pathways to reduce nitrite to NO; in the case of Hb and myoglobin (Mb) these have been proposed to include R-state catalysis, oxidative denitrosylation (of the intermediate NO-heme product formed), and the above nitrite anhydrase reaction (Gladwin, et al., 2009). Alternatively, a thiol group in Hb may become nitrosated during deoxygenation/reoxygenation, by a mechanism involving NO-heme formation from nitrite, to form a nitrosothiol (SNO-Hb) which may serve as NO-carrier (Angelo, et al., 2006), or that nitrite is reduced by the eNOS expressed in RBCs (Cortese-Krott, et al., 2012) under some conditions. Thus, even for some of the most extensively studied proteins the precise mechanisms involved remain unclear. As a result, proteins are typically categorised according to the principle reaction they are known to catalyse (which may not always match with the mechanism involved in nitrite bioactivation, but that is another matter), or the prosthetic group they carry. According to this principle, the proteins involved in nitrite reduction can be divided into two broad groups: heme-based proteins and molybdopterin-based oxidoreductases. The two best studied examples of the former are Hb and Mb, and both have been claimed to play major roles in nitrite bioactivation (Kim-Shapiro & Gladwin, 2013; Shiva, et al., 2007a; Totzeck, et al., 2012b), in particular under hypoxic conditions (Crawford, et al., 2006; Hendgen-Cotta, et al., 2014). Less well studied members of this group include neuroglobin and cytoglobin, cytochrome C and cytochrome C reductase, cytochrome P450, and the endothelial NOS isoform.

The most recent member of the heme-based nitrite reductases is cystathionine beta-synthase (CBS) (Gherasim, et al., 2014), a key enzyme of the transsulfuration pathway involved in homocysteine metabolism, glutathione production, and formation of hydrogen sulphide (H_2S). As with other heme-based reductases, NO-heme formation leads to autoinhibition of the enzyme, representing an interesting new facet of the NO/H_2S cross-talk. Members of the molybdopterin-based oxidoreductases shown to reduce nitrite to NO involve XO (Cantu-Medellin & Kelley, 2013), aldehyde oxidase (Li et al., 2008), and sulfite oxidase (Wang, et al., 2011). Many of the above proteins have been studied in relative isolation; their importance in mediating the vasorelaxant response to nitrite has been assessed using pharmacological inhibitors (of varying specificity) and, in some cases, knockout mice. Yet most papers are limited to the investigation of one specific pathway, and reading the literature one could easily get the impression that they are all equally important, or solely responsible for the effects of nitrite under some conditions. Figure 1 illustrates the nitrite-derived NO signalling in the vascular system.

Let us now revisit the discrepancy between the *in vivo* results presented by the groups of Lauer et al and Cosby et al. mentioned in section 6.3. Why did apparently identical solutions of sodium nitrite in saline produce no vasodilatation whatsoever in one case (Lauer et al, 2001) and marked increases in FBF in the other (Cosby et al, 2003) Notwithstanding minor differences in blood flow at baseline, intravascular nitrite concentrations achieved must have been of comparable magnitude. The explanation may be linked to differences in infusion times (and thus total amounts of nitrite delivered). If the same concentration of a nitrite stock solution is infused at the same rate into the vasculature then the total amount of bioactive drug (nitrite) scales with the duration of infusion. In the earlier studies by Lauer et al infusions were limited to 1 min (during which time no changes in blood flow were observed, consistent with a lack of direct vasodilator effect of nitrite) whereas in the Cosby study infusions continued for as long as 5 min. As nitrite delivery continues, increasing amounts of nitrite enter the systemic circulation, gaining access to tissues and blood cells; if the rate of administration exceeds that of elimination, nitrite and its metabolic products (including nitroso and nitrosyl species) begin to accumulate in the vasculature and relaxation ensues. A number of animal experimental results (Bryan et al, 2004; Nagasaka et al, 2008; Perlman et al, 2009) indicate that plasma concentrations of nitrite are uncoupled from those in tissues, suggesting the involvement of additional steps that regulate circulating nitrite concentrations. Thus, short-term infusions of nitrite may differ in effects on pressure and flow from those of longer lasting infusions, and total amounts of nitrite delivered may be as important as local concentrations achieved. If this was true, *in vivo* nitrite administration regimes might better be compared on the basis of cumulative amounts administered rather than circulating concentrations measured – time will tell.

While initial efforts focussed on the identification of potential nitrite bioactivation pathways (often under very specific reaction conditions with exclusion of oxygen), many more ‘leads’ emerged than anticipated and likely in operation under physiological conditions. There is a growing appreciation that reduction of nitrite to NO may be mediated by different pathways under different physiological conditions and that the effects of nitrite are sometimes mediated by metabolites other than NO. Moreover, most of the known chemistries proposed to be involved in these processes are rather slow, questioning their overall relevance for physiological regulation. Ultimately, confirmation for the involvement of candidate pathways of bioactivation will require *in vivo* experimentation. To this end, Hendgen-Cotta et al. (2014) have recently demonstrated a role for Mb in nitrite-mediated vasodilatation under hypoxic conditions. In addition, there is increasing evidence that nitrite reduction may occur *via* blood borne protein mediated mechanisms such as RBC eNOS, as demonstrated by Webb et al. (2008a) and Wood et al. (2013), as opposed to or in addition to the currently favoured vascular tissue-derived NO. Also of interest is recent work by Umbrello et al. (2014) who have demonstrated that short-term hypoxic vasodilatation may be mediated by bioactive NO metabolites rather than by free NO. These recent findings are of great interest in terms of the perceived “physiology” of nitrite but require further substantiation and independent confirmation by other groups. The above observations do not exclude the possibility that nitrite bioactivation occurs through as yet unknown enzymatic or chemical mechanisms. There is also much to be considered physiologically in terms of cross-talk between different enzymatic pathways, such as regulation of activity, which will impact on the effects of nitrite and rate of NO/metabolite production and clearance, and even other chemical entities (carbon monoxide and hydrogen sulfide, for example) much of which has yet to be discovered *in vivo*.

8. Potential therapeutic role of nitrite in acute heart failure

Acute emergence or deterioration of heart failure, with or without the associated development of acute pulmonary edema and the potential need for assisted ventilation, remains a frequent cause of hospital admission with associated morbidity and mortality. Furthermore, there is currently no consensus as to the optimal management of AHF, despite emergence of a large number of potential forms of pharmacotherapy. Many patients with AHF have pre-existent impairment of left ventricular systolic function, and decompensation may reflect intercurrent infection, onset of tachyarrhythmias, recurrent myocardial ischemia and/or poor compliance with prescribed therapy.

Traditionally, AHF has been treated with therapeutic regimens based on the use of diuretics, but it has emerged over the past 20 years that a therapeutic approach centred on the intravenous administration of NO donors to all patients with associated pulmonary edema, together with inclusion of NO donors in the treatment of patients with less severe hemodynamic decompensation, may have substantial advantages (Beltrame, et al., 1998; Cotter, et al., 1998). On this basis, intravenous infusion of organic nitrates such as GTN or isosorbide dinitrate is commonly utilized as a component of therapy for AHF with pulmonary edema.

While organic nitrate infusions are generally helpful in the immediate treatment of such patients, they impose a number of difficulties. Firstly, organic nitrates such as GTN are absorbed by the plastics material of common intravenous infusion set (bags and infusion tubing) (Cawello and Bonn, 1983; Hansen and Spillum, 1991). Hence, unless specialised infusion apparatus is used, it is uncertain whether patients are receiving appropriate GTN infusion rates. Secondly, all organic nitrates are potentially prone to the development of nitrate tolerance during long-term therapy: this is manifest as progressive attenuation of hemodynamic and anti-aggregatory responses to the administered nitrate, with cross-tolerance to other organic nitrates. While some investigators have suggested that nitrate tolerance is also associated with worsening of endothelial dysfunction and attenuation of responsiveness to endogenous NO (that is, cross-tolerance to endothelial NO), overall evidence is more consistent with the concept that nitrate tolerance is engendered primarily by failure of enzymatic release of NO from organic nitrates (Sage, et al., 2000). As the probability of emergence of nitrate tolerance is determined essentially by the combination of infusion rate and duration of exposure (Henry, et al., 1989), this imposes the need to utilize organic nitrate infusions briefly and with the lowest possible infusion rates.

On the other hand, patients with heart failure, whether acute or chronic, display tissue resistance to the effects of NO (for review see (Chirkov & Horowitz, 2007)), related primarily to dysfunction of sGC and “scavenging” of NO by O_2^- . Hence low organic nitrate infusion rates may not achieve ideal hemodynamic responses. Resorting to intermittent dosing regimes, as sometimes used for antianginal treatment, is impractical for AHF as it bears the risk of inappropriate therapeutic coverage during drug-free intervals.

Infusion of nitrite as a means of treatment for AHF offers a theoretical means of circumventing many of the problems associated with organic nitrate infusion. The relative selectivity for the (hypoxic) venous capacitance vessels and pulmonary vasculature would be an attractive profile in the management of decompensated heart failure. Furthermore the apparent lack of tolerance (Haas, et al., 1999; Dejam, et al., 2007) would be an additional advantage over organic nitrates (Sage, et al., 2000). However sustained high dose nitrite infusion can cause methemoglobinemia and hemolysis (Pluta, et al., 2011). To date there have been no large scale studies of nitrite infusion in heart failure.

9. Potential toxicity of nitrate and nitrite

Although the role of nitrate and nitrite in cardiovascular health is becoming increasingly apparent, the ingestion of these anions has also been linked to health concerns. There is an abundance of literature on the subject, so we only provide a few pointers for balance here. Dietary nitrate and nitrite can form N-nitrosamines, and countless animal studies have documented low-molecular weight N-nitrosamines to be carcinogenic in numerous organ systems when ingested orally over prolonged periods (Archer, 1989; Tricker, 1997; Mirvish, 1995). In humans, the role of dietary intake of N-nitrosamine compounds and their precursors in the development of cancer is of ongoing interest, but relies largely on observed associations between the intake of certain food classes and cancer risk/death; in many cases, nitrite and nitrate (or preformed N-nitroso compound) intakes were not quantified but estimated using food frequency questionnaires (considering the level of geographical and seasonal variations in nitrate content alone this is not without problems). Some more recent meta-analyses and careful re-assessments of cancer risks and dietary habits in larger cohorts find no association between nitrite and/or nitrate intake and cancer development, but risks may vary depending on cancer types and organs involved. Most likely, matters are much more complicated than hitherto assumed, with environmental and life-style related factors playing important modulatory roles. One very recent study (Delavalle et al, 2014) suggests that colon cancer, for example, develops not as a result of increased nitrate intake but is secondary to reduced antioxidant levels (vitamin C, for example, can inhibit nitrosation). Teleologically, it is difficult to see why moderate intake levels of nitrite and nitrate would be linked to cancer development as they occur endogenously in astonishing concentrations in some compartments (e.g. in saliva) and thus are part of our normal body physiology.

Another concern about nitrate is methemoglobinemia. Bacteria in the mouth and gut convert nitrate into nitrite, and nitrite reacts with Hb to produce methemoglobin, which is no longer able to transport and release oxygen effectively to tissues. Most cases of methemoglobinemia were reported in the 1940's where methemoglobinemia or "baby blue syndrome" was seen in infants fed formula with nitrate contaminated well-water (Powlson et al, 2008). It was later suggested that methemoglobinemia was not caused by nitrate but by fecal bacteria contamination in the well-water or bacterial nitrate reduction *in vivo* that may have caused the intestinal infection, and this may have been responsible for the nitrate-induced methemoglobinemia in the infants (Hanukoglu & Danon 1996; Ward et al, 2005). Of interest, nitrate was used in very high doses (often for weeks) at the beginning of the last century as a diuretic (Butler & Feelisch, 2008). Recent studies by Pluta and colleagues (2011) have investigated the safety and feasibility of long-term intravenous infusion of sodium nitrite in healthy subjects. The authors demonstrated that acute intravenous infusion of sodium nitrite was tolerated up to a maximum dose at 267 µg/kg/hour, and that the dose-limiting toxicity was reached at 446 µg/kg/hour. Toxicity included a transient asymptomatic decrease of MAP and an increase of methemoglobin (above 5%). Overall, the authors suggested that nitrite could be 'safely infused intravenously at defined concentrations for prolonged intervals'.

Finally, although there are reports to suggest that nitrate and nitrite are harmful when ingested in excess, the same is true for about every other substance essential to mammalian life including glucose, fat and oxygen. It is important to establish limits at which the harm may outweigh the potential benefits, and further research is warranted to investigate what these limits may be for nitrate and nitrite. The extensive monograph by L'hirondel & L'hirondel (2002) and several recent articles (Lundberg et al, 2004; Bryan et al, 2012; Kapil et al, 2014) provide a more detailed information for further reading about the potential harmful effects of nitrate and nitrite.

10. Summary and conclusions

Nitrite apppears to have considerable potential as a therapeutic agent to increase the bioavailability of NO under certain conditions such as in hypoxia, where endogenous NO production *via* the L-arginine-NOS-NO pathway may be compromised. Thus, nitrite could conceivably be applied in conditions such as heart failure due to its vasodilatory capacity, apparently without the risk of development of tolerance and headache as documented with organic nitrate treatment, making nitrite perhaps a more acceptable alternative. Oral nitrate administration would appear to represent an attractive vehicle for nitrite delivery *in vivo*. The beneficial vasodilatory effects apparent with nitrate and nitrite consumption through dietary sources may promote vascular health and ward off CVD. Nitrite therapy is a rapidly expanding area with great potential for improved clinical outcome in patients, however caution is advised in the translation of results obtained in animal experimental models to the clinical setting. A recent multi-centre, double-blind, placebo controlled clinical trial showed that nitrite was ineffective when administered intravenously immediately prior to PPCI in patients presenting with first acute STEMI (Siddiqi et al, 2014). Therefore, we need to be mindful of differences between animal and human physiology as well as inter-individual differences in responsiveness to nitrite between subjects. Moreover, the handling of nitrite (and nitrate) may differ between health and disease, posing additional challenges to effectiveness and applicability of the administered treatment.

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JCB, MF, JDH and MM have no conflict of interest to report. MF has an ownership interest in a “method of use” patent held for Perhexiline in heart muscle diseases.

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Figure and table legends

Figure 1. The role of nitrite-derived nitric oxide in the vasculature. During normoxia, L-arginine is metabolized in endothelial cells via nitric oxide synthase (NOS) to nitric oxide (NO) to induce vasodilatation. Under normal physiological pH and oxygen tension, nitrite (NO_2^-) is an endogenous substance produced *via* the oxidation of NO (principally by cytochrome c in cells and ceruloplasmin in blood). During hypoxia and acidosis, NO_2^- can be bioconverted to NO *via* multiple nitrite reductases, including deoxyhemoglobin, myoglobin, xanthine oxidoreductase, and endothelial NOS to mediate hypoxic vasodilatation. cGMP (cyclic guanosine monophosphate); sGC (soluble guanylyl cylase); NO_3^- (nitrate).

Table 1: Experimental studies investigating the efficacy of nitrite on vasorelaxation and blood pressure. A summary of *in vitro* and *in vivo* studies that have evaluated the efficacy as a vasodilator. Adenosine tri-phosphate (ATP), aldehyde deydrogenase 2 (ALDH2), blood pressure (BP), cyclic guanosine monophosphate (cGMP), deoxyhemoglobin (deoxyHb), endothelial nitric oxide synthase (eNOS), glyceryl trinitrate (GTN), hemoglobin (Hb), mean arterial pressure (MAP), myoglobin (Mb), nitric oxide (NO), oxyhemoglobin (oxyHb), red blood cells (RBC), soluble guanylate cyclase (sGC), systemic arterial pressure (SAP), xanthine oxidase (XO), xanthine oxidoreductase (XOR).

Table 2: Nitrite efficacy in humans and regulation of vasodilatation and blood pressure. A summary of recent studies that have evaluated the efficacy of nitrite as a vasodilator in healthy subjects, hypertensive and heart failure patients. Acetylcholine (ACh), blood pressure (BP), congestive heart failure (CHF), cyclic guanosine monophosphate (cGMP), deoxyhemoglobin (deoxyHb), diastolic blood pressure (DBP), flow-mediated dilatation (FMD), forearm blood flow (FBF), New York Heart Association (NYHA), nitric oxide (NO), nitric oxide synthase (NOS), mean

arterial pressure (MAP), red blood cells (RBC), systolic blood pressure (SBP), xanthine oxidoreductase (XOR).

Figure 1.

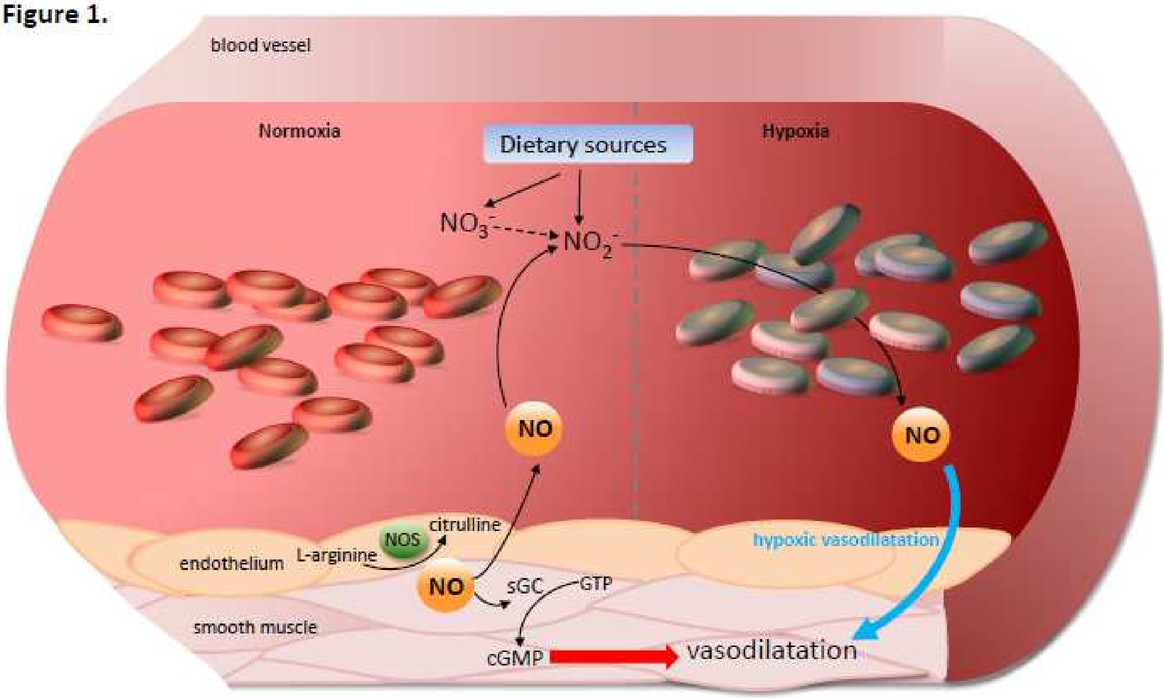
blood vessel
Normoxia
Dietary sources
Hypoxia
NO3-
NO2-
NO
NO
citrulline
NOS
endothelium
L-arginine
hypoxic vasodilatation
NO
sGC
GTP
smooth muscle
cGMP
vasodilatation

Table 1

Species	**Condition/model**	**Nitrite reductase**	**Dose/ route/timing administration of nitrite**	**Results**	**Reference**
Mouse	Hypertension model. Wild type compared to eNOS -/- mice	eNOS from RBC (non-endothelial)	Endogenous systemic nitrite levels	eNOS -/- mice displayed lower plasma nitrite concentrations compared to wild type. Provides evidence that circulating blood eNOS plays a role in nitrite homeostasis and BP regulation during physiological conditions	(Wood, et al., 2013)
Rat	Hypertension model. Used spontaneous hypertensive & normotensive Wistar Kyoto rats	XOR derived from RBC's	Potassium nitrite administered as bolus doses between 1-30,000 x 10^{-9} mol/kg	Nitrite decreased BP in a dose-dependent manner. The effect of nitrite was greater in spontaneously hypertensive rats compared to normotensive and was abolished with allopurinol (XOR inhibitor). The study showed that this effect was associated with an increase in erythrocytic XOR expression but not in the blood vessel wall	(Ghosh, et al., 2013)
Mouse	Assessment of hypoxia vasodilatation in Mb wild type and deficient mice	Mb	Assessed endogenous and exogenous nitrite. For exogenous studies 1.67 or 16.7 µmol/kg was used	Mb expression in vascular smooth muscle contributes to nitrite-mediated hypoxic vasodilatation *ex vivo* and *in vivo*. This effect was mediated via Mb-induced activation of	(Totzeck, et al., 2012a)

				NO/sGC/cGMP pathway	
Rat	Normotensive compared to hypertensive rats. Assessed whether gastric pH reduced hypotensive effects	-	2 protocols used: (1) oral (gavage) administration sodium nitrite (1 - 45 mg/kg) compared with (2) intravenous sodium nitrite (1-15 mg/kg)	Increased gastric pH caused by omeprazole reduced the hypotensive effect of nitrite in both normotensive and L-NAME-hypertensive rats. The study concluded that the hypotensive effect of sodium nitrite was partly due to bioconversion to NO under acidic conditions of the stomach	(Pinheiro, et al., 2012)
Mouse	Assessed the effects of dietary nitrate on XOR and eNOS in pulmonary hypertension model	eNOS and XOR	Supplementation of drinking water with either potassium nitrate (15 mmol/L or 45 mmol/L) or potassium nitrite (0.6 mmol/L)	Dietary nitrate, but to lesser extent dietary nitrite, causes pulmonary dilatation and prevents vascular remodelling and right ventricular hypertrophy. These effects were dependent on eNOS and XOR reduction of nitrite to NO	(Baliga, et al., 2012)
Ovine	Assessed the effects of nitrite on pulmonary and systemic arterial vascular resistance in newborn lambs	DeoxyHb	Inhalation of sodium nitrite via nebulizer (0.87 mol/L) compared to intravascular nitrite infusion 5 mg/kg/hour	Inhaled nitrite elicited pulmonary vasodilatation through NO mediated mechanism. Intravascular nitrite did not elicit pulmonary vasodilatation. Inhaled nitrite produces NO in the airway and parenchymal lung tissue	(Blood, et al., 2011)

				to mediate vasodilatation and this was independent of deoxyHb in the pulmonary circulation	
Mouse	Model of vascular endothelial dysfunction associated with age using old (26-28 month) mice compared to young (4-6 month) mice.	-	Supplementation of sodium nitrite in drinking water (50 mg/L) for three weeks	Sodium nitrite restored endothelium-dependent dilatation in old mice via increase in NO bioavailability. Nitrite reversed vascular endothelial dysfunction associated with age	(Sindler, et al., 2011)
Mouse	Assessed the effects of nitrite on vascular Mb	Mb	Sodium nitrite	Vascular Mb plays an essential role in nitrite-dependent vasodilatation.	(Ormerod, et al., 2011)
Rat	Assessed the role of GTN and sodium nitrite in the pulmonary vascular bed	ALDH2 and XOR	Intravenous injection sodium nitrite (10-100 µmol/kg)	Administration of GTN or sodium nitrite caused a decrease in pulmonary and SAP. Response to GTN or sodium nitrite was attenuated by cyanamide (ALDH2 inhibitor). The effect of sodium nitrite, but not GTN, was also attenuated by allopurinol (XOR inhibitor)	(Badejo, et al., 2010)
Rat	Assessed the mechanism of nitrite and RBC-mediated vasodilatation	-	Intravenous injection sodium nitrite 10 µmol/kg	Vasodilatation via nitrite could be mediated through nitrite enhancement of ATP release from RBC	(Cao, et al., 2009)

Rabbit	Assessed the effect of nitrite on NO-dependent and independent vasodilatation pathways during hypoxic conditions	Aldehyde oxidase, eNOS, XO	Nitrite	During hypoxia, nitrite-induced vasorelaxation was largely due to nitrite reduction by aldehyde oxidase to NO, but was also partly mediated via the cyclooxygenase pathway. XO or eNOS did not play a role in this study	(Pinder, et al., 2009)
Rat	Effect of carbonic anhydrase on nitrite-induced vasodilatation during normoxia and hypoxia	Carbonic anhydrase	Sodium nitrite	Carbonic anhydrase reacts with nitrite to produce NO during low pH conditions. This reaction induced vasorelaxation during normoxic and hypoxic conditions	(Aamand, et al., 2009)
Rat	Investigated the importance of oral microflora and dietary nitrate in regulation of BP and gastric mucosal defence	-	Supplementation of drinking water with either sodium nitrate 10 mM (~140 mg/kg/day) or sodium nitrite 1 mM (~14 mg/kg/day)	Mouthwash reduced nitrate-reducing oral bacteria and caused a reduction in circulating nitrite. BP reduction was observed after nitrate supplementation in the absence of mouthwash. Gastroprotectitive effect of nitrate was reduced in rats treated with mouthwash	(Petersson, et al., 2009)
Rat	Assessed the role of XOR and ALDH2 in nitrite-mediated effects on BP in rats	XOR and ALDH2	Intravenous administration of sodium nitrite	Nitrite decreased mean SAP. The decreases in mean SAP in response to sodium nitrite was attenuated by both XOR and ALDH2 inhibitors.	(Golwala, et al., 2009)

				The study suggested that both XOR and ALDH2 work in parallel to mediate nitrite conversion to vasoactive NO	
Rat	Assessed the role of XOR in response to sodium nitrite in the pulmonary vasculature	XOR	Intravenous injection of sodium nitrite 10 - 100 µmol/kg	Intravenous administration of nitrite decreased pulmonary arterial pressure and SAP The responses to nitrite was attenuated by allopurinol (XOR inhibitor). This study suggests that XOR is the major enzyme reducing nitrite to vasoactive NO, and that this mechanism is not modified by hypoxia	(Casey, et al., 2009)
Canine	Assessed the physiological effects of sodium nitrite and determined whether the effect was influenced by cell-free plasma Hb during intravascular hemolysis	Hb	Intravenous infusion of sodium nitrite 27.5 mg/hour for 6 hrs	Nitrite reductase activity of Hb caused an increased vasodilatory response to nitrite during low levels of hemolysis	(Minneci, et al., 2008
Rat	Assessed effect of nitrite on BP *in vivo* and vasorelaxation in isolated aorta *in vitro*	Hb	*In vivo:* intravenous injection of sodium nitrite (10 µM - 2 mM)	*In vivo*: Sodium nitrite caused a dose dependent decrease in BP *In vitro*: During aerobic conditions, XOR , mitochondrial electron transport, cytochrome P450 and NOS inhibitors	(Alzawahra, et al., 2008)

				did not effect nitrite-mediated vasorelaxation. The study suggested that heme proteins and/or sGC pathways are involved in nitrite mediated effects	
Non-human primates	Assessed the physiological and pharmacological effects of sodium nitrite on vasodilatory responses	XOR and deoxyHb	Sodium nitrite 12.5 µg/kg/min over 24 hours for 14 days	Sodium nitrite was a potent vasodilator at near-physiological concentrations. Nitrite was reduced to NO by intravascular reactions with deoxyHb. In contrast, XOR inhibition did not attenuate the nitrite-induced vasodilatation	(Dejam, et al., 2007
Rat	Assessed effect of Hb oxygen saturation on vasodilatation	OxyHb/ DeoxyHb	Sodium nitrite increasing concentration 0.01 to 1000 µM	Deoxygenation of Hb was associated with nitrite-dependent vasodilatation which was inhibited by NO scavenger (c-PTIO) under both normoxic and hypoxic conditions. In contrast, NO dependent vasodilatation (via NO donor NONOate) was inhibited by both oxyHb and deoxyHb suggesting unique interaction of nitrite with Hb. This study suggested that NO homeostasis was regulated by balance in NO	(Isbell, et al., 2007)

				scavenging/generating activity through oxygen saturation of Hb	
Rat	Effect of nitrite dependent vasodilatation in hypoxia (*in vitro*)	Hb, XO, mitochondrial *bc*1 complex	Sodium nitrite in cumulative additions 0.01 to 300µM	Nitrite induced a concentration dependent effect on vasodilatation. The vasoactive effect of nitrite during hypoxia was attenuated on inhibition of sGC but was unaffected by inhibition of xanthine oxidase or the mitochondrial *bc*1 complex. In addition, deoxygenation of Hb did not appear to enhance vasoactivity of nitrite	(Dalsgaard, et al., 2007
Rat	Hypoxic pulmonary vasoconstriction in isolated perfused lungs	Hb	Lungs perfused with sodium nitrite buffer increasing concentration (250 nM to 1 mM) with or without RBC's	In isolated perfused lungs, low concentrations of nitrite inhibited hypoxic pulmonary vasoconstriction, possibly due to the release of NO rather than the direct effect of nitrite on vascular smooth muscle. However, physiological concentrations of RBCs and free Hb prevented nitrite inhibition of hypoxic pulmonary	(Deem, et al., 2007)

				vasoconstriction thus raising doubts for the role of RBCs in nitrite-mediated vasodilatation in the pulmonary circulation	
Canine	*In vivo* model of acute pulmonary thromboembolism	-	Intravenous infusion of nitrite (6.75µmol/kg over 15 min then at 0.28µmol/kg/min for 120 minutes)	Infusion of nitrite increased plasma nitrite levels with a dose-dependent decrease in pulmonary vascular resistance index, systemic vascular resistance index, and MAP	(Dias-Junior, et al., 2006)
Rat and rabbit	Hypoxic vasodilatation in isolated thoracic aortas	Hb	Sodium nitrite 0.01 - 1000µM	This study showed that nitrite induced vasodilatation and supported the role of RBC Hb to redox regulate nitrite reductase activity during hypoxia	(Crawford, et al., 2006
Rat	Hypertension	Hb-NO complex	Acute: sodium nitrite (1, 3, 10 mg/kg) by oral gavage. Chronic: sodium nitrite supplemented in drinking water (100 mg/L or 1000 mg/L)	Orally administered nitrite is detectable in the circulation as HbNO. Nitrite treatment attenuates L-NAME induced hypertension in a dose-dependent manner	(Tsuchiya, et al., 2005)
Ovine	Assessed inhaled sodium nitrite by aerosol in hypoxia-induced pulmonary hypertension in newborn lambs	Hb and iron-nitrosyl-Hb	Inhaled nebulized sodium nitrite 15 mg/min for 20 minutes	Pulmonary vasodilatation was elicited by aerosol nitrite. This was deoxyHb, pH dependent and associated with increased	(Hunter, et al., 2004)

				blood levels of iron-nitrosyl-Hb	
Rat	Assessed the role of NO-modified Hb in isolated rat thoracic aortas	DeoxyHb	*In vitro* sodium nitrite	Sodium nitrite was associated with reduction of nitrite to NO by deoxyHb during hypoxic conditions	(Cosby, et al., 2003)
Swine	*In vitro* and *ex vivo* assessment of nitrite in isolated perfused and ventilated pig lungs	-	Lungs: perfusion of buffer containing 0.1 and 1 mmol/L nitrite anions from sodium nitrite	Nitrite anions at physiological concentrations act as a vasodilator	(Demoncheaux, et al., 2002)
Rat	Assessed effect of pH on nitrite-induced vasorelaxation in rat aorta	-	Cumulative addition of sodium nitrite 0.5 to 1000µM	Nitrite induced vasorelaxation, which was enhanced under acidic conditions and nitrite-derived NO was generated in a pH-dependent manner. Vasoactivity of nitrite was greatly reduced by inhibition of sGC	(Modin, et al., 2001)

Table 2

Condition/model	Nitrite reductase	Dose/ route/timing administration of nitrite	Results	Reference
Hypertension model. Grade 1 hypertensive patients	XOR derived from RBC's	Dietary nitrate *via* 250 ml beetroot juice containing ~3.5 mmol nitrate	Hypertensive patients showed a dose-dependent effect of plasma nitrite with decrease in BP and was associated with increased XOR activity. The study showed that this effect was associated with increase in erythrocytic XOR expression but not in the blood vessel wall	(Ghosh, et al., 2013)
CHF patients (NYHA class II-III) compared to healthy volunteers. Hemodynamic assessment of unstressed forearm venous volume and FBF	-	30 minutes intravenous infusion of sodium nitrite (0.31 - 7.8 µmol/min) in the forearm brachial artery	FBF increased markedly in CHF patients when compared to normal subjects. Unstressed forearm venous volume increased in both CHF and normal subjects, with CHF being being hyporesponsive when compared to healthy subjects. CHF patients showed accelerated transvascular clearance of nitrite suggesting increased conversion to NO in these subjects	(Maher, et al., 2013)
Healthy subjects	-	Dietary nitrate via 200g beetroot bread containing 100g beetroot (1.1 mmol nitrate)	Beetroot bread increased vasodilatation and decreased DBP	(Hobbs, et al., 2013)

Healthy subjects. Assessment of inorganic nitrate on endothelial function by FMD and BP	-	Potassium nitrate oral capsules 8 mmol	Inorganic nitrate supplementation had no effect on endothelial function despite increases in plasma nitrite levels. However, inorganic nitrate decreased SBP and aortic pulse wave velocity, but had no effect on DBP	(Bahra, et al., 2012)
Healthy subjects	-	Intravenous infusion of sodium nitrite for 48 hours from 4.2μg/kg/hour to 533.8μg/kg/hour	Nitrite induced a decrease in MAP in healthy subjects. The maximum tolerated dose of nitrite was 267μg/kg/hour with toxicity occuring at 446μg/kg/hour. Concluded that nitrite was safe to infuse for a prolonged period given the correct dose	(Pluta, et al., 2011)
Healthy subjects. Assessment of dietary nitrate and supplementation on BP, and sex differences in response to nitrate	-	Supplementation with potassium nitrate (capsules - 4 to 24 mmol) or beetroot juice 250 ml containing 5.5 mmol nitrate	Nitrate supplementation or beetroot juice caused an increase in plasma nitrite and cGMP levels, and was associated with decreased BP in healthy subjects. Sex difference in sensivitity to nitrate was dependent on baseline plasma nitrite concentration and BP, whereby males had lower baseline nitrite levels and higher BP than females. Following nitrate supplementation males had significantly greater reduction in BP than the females, thus suggesting	(Kapil, et al., 2010a)

			differences in nitrate conversion to nitrite between the two sexes	
Healthy subjects. Assessed acute and chronic supplementation of nitrate on BP and exercise	-	Dietary nitrate via beetroot juice 500 ml (5.2 mmol nitrate)/day for 15 days	Nitrate supplementation elevated plasma nitrite concentrations both short term (2.5 hours) and long term (15 days). Increased plasma nitrite concentration was associated with decreased BP and the O_2 cost of moderate intensity exercise	(Vanhatalo, et al., 2010)
Healthy subjects. Effect of nitrate from Japanese diet on BP	-	Dietary nitrate provided via Japanese traditional diet. Estimated nitrate intake 18.8 mg/kg/day	Consumption of Japanese traditional diet over a period of ten days increased plasma and salivary levels of both nitrate and nitrite. Japanese traditional diet was associated with a decrease in BP in healthy normotensive subjects	(Sobko, et al., 2010)
Healthy subjects. Effect of low-dose nitrite on hypoxic pulmonary vasodilatation	-	Sodium nitrite infusion into brachial artery 1 µmol/min for 30 min	During hypoxia sodium nitrite increased FBF and reduced pulmonary arterial pressure. No effects were observed during normoxia	(Ingram, et al., 2010)
Investigated the effects of sodium nitrite on FBF in patients with sickle cell disease. Compared to healthy controls (Cosby et	-	Intravenous infusion of sodium nitrite (brachial artery: 0.4-40 µmol/min)	Nitrite infusion increased plasma nitrite in a dose-dependent manner which was associated with an increase in FBF in both	(Mack, et al., 2008)

al., 2003)			healthy controls (Cosby et al., 2003) and patients with sickle cell disease. However, the response/sensitivity to nitrite was reduced in patients with sickle cell disease	
Healthy subjects. Assessed effect of dietary nitrate on BP		Dietary nitrate via beetroot juice 500 ml (nitrate content ≈45.0 mmol/L)	Beetroot juice caused an increase in plasma nitrite levels and decrease in BP. This effect was abolished by the interruption of enterosalivary reduction of nitrate to nitrite via spitting. The study demonstrated that the conversion of nitrate to nitrite is essential for vasoactivity	(Webb, et al., 2008b)
Assessment of FBF and forearm venous volume during normoxia and hypoxia in healthy subjects	-	Brachial artery infusion of sodium nitrite (40 nmol/min to 7.84 µmol/min)	Nitrite was a potent venodilator during normoxic and hypoxic conditions. Whilst nitrite had a modest vasodilatory effect in the resistance vessels during normoxia, the effect was potent during hypoxia	(Maher, et al., 2008)
Assessed the physiological and pharmacological effects of sodium nitrite on vasodilatory responses	XOR and deoxyHb	0, 7, 14, 28, 55 and 110 µg/kg/min 5 mins each dose	Sodium nitrite was a potent vasodilator at near-physiological concentrations. Nitrite was reduced to NO by intravascular reactions with deoxyHb. In contrast, XOR inhibition did not attenuate the nitrite-induced vasodilatation	(Dejam, et al., 2007)

BP in healthy subjects	-	3 day dietary supplementation with sodium nitrate (0.1 mmol/kg/day)	Short term dietary nitrate supplementation increased plasma nitrite levels and reduced DBP and MAP, but did not affect SBP in healthy subjects	(Larsen, et al., 2006)
Subjects with endothelial dysfunction compared to healthy subjects	-	Endogenous plasma nitrite	Subjects with endothelial dysfunction displayed lower levels of plasma nitrite and lower FMD levels than healthy subjects	(Kleinbongard, et al., 2003)
Assessed the vasodilatory properties and bioactivation of nitrite in forearm (via FBF) before and during exercise	DeoxyHb	Infusion of sodium nitrite 0.36 and 36µmol/min before and during exercise	Sodium nitrite induced vasodilatation in humans and was associated with reduction of nitrite to NO by deoxyHb during hypoxic conditions	(Cosby, et al., 2003)
Assessed whether changes in NOS concentrations are reliable marker for NO production and whether physiological concencentration of nitrite is vasoactive	eNOS	Intra-arterial infusion of sodium nitrite 0.01-36µmol/min	eNOS stimulation with ACh dose-dependently increased venous nitrite levels and this effect was associated by an increase in FBF. Intra-arterial infusion of nitrite had no effect on FBF	(Lauer, et al., 2001)