CLINICAL TRIAL PROTOCOL

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A randomized double-blind placebo-controlled crossover trial of sodium nitrate in patients with stable angina INAS

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In an aging western population, a significant number of patients continue to suffer from angina once all revascularization and optimal medical treatment options are exhausted. Under experimental conditions, oral supplementation with inorganic nitrate was shown to exhibit a blood pressure-lowering effect, and has also been shown to promote angiogenesis, improve endothelial dysfunction and mitochondrial efficiency in skeletal muscle. It is unknown whether similar changes occur in cardiac muscle. In the current study, we investigate whether oral sodium nitrate treatment will improve myocardial ischemia in patients with stable angina.

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Aim

In 2013, the British Heart Foundation reported that 2.3 million patients (3.5% of the population) were registered with the diagnosis of angina in the UK [1]. Despite impressive advances in revascularization options and optimal medical treatment over the last two decades, a significant number of patients continue to suffer from limiting angina. With improving survival and active lifestyle, clinicians increasingly encounter patients 10–20 years after their initial revascularization procedure in whom repeat revascularization is not possible or only to a limited extent. Current first-line antianginal drugs are very effective, but in some patients their use can be precluded due to side effects (especially in pre-existing bradycardia or hypotension).

Over the last decade, inorganic nitrate (putatively via the nitrate–nitrite–nitric oxide pathway) has been at the center of considerable interest as a potential therapeutic option for cardiovascular diseases [2,3]. The human body is able to produce endogenous nitrite and nitrate via oxidation of nitric oxide originating from nitric oxide synthases. However, the major source of the body storage pool comes from diet. Beetroot and leafy green vegetables are especially rich in inorganic nitrate. Inorganic nitrate is actively transported into the salivary glands and secreted into the saliva. Salivary bacteria reduce the nitrate into nitrite. This in turn is reduced to nitric oxide in the stomach, an effect that is facilitated by the presence of low pH. This effect of oral nitrate load on stomach nitric oxide production has been

KEYWORDS

- angina
- inorganic nitrate
- randomized controlled trial

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elegantly demonstrated by Spiegelhalter *et al.*, Benjamin *et al.* and Lundberg *et al.* [4–6]. The nitric oxide and some of the remaining nitrite is absorbed in the upper small intestine and reaches all tissues via circulation presumably via conversion back to nitrite, which is more stable. Intravenous nitrite (the main metabolite of inorganic nitrate) is a potent vasodilator under hypoxia, but only a modest vasodilator under normoxia. Cosby *et al.* demonstrated that nitrite-induced vasodilation was associated with reduction of nitrite to nitric oxide by deoxyhemoglobin [7]. The effect of hypoxia on nitrite–nitric oxide vasodilation was later confirmed by others. [8,9]. Nitrite reduces the increase in pulmonary arterial pressure induced by hypoxia in healthy volunteers, an effect that persisted even 1 h after cessation of nitrite infusion when plasma levels returned back to the baseline [9]. A single dose of oral sodium nitrate elevated angiogenic markers and recruited circulating angiogenic cells in healthy human volunteers [10]. Improved angiogenesis was confirmed in an experimental animal model of chronic hind limb ischemia following chronic oral supplementation [11]. Recently, 4-week supplementation with sodium nitrate resulted in improved endothelial dysfunction when assessed by brachial artery flow mediated vasodilation and also reduce arterial stiffness in an elderly population [12]. A beneficial effect of inorganic nitrate or beetroot supplementation on endothelial function was reported by a recent meta-analysis [13]. Another recent meta-analysis (total number of participants $n = 254$; 7–30 participants per study) suggests that a dose of 300–600 mg of sodium nitrate modestly reduces blood pressure [14]. In this meta-analysis of inorganic nitrate and beetroot supplementation was associated with greater mean changes in systolic BP (-4.4 mmHg; $p < 0.001$) than diastolic BP (-1.1 mmHg, $p = 0.06$). Oral inorganic nitrate supplementation was shown to reduce the oxygen cost of submaximal exercise in healthy volunteers [15–17], to improve skeletal muscle contractile function [16,18] and skeletal muscle mitochondrial ATP production efficiency [19]. Recently, improved skeletal muscle contractile function was documented following a single dose of oral inorganic nitrate load (11.2 mmol beetroot juice) in patients suffering with systolic heart failure [20]. It is unclear whether these effects in skeletal muscle also occur in cardiac muscle. However, these vascular and myocyte properties would potentially be of therapeutic value in patients suffering from angina.

Hypothesis

The main hypothesis is to assess any potential anti-ischemic effects of oral sodium nitrate treatment in patients with stable angina treated with background cardiovascular and antianginal medication.

• Primary outcome

- Time to 1 mm ST depression (exercise treadmill test).

• Secondary outcomes

- Time to chest pain onset (exercise treadmill test);
- Total exercise time (exercise treadmill test);
- Angina and glyceryl trinitrate (GTN) use frequency;
- Modified Seattle questionnaire (SQ);
- Nitrate and nitrite plasma levels, angiogenic markers;
- Dobutamine stress echocardiography
 - tissue doppler imaging;
- Myocardial contractility assessment by peak systolic velocity.

Previously, the best validated primary outcome was time to 1 mm ST depression in several clinical crossover design studies assessing the effects of pharmacological intervention in patients suffering from stable angina [21–25]. Some studies report several treadmill ‘main outcomes’ including time to change in total exercise time, time to chest pain onset and time to 1 mm ST depression [26,27]. The treadmill test results will be the best validated set of data. These results will be least likely to vary due to daily life challenges of patients when not under standardized research facility observation. However, we will report also the other above secondary outcomes including the angiogenic marker and dobutamine stress echocardiogram results that may contribute to the mechanistic explanation of potential antianginal benefits.

Methods

• Design

The trial has a randomized, placebo-controlled, double-blind, crossover design. The study is approved by the Scotland A Research Ethics Committee, subject to Medicine and Healthcare

Regulatory Agency regulation and ran in accordance with the Declaration of Helsinki. All patients will sign an informed written consent.

• Patient selection & protocol

Patients will be recruited from several sources: Aberdeen Royal Infirmary cardiology department, General Practitioner surgeries via Scottish Primary Care Research Network, patients attending the Heart Health Community Study in Aberdeen and by posters in public places and adverts in local newspapers.

Patients aged 18 and over with chronic exertional angina (≥ 2 months duration) will be interviewed, examined and asked to give a written informed consent. Entry criteria will be positive ECG treadmill test (ETT) and either angiographic evidence of obstructive coronary artery disease or if not available a positive dobutamine stress echocardiogram or a positive myocardial perfusion scan. Patients will be screened with two modified Bruce Protocol ETTs on separate days and enrolled only if they have replicable exercise-induced ECG evidence of ischemia ($\leq 15\%$ difference in time to 1 mm ST segment depression at the J + 80 ms point between the first- and the second-baseline ETT [25], **Figure 1**.

Exclusion criteria will be inability to perform an exercise treadmill test, women of childbearing potential, G6PD deficiency, ejection fraction of the left ventricle (LV) $< 45\%$ or New York Heart Association heart failure class three or four, myocardial infarction or revascularization within the last 2 months, resting ST depression ≥ 1 mm or left bundle branch block. Additionally, patients in nonsinus rhythm and significant valvular disease will not be included in the study as these may render the data interpretation unreliable.

Patients will be able to continue their regular antianginal medication at a fixed dose apart from long-acting organic nitrates that will be stopped in all patients at least 72 h prior to enrollment. Patients undergoing a concomitant dobutamine stress echocardiogram will be asked to omit their β -blocker for 48 h prior to their visits in order to facilitate the dobutamine response, unless clinically contraindicated, in which case the β -blocker treatment may continue uninterrupted. This decision will be at the discretion of the researcher (mainly depending on the severity of symptoms) and the elected strategy will be kept fixed throughout all subsequent patient's visits. Patients will be allowed to continue short-acting sublingual GTN use

and other background angina medication at a fixed dose.

• Treatment & randomization process

The rationale for the dose used in the study (600 mg [7 mmol] sodium nitrate per day) is based on evidence from previous studies using similar or smaller doses (often given in a single bolus) when assessing blood pressure lowering effects [14,28] and exercise capacity studies [17]. Doses as low as 3.5 mmol of nitrate (beetroot juice) were effective to lower blood pressure when given to drug-naïve grade one hypertensive volunteers [29]. A single dose of 4 mmol nitrate (potassium nitrate) in a single oral dose was sufficient to lower the blood pressure in healthy volunteers [28].

A recent meta-analysis of 17 studies showed that doses ranging from 300 to 600 mg of inorganic nitrate (either in form of beetroot juice or sodium nitrate) ranging from single bolus to 15 day supplementation, showed a moderate benefit on improved time to exhaustion in healthy volunteers. [17]. Larsen *et al.* demonstrated in young healthy volunteers that a dose of 0.1 mmol/kg (7 mmol = 600 mg for 70 kg) split in three doses over the day given for 3 consecutive days can improve mitochondrial efficiency in the skeletal muscle [19]. Kenjale *et al.* gave single dose of 9 mmol inorganic nitrate in form of a 750-ml beetroot juice showing improvement in claudication onset [30]. The dose used in our study (600 mg, 7 mmol) is several times higher than an average western diet intake that contains approximately 100 mg/d [31], but this should be safe as similar or even higher nitrate content can be achieved by nitrate-rich Mediterranean diet or the fruit- and vegetable-rich dietary approaches to stop hypertension diet, which confer health benefits [2,31,32].

The trial medication will be manufactured and placed into packs containing two bottles labeled one (first-treatment visit) and two (second-treatment visit) at the Western Glasgow Infirmary Pharmacy. Sodium nitrate powder will be purchased from Merck KGaA, Darmstadt, Germany. Each bottle will include 14 capsules and contains either 600 mg (7 mmol) of sodium nitrate or placebo (lactose monohydrate) filled in opaque matching hard gelatin capsules.

The sequence of treatment randomization to bottles one and two will be decided according to a list provided by Aberdeen Randomization Service (CHaRT, University of Aberdeen). At no point during the study will the research team or

Figure 1. Randomized double-blind placebo-controlled crossover design.

[†]Patient will be excluded if DSE or MPI positive, but they have recent angiographic evidence of non-obstructive coronary artery disease.

DSE: Dobutamine stress echocardiogram; ETT: ECG treadmill test; MPI: Myocardial perfusion imaging; STD: ST segment depression.

the patient know which bottle contains which treatment. Following treatment enrollment, the patient will be handed out the first bottle and start treatment with one capsule a day for a period of 7–10 days before undergoing a treadmill test and/or dobutamine stress echocardiogram (DSE) and/or blood tests and a second bottle will be handed out. After a 2-week wash out period, the second bottle will be started for 7–10 days and same tests performed on the last day. After each arm, the patient will returned the bottle with the remaining capsules for compliance assessment and returned to pharmacy. The 2-week wash-out period should be sufficient to avoid any confounding carryover effects of nitrate treatment as its plasma half-life ranges from 5 to 8 h.

Following verbal instruction, patients will be handed out a written diet advice sheet and asked to follow a low nitrate and nitrite diet, to limit caffeine intake and avoid use of antibacterial mouthwash during the treatment weeks. The latter is in order to prevent the loss of nitrate to nitrite bacterial bioconversion that occurs in the oral cavity and forms an integral part of the nitrate/nitrite enterosalivary circulation [33–36]. On the morning of the test, the patients will be asked to avoid any caffeine intake and take the last study capsule approximately 2 h prior to their visit.

• Exercise treadmill test

Seventy patients will undergo an ECG treadmill test following each treatment arm. They will be

performed approximately 2 h following ingestion of the last study capsule to ensure the nitrate to nitrite bioconversion can take place. Automated blood pressure monitoring and 12-lead ECGs will be recorded at rest in standing position and during a modified Bruce Protocol (at the end of each stage, at the time of first 1 mm ST depression, at time of first chest pain onset, at peak exercise and every 3 min into recovery). In patients with minor resting ST depression (<1 mm), the time to 1 mm ST change will be defined as additional ST depression of 1 mm below the resting value as digitally displayed at J point + 80 ms [26]; see **Figure 2**.

• Dobutamine stress echocardiography

All patients with a positive ECG treadmill test will be invited for a screening contrast DSE. Only patients with evidence of inducible regional wall motion abnormalities, satisfactory echo windows, tolerating well the baseline scan will be enrolled into the DSE arm. All tests will run 2 h following finish of the ETT and approximately 5 h following the last capsule ingestion (to allow optimal treatment plasma levels).

A standard protocol will involve resting for 20 min, baseline acquisition, loading with dobutamine 10 µg/kg/min for 5 min and then 20, 30 and 40 µg/kg/min each for 3 min. The predefined end points will be: inducible regional wall motion abnormality, significant chest pain, ST depression >2 mm or ST elevation, persistent arrhythmia and symptomatic BP (blood pressure) fall. In patients with poor heart rate rise without any other predefined end points, atropine (up to total of 1.2 mg) can be added from 30 µg/kg/min stage onward to reach at least 85% of age predicted target HR ($0.85 \times 220 - \text{age}$) [37]. LV contrast agent will be used as this was previously shown to significantly improve detection of inducible regional wall motion abnormalities [38]. Six views (apical four chamber, two chamber, three chamber, parasternal short axis at base, mid ventricle and apex) will be routinely obtained.

Patients with an evidence of inducible regional wall motion abnormality on screening will be enrolled into the DSE arm and undergo two further tests, one following each treatment arm. These on-treatment DSEs will run using exactly the same individual pharmacological protocol (dobutamine stage ± fixed atropine dose) as defined during the patient's screening exam. Images will be obtained without contrast

using Doppler tissue velocity imaging (TVI; Q-stress) in apical four-chamber, two-chamber and three-chamber view only. Image depth, width, color tissue Doppler velocity scale and frame rate will be optimized to avoid aliasing and aim at >120 frames/s. During passively held end expiration, three loops will be recorded in each view in the last minute of each stage. Digitized images will be later analyzed off line. Longitudinal basal segment peak systolic velocity (Sp) is the most reproducible tissue Doppler parameter, sensitive to ischemia and related to blood flow [39,40]. Sp will be measured in six segments: basal inferoseptum, basal lateral, basal inferior, basal anterior, basal posterior and basal anteroseptum as previously described [41]. Sp will be measured as the maximal velocity following isovolumic contraction averaged from three cycles (**Figure 2**).

• Bloods

Twenty patients will be invited additionally to take part in the blood subgroup. These patients will have blood taken on three occasions: their final screening visit and the two on-treatment visits. All will attend fasting from midnight, but clear water will be allowed with their morning medication. Patients will be advised to take the last study capsule approximately 2 h prior to their study visit. Diabetic patients taking either tablet or insulin treatment will not be included in this substudy in order to avoid hypoglycemia when fasting and exercising. Blood will be taken prior to the treadmill test and samples for angiogenic markers: sFlt-1, PlGF and VEGF in Li-Heparin tubes and nitrate/nitrite aliquots will be sampled into Ethylenediaminetetraacetic acid tubes that will be supplemented with *N*-Ethylmaleimide.

Nitrite/nitrate plasma levels

All samples will be spun immediately for 5 min at 1000 g at room temperature, supernatant will be saved and snap frozen in liquid nitrogen and stored by -80°C. Nitrite/nitrate levels will be analyzed at the University of Southampton [42]. Frozen plasma samples will be thawed in the presence of *N*-Ethylmaleimide (10 mM final concentration) and deproteinized by methanol precipitation immediately prior to analysis. Plasma nitrite and nitrate will be measured by high-pressure liquid ion chromatography with postcolumn derivatization using a dedicated analysis system (ENO-20 with Gilson

Figure 2. Proposed data analysis method. (A) Example of ECG exercise treadmill test and time to 1 mm ST depression end point. (B) Dobutamine stress echocardiography, top – screening contrast dobutamine stress echocardiography (different stages of dobutamine stress, four-chamber view); bottom – example of native dobutamine stress echocardiography with tissue velocity imaging systolic velocity measurements, three-chamber view example). STD: ST segment depression.

234 autoinjector, EPC-500 data processor and PowerChrome software; Eicom).

Angiogenic markers

VEGF and its receptor (VEGFR-1 = Flt-1) play a central role in maintaining endothelial cell integrity and in the promotion of angiogenesis and lymphogenesis. Soluble Flt-1 (also known as soluble VEGFR-1 or sFlt-1) is derived from the ligand binding region of VEGFR-1/Flt-1 and its main function is believed to be in the regulation of VEGF bioavailability and hence suppression of VEGF signaling [43].

• Modified Seattle questionnaire, GTN use & angina frequency

The SQ was developed in the 1990s as a 19-item quality of life questionnaire assessing five dimensions of patients suffering from angina: physical limitation, angina stability, angina frequency, treatment satisfaction and disease perception [44].

It is widely used and was validated as a functional instrument in cardiovascular research outcome [45–49]. We modified the questionnaire to reflect the short treatment period of 1 week in our study when compared with the original SQ which, in contrast, interrogates over a period of the last 4 weeks. The higher the score the better is the quality of life, angina control and disease perception. Patients will be handed out a checklist where they will document the frequency of their angina attacks and GTN use during their treatment weeks.

• Statistical analysis

Based on data from several previous randomized controlled studies testing the efficacy of antianginal medication with ECG exercise treadmill tests, the mean improvement in time to 1 mm ST depression between the active and placebo groups was around 50 s (36 s with amlodipine [23,50], 60 s with organic nitrates [24],

46 s with atenolol and ranolazine [51], 46 s with ivabridine [52] or 43 s with allopurinol [25]). The standard deviation in crossover studies ranged approximately 80–90 s [21–24]. Projecting an expected absolute mean treatment difference between the two arms of 30 s and a SD of 80 s and allowing for a significance of 0.05 at 80% power in a paired crossover trial design, would require a sample size of 58 patients. To allow for dropouts, we planned to randomize 70 patients.

For the secondary dobutamine stress echocardiogram, end point of tissue Doppler velocity-derived peak Sp, we aim to invite all eligible patients, but we recognize that many patients may not participate either due to contraindications, not tolerating the baseline scan or frequently their personal choice to opt out of this subgroup as the research visits will last significantly longer and often may interfere with their social or working life. We will aim to recruit a minimum of twenty patients based on a previous study by Ingram *et al.* who showed that single intravenous nitrite infusion (30 $\mu\text{mol NaNO}_2$) increased peak Sp in ischemic segments when compared with saline infusion ($n = 10$; 9.5 ± 0.5 vs 12.4 ± 0.6 cm/s; $p < 0.001$ [53]). A sample size of 16 patients would be necessary to observe 1.0 cm/s velocity difference Sp and a standard deviation of 1.0 cm/s (two tailed, paired, power 0.95 and $p = 0.05$).

The primary end point (time to 1 mm ST depression) is assumed to follow a normal distribution. The analysis will follow that recommended by Senn [54] for the analysis of a two-treatment, two-period crossover trial. A general linear model will be constructed with the following terms included: participant (as a random effect), period and treatment (both as fixed effects). Baseline terms will not be included as baseline data is not available for both treatment periods. Baseline data will, however, be tabulated and described, by randomized group (i.e., by treatment sequence). Treatment efficacy will be estimated as the treatment effect estimate from the general linear model with a 95% CI constructed and the hypothesis of zero effect tested (at the 5% significance level).

Secondary end points will be analyzed in the same manner. For some end points (for example number of angina attack episodes), the assumption of a normal distribution is unlikely

to hold and an appropriate transformation will be carried out prior to analysis (for example a logarithmic or square-root transformation). The residuals from each model will be checked to follow and approximate normal distribution. The trial statistician will conduct and report the analyses blind, that is, simply comparing treatment 'A' to treatment 'B' according to the randomization schedule provided, without knowing which treatment is active or placebo. All analyses will be carried out in SAS version 9.3.

• Trial oversight

A trial steering committee will oversee, monitor and supervise the progress of the study and will be responsible for the scientific integrity of the research. A data-monitoring committee will monitor the safety of the study and research validity of its conduct. The Research and Development department of the University of Aberdeen will act as the sponsor and monitor of the study. The study is registered and underwent regulatory approvals by the Medicine and Healthcare Regulatory Agency, NHS-Grampian Research and Development department and the Research Ethics Committee.

Conclusion

In the aging population, an increasing proportion of patients with advanced coronary disease survive to the stage when no more revascularization is possible and first-line antianginal treatment options are exhausted. Inorganic nitrate treatment offers, via nitrate–nitrite–nitric oxide treatment pathway, a unique antianginal strategy by theoretically improving selective vasodilation in hypoxic territories, promoting vasodilation or improving mitochondrial efficiency. While sound in experimental animal studies and pilot studies on healthy volunteers, this study proposes to investigate potential antianginal benefits of sodium nitrate in elderly population of patients suffering from angina and known advanced atherosclerotic disease who are on background polypharmacy.

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EXECUTIVE SUMMARY**Background**

- Angina remains a therapeutic challenge in the era of an aging western population once all revascularization options are exhausted and the use of first-line antianginal drugs is limited due to their side effects.
- Oral inorganic nitrate supplementation was shown to selectively vasodilate under hypoxic conditions, lower blood pressure, improve endothelial dysfunction, promote angiogenesis and improve mitochondrial efficiency in skeletal muscle.

Aim

- We hypothesize that, if similar effects occur in heart, oral sodium nitrate could improve markers of myocardial ischemia in patients suffering from stable angina.

Methods

- Design, treatment protocol and proposed analysis methods are reviewed in this trial protocol paper.

Conclusion

- This study proposes to investigate potential antianginal benefits of sodium nitrate in elderly population of patients suffering from angina and known advanced atherosclerotic disease who are on background polypharmacy.

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