

## Cardiovascular prevention by dietary nitrate and nitrite

Jon O Lundberg

Running title: Nitrite prevents vascular inflammation

Correspondence to: Prof Jon Lundberg, Dept. Physiology and Pharmacology

Karolinska Institutet, S-171 77, Stockholm, Sweden

Tel: +46852487952

Fax: +468332278

e-mail: [jon.lundberg@ki.se](mailto:jon.lundberg@ki.se)

Over the past half century dietary nitrate ( $\text{NO}_3^-$ ) and nitrite ( $\text{NO}_2^-$ ) have become notorious because of their proposed association with the development of disease, most notably gastric cancer (28, 38). This has led to very strict regulations of nitrate and nitrite levels in food and drinking water. In the early 1980s it was surprisingly shown that, in addition to dietary exposure, nitrate and nitrite are also generated endogenously in our bodies (9). Soon after this, the entire L-arginine-nitric oxide pathway was revealed and this pathway was found to be the major source of endogenous nitrate and nitrite, since NO is quickly oxidized to these higher nitrogen oxides (11, 30, 37). Until only recently biologists have considered nitrate and nitrite merely as inactive end-products of NO metabolism but this view is now changing rapidly. It turns out that they undergo a serial reduction in vivo and again form bioactive nitrogen oxides, including NO (7, 24-26, 42, 44). A picture is now emerging suggesting important physiological as well as therapeutical roles for the Nitrate-Nitrite-NO pathway, especially under hypoxic conditions when the oxygen-dependent NO synthases may be dysfunctional (26). Thus, instead of just wasting oxidized NO, our bodies are actively recycling it. Nitrite reduction to NO was first described in the stomach, where salivary nitrite forms NO non-enzymatically via acid-catalysed reduction (2, 27). Soon after this Zweier described NOS-independent nitrite reduction in the ischemic and acidic heart (45). Subsequent studies show that a variety of enzymes and proteins can catalyse the one-electron reduction of nitrite to NO in blood and tissues. These include deoxygenated heme proteins (4, 31), xanthine oxidase (43),

and components of the mitochondrial respiratory chain (19). In addition, reducing agents such as vitamin C (29) and polyphenols (6) greatly accelerate nitrite reduction.

The therapeutic effects of nitrite have been elucidated in the cardiovascular system and in the gastrointestinal tract (26). Systemic delivery of nitrite at remarkably low doses, have been shown to protect against ischemia-reperfusion injury in various organs, including heart (5, 40), brain (17), kidney (39), liver (5) and limb (20). Both nitrate and nitrite are active also when given orally. Nitrate is first reduced by oral commensal bacteria (8) or by mammalian enzymes (13) to form nitrite which then can enter the systemic circulation (23). In humans blood pressure is reduced after ingestion of nitrate in doses equivalent to a diet rich in vegetables (21). Moreover, dietary nitrate also prevents endothelial dysfunction after an acute ischemic insult in humans and inhibits ex vivo platelet aggregation (41). In animal studies dietary nitrate (3) and nitrite (3, 34) protect against ischemia reperfusion injury. Jansson et al (14) and Petersson et al (32) recently described anti-inflammatory effects of dietary nitrate and nitrite in the gastrointestinal tract but until today there have been no reports on the effects of nitrate or nitrite on vascular inflammation.

In this issue of *the Journal* Stokes and colleagues studied the effects of dietary nitrite supplementation on the vascular events associated with a high cholesterol diet (36). Mice fed a cholesterol-enriched diet for 3 weeks developed clear signs of vascular pathology, including elevated leukocyte adhesion and emigration as well as impaired endothelium-dependent vasorelaxation. Remarkably, the addition of nitrite to the drinking water prevented these events. This might be of significant importance since inflammatory events are thought to be central in the development of

atherosclerosis. The exact mechanism for these beneficial effects remains to be studied although the reduction of nitrite to NO and other closely related nitrogen oxides (e.g S-nitrosothiols) is a likely first step. Indeed NO has anti-inflammatory, anti-adhesive and vasodilatory properties, and a reduced NO bioavailability is a central even in development of cardiovascular disease including atherosclerosis and hypertension (12). Interestingly, Stoke and colleagues noted a sparing of reduced tetrahydrobiopterin (BH4) with nitrite treatment. This co-factor is vital for NO generation by NOS, which would indicate that nitrite can function not only as a direct substrate for NO generation, but in addition it might also enhance NO generation from the classical NOS pathway and prevent potentially harmful uncoupling of NOS.

Stoke and colleagues also found that the levels of C-reactive protein (CRP) were lower in cholesterol-fed mice treated with nitrite. This is very interesting especially in light of recent studies in humans, showing that this marker of vascular inflammation is coupled to clinical outcome (35). In addition, there might be a direct link to the NO system since C-reactive protein has been shown to induce endothelial dysfunction and uncoupling of eNOS in vivo (10). In a recent large scale clinical study, apparently healthy subjects with increased high-sensitivity CRP but without hyperlipidemia, were treated with a statin (33). The treatment was associated with a reduction in CRP and a markedly reduced risk for major cardiovascular events. Although, this does not prove a causal link between CRP and cardiovascular events, it indicates that suppression of chronic vascular inflammation has long-term beneficial effects.

Surprisingly, there was no effect of nitrite on blood pressure in the current study, despite a great increase in circulating and tissue levels of nitrite and

nitroso species. In humans and rats even a modest increase in plasma nitrite (induced by ingestion of nitrate) is associated with a significant blood pressure reduction (21, 32, 41). The reason for this difference is not clear but we cannot exclude that nitrate and nitrite have different pharmacodynamic profiles. However, a more likely explanation for these diverging results is related to technical issues. Stoke and colleagues measured blood pressure in anesthetized animals while Larsen measured in awake subjects and Petersen used telemetric measurements in awake rats. Anesthetic drugs have major effects on the cardiovascular system and this will interfere with blood pressure readings.

The perhaps most interesting aspect of Stokes study and other recent reports, is the nutritional implications. A picture is now emerging suggesting that dietary nitrate is bioconverted in vivo to form nitrite and then bioactive nitrogen oxides including NO (23). The vast majority (>80%) of nitrate in our diet comes from vegetables, some of which are extremely rich in this anion (22). It is clear that a diet rich in vegetables is associated with cardiovascular protection, including lower blood pressure and reduced risk of myocardial infarction and stroke (1, 15, 16). However, the active ingredient(s) responsible for these effects has not been pinpointed. It has been suggested that nitrate contributes to the well-known cardioprotective effects of diets rich in vegetables such as the mediterranean diet (22, 26). For the current study such nutritional claims would have been stronger if the authors had tested lower nitrite doses or added a group receiving moderate nitrate supplementation (resembling a diet rich in vegetables). Nevertheless, the fact that there was no dose-response whatsoever in nitrite effects suggests that maximum effects were

seen already at the lower dose (50 mg nitrite/liter drinking water) or even below this dose. Thus, a non-pharmacological dose of nitrite would likely also have had effects. Indeed, this is supported by a recent study by Kanematsu who studied dietary nitrite and renal function in rats (18). They found that nitrite at a 50-fold lower dose could completely prevent the renal injury associated with long term treatment with a NO synthase inhibitor. Thus, it seems as if the nitrate-nitrite-NO pathway can compensate for disturbances in endogenous NO generation from NO-synthases. We need further studies in animals and later in humans to find out if nitrate and nitrite can offer long-term protection against development of atherosclerosis.

Clearly, much more research is needed before we can start encouraging people to increase their daily nitrate intake in order to achieve better health. Nevertheless, with the emerging evidence for beneficial effects of dietary nitrate on the cardiovascular system the time has come to move the discussion to a new level. Researchers and politicians advocating harmful health effects of nitrate now also have a responsibility. Fifty years of continuous fear mongering has had a major negative impact on people's view on nitrate. Is this propaganda of overall benefit to the population, or is it holding back the consumption of an essential nutrient? In the end the potential benefits of reducing our overall nitrate intake in terms of lower cancer incidences need to be weighed against its positive effects on the cardiovascular system. The acceptable daily intake (ADI) for nitrate is set to 3.7 mg/kg bodyweight which is around 250 mg in an adult. Ironically, one glass of fresh beetroot juice (41) or a plate of fresh rucula contain at least twice this amount of nitrate.

While compelling epidemiological evidence suggest no harmful effects of dietary nitrate, we need to be somewhat more careful when we discuss dietary nitrite. Nitrite is added to certain meat products to enhance its appearance and to protect against food borne infections. Although there is no direct evidence for a harmful health effect of nitrite in meat, preformation of potential carcinogens such as nitrosamines might occur during the preparation of food. However, it must also be noted that the major dietary source of nitrite is in fact vegetables. Much of the nitrate in these foodstuffs is converted to nitrite in our bodies via the action of oral nitrate reducing bacteria (23) and also by mammalian processes as described recently (13). Moreover, even if we were to exclude all nitrate and nitrite from our diet we would still be exposed to considerable amounts of these anions, since they are generated endogenously from the L-arginine/NOS pathway.

In conclusion, Stoke and colleagues demonstrate impressive anti-inflammatory effects of dietary nitrite in the vascular system. This report adds to the growing number of studies now suggesting that exposure to dietary nitrate and nitrite might not necessarily be a threat to human health. Instead, in some years we might even consider them as essential nutrients.

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