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Original article

Effect of a plant-based bioequivalent inorganic nitrate (NO_3^-) complex with vitamins, antioxidants and phytophenol rich food extracts in hypertensive individuals - A randomized, double-blind, placebo-controlled study

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SUMMARY

Background: This study assessed efficacy of plant based bioequivalent nitrate complex, consist of vitamins, natural antioxidants and phytophenol rich food extracts to elevate nitric oxide (NO) bioavailability as determined by saliva conversion of nitrate (NO_3^-) to nitrite (NO_2^-) a required step to produce NO, in relationship to lowering blood pressure (BP) in both men and women.

Methods: 67 individuals (26 men; mean age of 59.3 ± 9.0 yrs) with mean baseline systolic and diastolic BP >120 and 80 mmHg respectively were randomized to receive daily dosing of 314 mM NO_3^- or NO_3^- free (placebo) tablets in double-blinded study for 12 weeks (wks). Inorganic NO_3^- tablets consist of NO_3^- rich beetroot extract, thiamine nitrate, and potassium nitrate in the presence of ascorbic acid, to facilitate NO bioavailability.

Results: The primary endpoint of the study was reduction in BP at 12 wks by improving endothelial function. At study conclusion, mean ± SD reduction in systolic BP (SBP) in the inorganic NO_3^- group was 12.5 ± 13.3 mmHg ($p = 0.0007$), as compared to 6.19 ± 11.39 mmHg ($p = 0.004$) in the placebo group, for a placebo-corrected reduction of −6.31 mmHg (95% CI 10.89–2.31, $p = 0.04$). NO_3^- also reduced diastolic BP by 4.7 ± 10.3 mmHg ($p = 0.01$), while no significant reduction in placebo group (1.98 ± 9.38 mmHg, $p = 0.24$) was noted. Endothelial function improved at 12 weeks by 0.8 ± 3.1 ($p = 0.03$) in active group when compared to 0.1 ± 1.8 ($p = 0.82$) in placebo group.

Conclusion: Endothelial function improved robustly reducing both systolic and diastolic BP in hypertensive individuals with daily supplementation of dietary NO_3^-.

ClinicalTrials.gov ID: NCT03909789.

1. Introduction

Cardiovascular disease (CVD) is the leading cause of mortality in developed countries, accounting for approximately 17.9 million deaths each year and 31% of all deaths worldwide [1,2]. Hypertension (HTN) is now defined as a blood pressure (BP) of >120/80 mmHg and is the most common risk factor for global disease burden and is estimated to cause 10 million cardiovascular deaths per year [3]. Approximately 80 million (1 in 3) adults are hypertensive in the US, and in 2011 it was estimated that only 54% of these individuals have the condition under control [4]. Globally, it is estimated that the number will increase to more than 1.5 billion people with HTN by 2025, making it the most common non-communicable disease in the world [5]. HTN and several other modifiable risk factors of CVD such as hyperlipidemia, diabetes mellitus, smoking and poor consumption of a plant-based diet are

Abbreviations: CVD, cardiovascular disease; NO_3^-, Nitrate; NO_2^-, Nitrite; NO, Nitric oxide; GCE, green coffee bean extract; NOS, nitric oxide synthase; ACE, angiotensin converting enzyme; BP, Blood Pressure.

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associated with endothelial dysfunction, which is an early step in the pathogenesis of atherosclerosis [6–12]. Endothelial cells have both vasodilatation and vasoprotective properties by releasing the substances called endothelium-derived relaxing factor and nitric oxide (NO) [13].

NO is a potent vasodilator and also protects the vessel wall against the development of atherosclerosis and thrombosis by inhibiting platelet aggregation, smooth muscle cell migration and proliferation, monocyte adhesion and adhesion molecule expression [13–15]. It has recently emerged that these cardioprotective effects may be attained by naturally occurring dietary inorganic nitrate (NO_3^-), via increasing the bioavailability of NO. Green leafy vegetables are a great dietary source for this inorganic NO_3^- [16–18]. There are numerous initiatives designed to promote increased fruit and vegetable intake, including the Dietary Approaches to Stop Hypertension (DASH) diet in the US [18]. DASH diet, sodium restriction, and exercise have been shown to reduce SBP by 8–14 mmHg, 2–8 mmHg and 4–9 mmHg, respectively [19]. In addition, recent studies showed that dietary supplements such as aged garlic extract, antioxidants like ascorbic acid, polyphenol rich foods i.e. pomegranate juice and green coffee bean extract (GCE) have been known to have a positive effect on CVD risk factors, including BP, cholesterol, and endothelial function by increasing the bioavailability of NO [10,20–23]. Unfortunately, despite attempts to promote vegetable consumption via such initiatives, compliance to these diets is poor. Even after dietary counseling in a 3-month randomized, intervention study, only 21% of participants achieved a suitable daily consumption of fruit and vegetables [24]. This poor compliance is related, in part, to the time required to prepare these foods and/or the cost implications [24]. While more convenient nitrate containing vegetable juices are readily available, many individuals avoid these due to their cost and undesired taste. Consequently, there is a need for a readily available, affordable and convenient inorganic NO_3^- containing supplement alternative, capable of providing the desired BP lowering effect by increasing the bioavailability of endogenous NO.

Previous studies have demonstrated that NO potent vegetables, such as leafy greens and beets, rich in inorganic NO_3^- increased the bioavailability of NO, lowering blood pressure as effectively as hypotensive medication [7,16–18,25–28]. For many years there has been considerable uncertainty and controversy surrounding the non-pharmacological approaches to lower BP, such as dietary supplements. Our study investigates whether plant-based bioequivalent dietary inorganic NO_3^- supplement can play a preventive role in reducing blood pressure by restoring the endothelial function in HTN individuals to reduce the risk of CVD.

2. Methods

2.1. Study population and randomization

Our study is a randomized placebo-controlled single center double–blinded study (NCT03909789). The research study is approved by Institutional Review Board (IRB) of the Lundquist Institute for Biomedical Innovation at Harbor-UCLA Medical Center. 67 eligible patients of men and women were enrolled after signing the informed consent forms after careful explanation and instructions about the study. 5 participants (active = 2 and placebo = 3) were lost to follow-up. Eligible participants were 40–75 years of age who have BP >120/80 mm Hg and on stable hypertensive regimen. Patients were randomized at a 1:1 ratio to receive daily dosing of 314 mM inorganic NO_3^- tablets or placebo and the duration of administration of study drug was 12 wks. Patients were counseled to refrain from NO_3^- containing foods like all greens. After randomization, participants will return at 2 wks and 12 wks to assess for any side effects. Between the visits, we have an inter-trial phone visit to ensure study medication adherence and compliance.

2.2. Exclusion criteria

We excluded patients with known history of coronary artery disease (CAD) ($n = 2$), myocardial infarction (MI) ($n = 1$), stroke or life-threatening arrhythmia within the prior 6 months ($n = 1$), New York Heart Association Functional Classification II–IV heart failure ($n = 2$), renal impairment (serum creatinine > 1.4 mg/dL) ($n = 3$), current tobacco use ($n = 2$), history of bleeding disorders or use of anticoagulants ($n = 1$), hypertensive encephalopathy or cerebrovascular accident ($n = 1$), or who were currently enrolled in another placebo-controlled trial ($n = 0$).

2.3. Evaluation of cardiovascular risk factors

At baseline family history of heart disease, smoking history, medical history and current medications were collected in all the participants. Systolic blood pressure (SBP) and diastolic blood pressure (DBP) were recorded after resting for 5 min with back support, feet flat and arm bared at heart level prior to any blood pressure measurements and Body Mass Index (BMI) was calculated. Three BP readings were recorded on both right and left arms and the average BP of both arms was considered for eligibility criteria at baseline. Similar measurements of BP readings were recorded after 2 h (post-dosing of study IP), 2 wks and at 12 wks with daily administration of active or placebo. Plasma and saliva NO_3^- and NO_2^- were measured along with BP readings.

All participants will be instructed to abstain from smoking, alcohol, use of mouthwash and food intake, e.g., overnight fasting or at least 10 h before sample drawing. Sample were stored at −70 °C and analyzed for c-reactive protein (CRP), creatinine, serum glucose, hemoglobin A1c (HbA1c) and a lipid profile, including serum LDL cholesterol, HDL cholesterol, and TGs, with the use of automated diagnostic equipment (DLS Laboratories).

Plasma and salivary samples were collected and frozen at −80 °C for determination of NO_3^- and NO_2^- using a modified chemiluminescence technique as previously published [29]. NO_3^- and NO_2^- were determined by the reduction of NO metabolites ($NO = NO_3^- + NO_2^-$). Plasma and saliva samples were obtained at baseline (fasting) and at 2 h, 2 wks and 12 wks after administration of inorganic nitrate/placebo with food and water.

Saliva Nitric Oxide Test Strips was provided by The Berkeley Saliva Nitric Oxide Test Strips (Chicago, IL) and reader or manual log along with appropriate training and instructions on use, were provided to all participants. Berkeley strips are based on the modified Griess reagent reaction which correlated with the presence of salivary NO_2^-. NO_3^- is not measured by the strips, whereas the conversion of NO_3^- to NO_2^- in oral cavity is detected by the strips. Saliva Strip test was conducted twice daily: upon awakening and 2 hours' post-administrations of the active/placebo dietary NO_3^- tablet by participants. In short, the test strips require the participant to place the test strip on the surface of the tongue for 3–5 seconds (s). The test strip then change color within 10 s, with a deep red color indicating high NO_2^- concentration and a light pink color indicating a low NO_2^- concentration or the bioconversion does not occur. The Salivary strips values were adjusted as 1- depleted, 2- low, 3- threshold, 4- target, and 5- high; which were translated to 21, 108, 217, 434, and 869 μM NO, respectively for values 1,2,3,4,5 as previously described [30]. Participants recorded the value via

color scale on manual log and return the logs to site at 2 weeks and 12 weeks.

2.4. Measurement of brachial flow-mediated dilatation (bFMD)

The bFMD measurement was performed according to a previously defined protocol [31,32], by using ultrasound (UNEXEF18G, Unex Co Ltd, Nagoya, Japan) using a 10-MHz linear-array transducer, longitudinal images of the brachial artery above the antecubital fossa were recorded. Imaging was performed in a dark, quiet room at 25 °C. Patients rested supine for at least 5 min before the first scan and remained supine until the final recording was acquired. bFMD was calculated as the percentage change of the arterial diameter from baseline to maximum dilation. bFMD is measured at baseline, 2 h (post-dosing of study IP) and at 12 wks with daily administration of placebo or 314 mM inorganic NO_3^-.

2.5. Inorganic nitrate NO_3^- tablets

The inorganic nitrate NO_3^- tablets provided by Berkeley Life (Chicago, IL), consisted of nitrate rich beetroot extract 20 mg, thiamine mononitrate 90 mg, potassium nitrate 480 mg, ascorbic acid 150 mg, folic acid 200 mcg, methyl cobalamin 200 mcg, calcium 115 mg, pomegranate fruit extract 5 mg and 115 mg of green coffee bean extract (*Coffea canephora*; standardized to 50% total polyphenols, 45% chlorogenic acids and 10% 5-caffeoylquinic acid) to facilitate NO formation. The constituents of the pills were verified by mass spectroscopy. The placebo and the study drug appeared to be of similar size and color (color: beige; Shape: modified oblong; tablet length: 0.687 inches; tablet width: 0.281 inches); however, the placebo pill did not contain any active ingredient. The study drug was packaged in the containers by the sponsor as per the randomization list and sent them to the site with printed label containing only the number of the patient. The randomization key was provided to the Principal Investigator and was opened only after completion of the study. All participants were advised to take 2 active/placebo tablets once daily with water for 12 weeks in addition to their anti-hypertensive regimen. Each active tablet consists of 157 mg of NO_3^- and received a total of 314 mM of NO_3^- per day. The product used in this clinical trial is commercially available.

2.6. Statistical analysis

Continuous normally distributed data were expressed as means ± SDs, while categorical variables are stated as counts and percentages. A Students' t-test or Chi-square test was used to assess differences in all baseline parameters between the placebo and active. The analysis included comparisons for BP and bFMD at both baseline and 12 wks and between groups following the use of the ANOVA modeling with the Tukey approach *P*-value and ANCOVA modeling was also used to adjust for clinically significant variables. As none of these modeling techniques were significant, we thus chose to focus on the simple progression of BP in the placebo group and regression in the active group. BP differences between the placebo and active were assessed by the Wilcoxon rank-sum test. Saliva and plasma NO_2^- and NO_3^- changes at 2 h, 2 wks, 12 wks from baseline values were analyzed with the Wilcoxon Signed Rank test from SAS and expressed as median ± SDs within each group (i.e., inorganic nitrate and placebo). The Wilcoxon Signed Rank test was also used to analyze salivary strip test differences and were expressed as median ± SDs. A value of $P < 0.05$ was considered statistically significant. All statistical analyses were performed with SAS 9.4 software.

2.7. Power calculation

Prior literature on inorganic NO_3^- supplement therapy and its effects on blood pressure provided reasonable estimates to construct a robust power calculation for this study [7]. To detect a decrease of 6 mmHg in SBP, assuming 80% power, and an alpha of 0.05, 56 subjects are necessary to evaluate our primary endpoint. Assuming a 20% dropout rate, 67 subjects were enrolled in this study.

3. Results

3.1. Baseline characteristics

62 participants (26 men and 41 women; mean age 59.3 ± 9.0 yrs) completed the study visits, where baseline and follow up BP indexes were assessed. Of 62 patients, 30 were randomized to the treated group (18 females, mean age 57.5 ± 9.4 years) and 32 to the placebo group (23 females, mean age 60.9 ± 8.5 years). Patients were on current medications such as aspirin, hypertensive or hyperlipidemia medication, and did not change medications during the period of the study. None of the participants were on any nitrates such as isosorbide mono or di nitrate. The interventions were well tolerated without adverse effects. Baseline characteristics are shown in Table 1 and both the groups had similar demographic characteristics. Table 2 shows the labs collected at baseline and 12 weeks.

3.2. BP and bFMD analysis

The change in value of BP over 12 wks in the placebo and active groups is shown in Table 3. There was a significant reduction in mean SBP and DBP at 12 wks compared to baseline in active NO_3^- group [mean ± SD 12.5 ± 13.3 ($p = 0.0007$) and 4.7 ± 10.3 ($p = 0.01$) respectively]. The placebo group exhibited a mean reduction in SBP but at half the rate seen in the active group, 6.2 ± 11.4 ($p = 0.12$) and not a significant change in DBP at 12 wks (2.0 ± 9.4 mm Hg, $p = 0.24$). The placebo-corrected BP reduction is of −6.31 mmHg (95% CI 10.89–2.31, $P = 0.04$).

In placebo group, bFMD was 3.0 ± 1.9% at baseline, with subsequent measures of 3.3 ± 2.1% at 2 h and 3.1 ± 1.8% at 12 wks ($p = 0.82$). The active treatment group, bFMD increased from 2.9 ± 2.0% at baseline to 4.0 ± 2.4% at 2 h and to 3.7 ± 4.2% at 12 wks ($p = 0.03$) (Fig. 2).

Table 1
Baseline characteristics among participants.

	Placebo ($n = 32$)	Active ($n = 30$)	*P* value
Age, (mean ± SD), yrs	60.9 ± 8.5	57.5 ± 9.4	0.14
Female, *n* (%)	23 (71.8)	18 (60)	0.33
Hispanic, *n* (%)	19 (59.3)	17 (56.6)	0.83
BMI, (mean ± SD), kg/m^2	32.24 ± 6.56	33.63 ± 9.21	0.49
Taking antihypertensive medication, *n* (%)	30 (93.7)	29 (96.6)	0.60
Hyperlipidemia, *n* (%)	20 (62.5)	18 (56.2)	0.61
Taking antihyperlipidemic medication, *n* (%)	17 (53.1)	13 (43.3)	0.44
Taking anti-diabetic medications, *n* (%)	20 (62.5)	21 (70)	0.53
Taking aspirin, *n* (%)	19 (59.3)	17 (56.6)	0.83
Family history of heart disease, *n* (%)	13 (40.6)	11 (36.6)	0.74
Post-menopausal, *n* (%)	12 (37.5)	10 (33.3)	0.73
Previous smoker, *n* (%)	10 (31.2)	12 (40)	0.47

Values are means ± SDs or *n* (%). Yrs, years; BMI, body mass index.

Table 2
Labs collected at baseline and 12 wks between 2 groups.

Variable		*n*	Baseline (mean ± sd)	12 weeks (mean ± sd)	Within Group Δ V1 V3 (mean ± sd)	Between Group	
						(mean ± sd)	*P* value
C-reactive protein (CRP) (mg/L)	Active	30	8.03 ± 8.29	6.64 ± 6.12	1.39 ± 6.39	1.94 ± 4.91	0.12
	Placebo	32	4.79 ± 3.48	5.34 ± 4.31	0.55 ± 2.92		
Hemoglobin A1c (%)	Active	30	7.75 ± 2.54	7.46 ± 2.28	0.29 ± 0.66	0.22 ± 0.87	0.32
	Placebo	32	7.02 ± 2.18	6.94 ± 1.95	0.08 ± 1.02		
Mean plasma glucose (mg/dL)	Active	30	173.07 ± 72.20	167.27 ± 65.28	5.80 ± 24.10	3.46 ± 27.03	0.61
	Placebo	32	154.84 ± 62.69	152.50 ± 55.74	2.34 ± 29.50		
Creatinine (mg/dL)	Active	30	0.86 ± 0.32	0.88 ± 0.31	0.02 ± 0.12	0.014 ± 0.11	0.60
	Placebo	32	0.82 ± 0.19	0.83 ± 0.19	0.01 ± 0.09		
Total cholesterol (mg/dL)	Active	30	163.97 ± 53.48	164.97 ± 52.92	1.00 ± 36.51	−3.75 ± 35.47	0.67
	Placebo	32	174.88 ± 46.71	179.63 ± 52.31	4.75 ± 34.47		
HDL cholesterol (mg/dL)	Active	30	49.57 ± 11.54	52.23 ± 14.25	2.67 ± 7.71	1.64 ± 7.06	0.36
	Placebo	32	54.03 ± 14.85	55.06 ± 12.82	1.03 ± 6.40		
LDL cholesterol (mg/dL)	Active	30	88.10 ± 45.51	81.00 ± 42.88	7.10 ± 32.93	7.69 ± 30.96	0.33
	Placebo	32	91.78 ± 43.30	92.38 ± 46.29	0.59 ± 28.99		
Triglycerides (mg/dL)	Active	30	131.60 ± 85.26	158.30 ± 115.59	26.70 ± 44.92	14.23 ± 46.01	0.22
	Placebo	32	145.34 ± 83.95	157.81 ± 101.48	12.47 ± 47.02		
VLDL (mg/dL)	Active	30	26.30 ± 17.04	31.73 ± 23.16	5.43 ± 8.96	4.18 ± 14.25	0.24
	Placebo	32	30.94 ± 20.72	32.19 ± 23.07	1.25 ± 17.83		

Table 3
Blood pressure reduction stratified by placebo and active treatment.

Variable		Baseline	2 h	2 weeks	12 weeks	Difference with in the group		Between Group	
						Mean + sd	*P* value	Mean ± sd	*P* value
SBP (mmHg)	Active	143.1 ± 10.6	136.85 ± 12.49	134.05 ± 12.94	130.6 ± 12.5	12.5 ± 13.3	0.0007	6.3 ± 12.4	0.04
	Placebo	143.2 ± 10.6	137.63 ± 14.01	133.39 ± 11.37	136.3 ± 14.5	6.2 ± 11.4	0.004		
DBP (mmHg)	Active	81.0 ± 11.0	79.13 ± 9.51	79.68 ± 9.44	76.3 ± 7.1	4.7 ± 10.3	0.01	2.7 ± 9.7	0.26
	Placebo	80.5 ± 10.6	77.70 ± 10.7	76.64 ± 10.51	78.8 ± 9.0	2.0 ± 9.4	0.24		

Data present by mean ± SD; Abbreviations: SBP, systolic blood pressure; DBP, diastolic blood pressure.

3.3. Nitrate and nitrite analysis

Plasma and saliva NO_3^- and NO_2^- increased with the daily NO_3^- supplement group when compared to the placebo group over 12 weeks (Figs. 3 and 4). The median ± sd values are presented in Table 4.

3.4. Salivary strip analysis

At 12 weeks, the bioconversion of NO_3^- to NO_2^-/ NO after 2 h post-ingestion of inorganic NO_3^- supplement showed salivary strip levels of <21 μM NO_2^-/NO after receiving placebo tablets, as compared to an average range of 217–869 μM NO_2^-/ NO ($p < 0.0001$) among the 30 participants that received the inorganic NO_3^- supplement (Table 4).

4. Discussion

Our study demonstrated that there is reduction in SBP (−12.5 ± 13.3 vs −6.2 ± 11.4 mmHg) and DBP (−4.7 ± 10.3 vs −2.0 ± 9.4 mmHg) in patients who received daily 314 mM of dietary inorganic NO_3^- supplement when compared to placebo (Table 3). The SBP demonstrated a significant reduction in individuals with administration of inorganic NO_3^- supplementation ($p = 0.04$) when compared to DBP ($p = 026$). Several prospective metanalysis studies comparing SBP and DBP found that SBP is a stronger predictor of cardio metabolic risk than DBP [33,34]. SBP progressively increases with age up to 80 years (via mechanism of arterial stiffness), whereas DBP increases with age up to 55 years and decreases thereafter [34]. So, individuals with elevated SBP combined with either high or low DBP are at higher risk for cardiovascular mortality. For every 2 mm increase in SBP, risk of fatal stroke increases by 7% and coronary heart disease by 5%, as suggested by a large observational study [35].

Vascular aging is the major risk factor for CVD which is attributed to the loss of vascular elasticity and its ability for NO synthesis. The vascular endothelial cells are activated during the regular exercise to increase the production of eNOS and NO bioavailability. Increased consumption of NO_3^- rich foods are required in conjunction with the exercise to delay the vascular aging due to reduced natural synthesis of NO with aging [36]. Previous studies demonstrated the advantages of dietary NO_3^- supplementation on exercise performance and also noted its beneficial effect on BP with elevation of plasma NO_2^-. The chronic effect of dietary NO_3^- was observed with continued supplementation over 5 and 15 days [37–39]. Webb et al. [40] observed the decreased SBP by 10 mmHg and DBP by 8 mmHg among 14 healthy individuals with the elevated plasma NO_2^- concentration in 3 h following beetroot juice intake. Our study results were consistent with the prior studies showing a significant reduction in BP of 4–11 mmHg and a restoration of endothelial function with daily administration of inorganic NO_3^- in hypertensive individuals [7,25,27,41].

The current study demonstrated the improvement in endothelial function measured by bFMD among 30 individuals with oral administration of NO_3^- supplement. Endothelial dysfunction (ED) measured by bFMD significantly related to coronary atherosclerosis and development of CVD [32,42]. ED is characterized by proinflammatory and prothrombic state by reducing the eNOS activity and altering the vascular homeostasis. Oral NO_3^- supplementation were reported to have a protective effect on endothelial function and NO_2^- significantly demonstrated the marked protection against ischemia/reperfusion (I/R) injury in the myocardial, hepatic, renal,

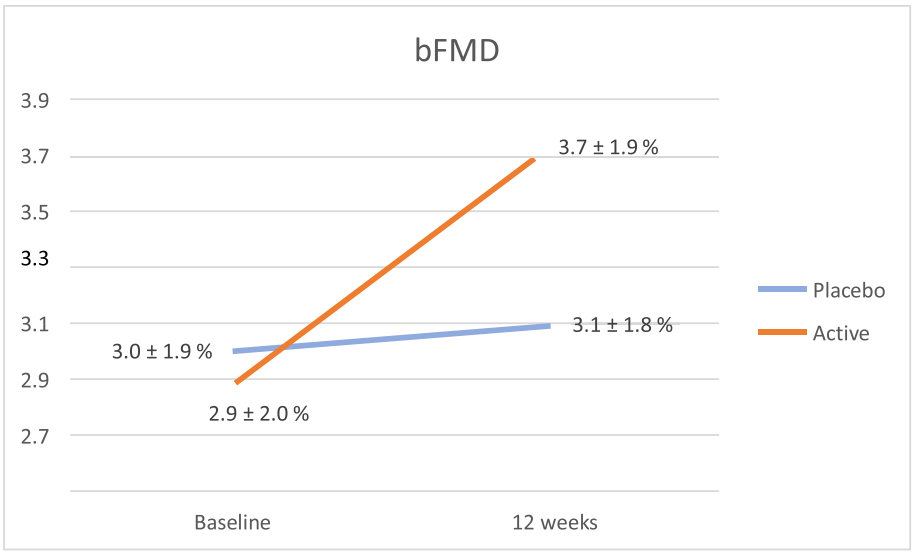

Fig. 1. Change in brachial flow-mediated dilatation (bFMD) between two groups (mean ± SD).

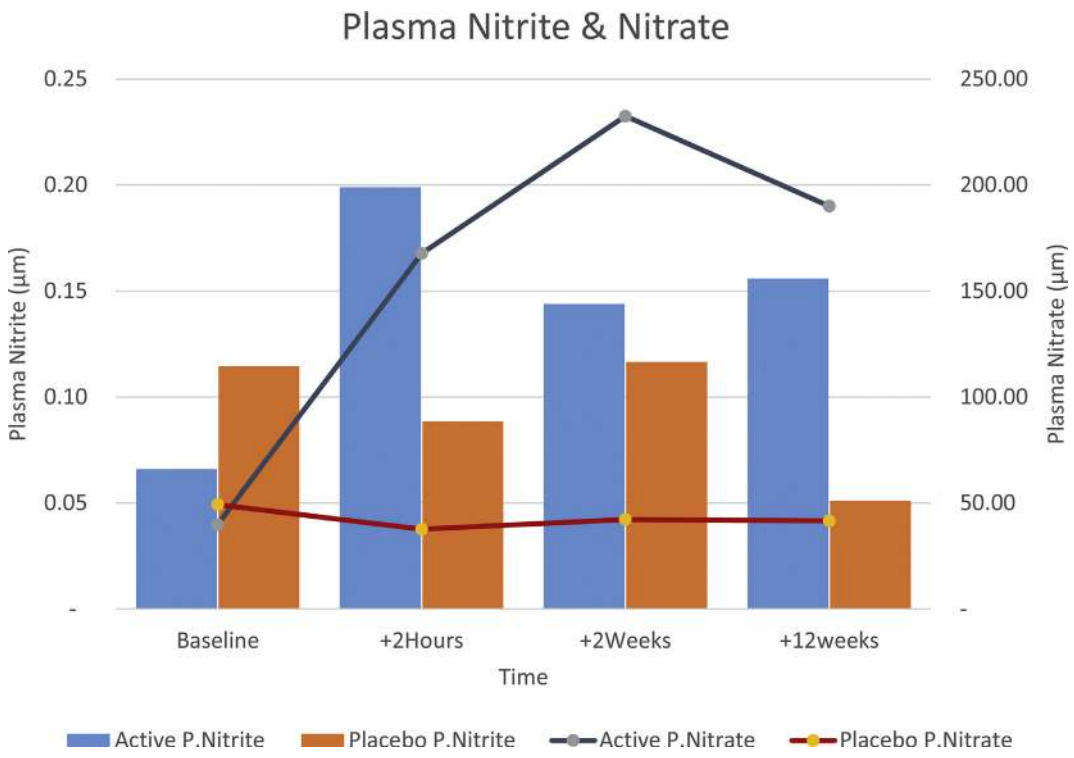

Fig. 2. Median plasma nitrite (µm) and nitrate (µm) values between active and placebo group at each study visit.

pulmonary and cerebral vasculature. Webb et al. [40] found that the I/R in the forearm of healthy volunteers significantly reduced FMD by approximately 60% ($p < 0.001$) whereas, the beetroot-juice protected the endothelium against the IR and did not alter pre-ischemic FMD ($p < 0.05$). Cosby et al. have shown that the NO_2^- represents a major NO bioavailability and NO_2^- mediated vasodilatation in the brachial artery is dose-dependent maintaining normal cardiovascular homeostasis [43]. Inorganic NO_3^- formulation in the current study is consistent with the work of Kapil et al. that dietary NO_3^- represents a valuable, safe and effective intervention to provide a sustained lowering of BP and improvement in vascular health in the population including hypertensive individuals and those at risk of becoming hypertensive [28].

Discovery of endothelial-mediated pathway for release of NO has prompted focus on dietary NO_3^-, and their role in the success of the DASH diet as an alternative to drug therapy. For prevention and/or treatment of HTN, it is desired to use plant based bioequivalent food extracts to avoid the adverse effects of synthetic drugs and high cost of drug therapies. Endogenous NO has therapeutic effects on HTN and various cardiovascular risk factors. NO is a potent vasodilator, which is generated by 2 pathways: by reducing dietary NO_3^- – NO_2^- – NO and by oxidation of L-arginine in vascular

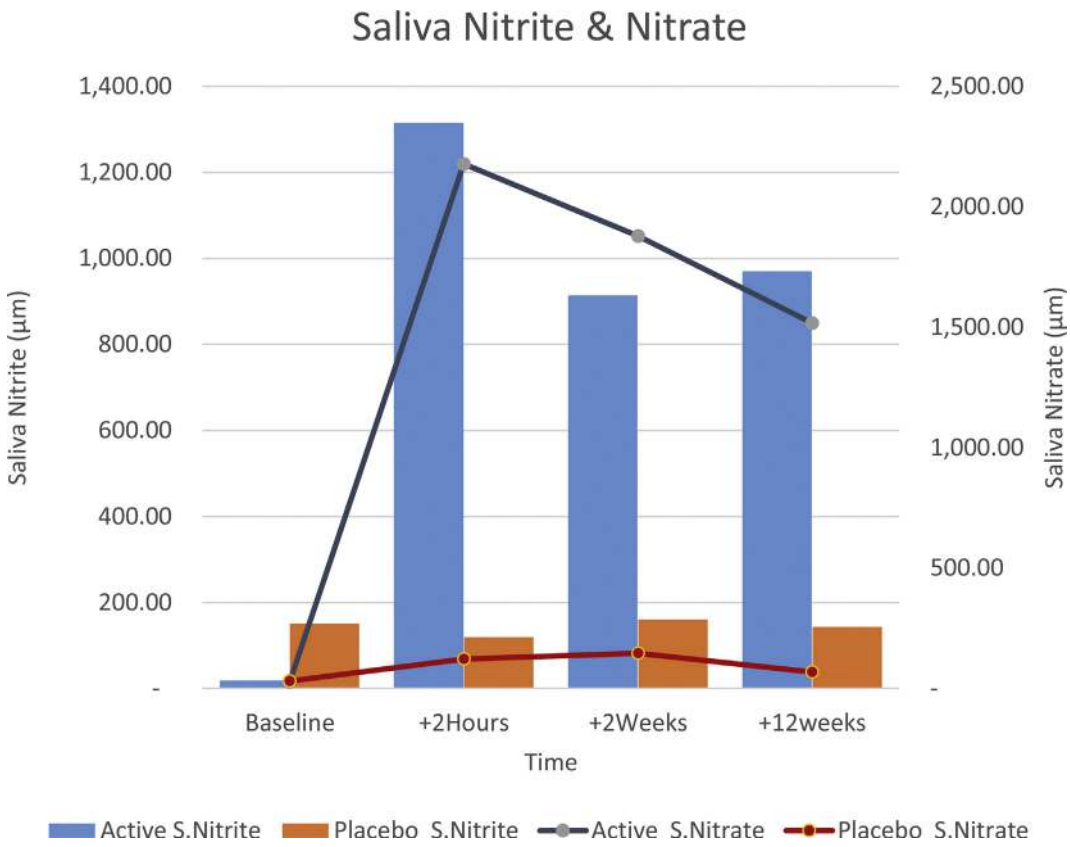

Fig. 3. Median salivary nitrite (µm) and nitrate (µm) values between active and group at each study visit.

endothelium by endothelial isoform of nitric oxide synthase (eNOS) pathway as previously published [40,44,45]. Fig. 1 shows the schematic presentation of inorganic NO_3^- mediation of cardiovascular protection by vegetable-rich diets. The oral bioavailability of ingested NO_3^- is nearly 100% and transported in to the plasma [46]. The bioconversion of NO_3^- to NO_2^- in the salivary gland through commensal microflora is a necessary and required step for the production of NO. Systemic NO_3^- and NO_2^- circulate between the saliva, blood and tissues, and only 25% of the ingested NO_3^- is transported into the saliva, where the concentration of salivary NO_3^- was reported to be 10 times of that of the plasma NO_3^- as a result of bioconcentration [47]. Only 20% of the saliva NO_3^- is reduced to NO_2^- through oral microflora. The majority of the swallowed saliva with NO_2^- enters the circulation and various tissues of the body. The NO_2^- to NO reduction was greatly influenced by the hypoxia or oxidative stress. It is NO that is the endogenous signaling molecule known to relax blood vessels, which can lead to reduce blood pressure. The half-life of NO is less than a second, however it can be sustained in the blood and tissues by oxidation to NO_3^- and NO_2^- and converted back to NO under the pathophysiological conditions to minimize the tissue dysfunction or injury.

In the present study, we examined the oral bioconversion through the salivary strip test with a corresponding salivary and plasma NO metabolite. At 2 h post-ingestion of inorganic NO_3^- supplement, we found that the salivary strip values were significantly improved when compared to the baseline (20.0 ± 53.36 vs 435.0 ± 332.24 µm; $p < 0.0001$). Similarly, after 2 h there is a significant rise in NO_3^- (salivary and plasma) and NO_2^- (salivary and plasma) providing the evidence for bioconversion of NO_3^- to NO_2^-. In addition, these findings not only suggest the bioconversion of NO_3^- to NO_2^- but also the entero-salivary circulation and oral microflora reduction. Following 2 wks and 12 wks of inorganic NO_3^- administration was found to have the consistent elevation of NO_3^- and NO_2^- concentration (salivary and plasma) with corresponding salivary strip results. However, there is significant increase in the values of plasma NO_2^- and salivary strip values in patients who received placebo might be attributed to their dietary habits rich in NO_3^- (drinking water/green leafy vegetables). The current study results were consistent with the previous studies demonstrated a significant elevation of NO_3^- to NO_2^- in both saliva and plasma following the oral administration of inorganic NO_3^- [28–30,40,46,48].

4.1. Role of plant-based bioequivalent inorganic NO_3^- supplementation and its constituents

Beetroot extracts are rich in high inorganic NO_3^- content with betalains pigment and a good source of phytochemical compounds such as ascorbic acid, carotenoids, phenolic acids and flavonoids which are known for its anti-oxidant and anti-inflammatory properties [49]. Previous studies have demonstrated the effects of beetroot supplementation on endothelial function, peripheral arterial stiffness and BP by increasing the NO bioavailability [28,30,37,40,49]. *Thiamine mononitrate*, thiamine is a water-soluble vitamin and is an essential coenzyme for carbohydrate metabolism. Thiamine-rich environment was demonstrated to improve the endothelial function by reducing the oxidative stress and activated the eNOS to improve the generation and bioavailability of NO. Additionally, thiamine reduces the secretion of von Willebrand factor (vWF), which is a marker of endothelial cell damage and vWF-mediated platelet adhesion to the injured endothelial is the

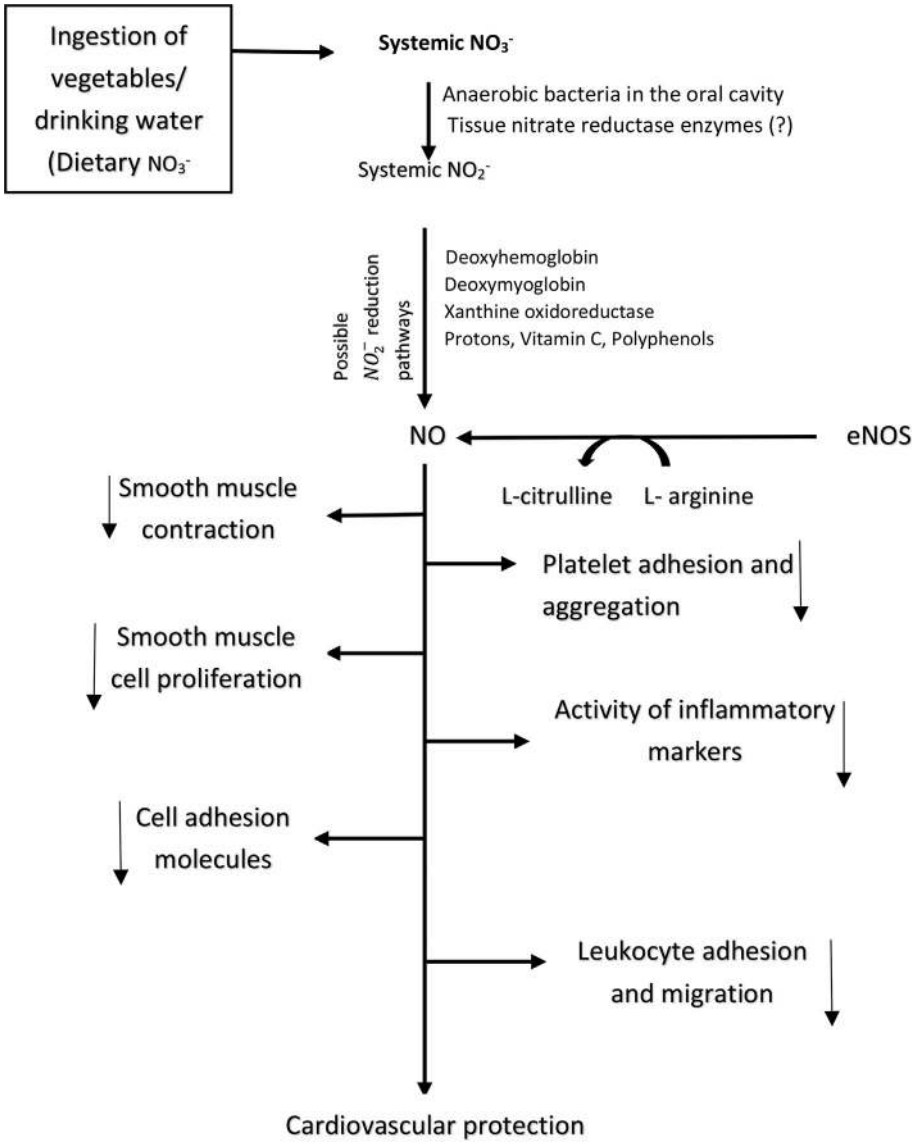

Fig. 4. Schematic presentation of inorganic nitrate (NO_3^-) mediation by vegetable-rich diets by Machha et al. [47]. After ingestion of vegetables/drinking water, NO_3^- reduced to nitrite (NO_2^-) and then to nitric oxide (NO) responsible for cardioprotective properties. NO_3^- and NO_2^- reduction pathways and classical L-arginine endothelial nitric oxide synthase (eNOS) pathway for NO production in the body.

Table 4
Significant plasma and salivary nitrate and nitrite/salivary strip test values in active and placebo groups at each study visit.

Group		Baseline	2 h	*P*-value[a]	2 weeks	*P*-value[a]	12 weeks	*P*-value[a]
Placebo ($n = 32$)	Plasma nitrate (μm)	49.16 ± 27.22	37.61 ± 95.16	0.47	42.26 ± 99.28	0.65	41.60 ± 68.88	0.45
	Plasma nitrite (μm)	0.11 ± 0.13	0.09 ± 0.12	<0.0001	0.12 ± 0.22	<0.0001	0.05 ± 0.13	<0.0001
	Salivary nitrate (μm)	31.34 ± 275.30	122.74 ± 960.13	0.01	146.56 ± 446.92	0.06	68.70 ± 1503.39	0.88
	Salivary nitrite (μm)	150 ± 277.98	119.43 ± 651.37	0.74	160.59 ± 841.32	0.30	143.39 ± 362.90	0.86
	Salivary strip (μm)	20.00 ± 64.51	110.00 ± 131.24	<0.0001	110.00 ± 245.48	0.0006	110.0 ± 163.28	0.001
Active ($n = 30$)	Plasma nitrate (μm)	39.75 ± 26.52	167.67 ± 107.27	0.01	232.54 ± 139.11	0.00	190.06 ± 170.37	0.003
	Plasma nitrite (μm)	0.07 ± 0.22	0.20 ± 0.36	<0.0001	0.14 ± 0.26	<0.0001	0.16 ± 0.42	<0.0001
	Salivary nitrate (μm)	27.75 ± 284.01	2177.62 ± 4.352.13	<0.0001	1878.47 ± 6218.14	<0.0001	1,516,65 ± 3625.42	<0.0001
	Salivary nitrite (μm)	19.09 ± 670.04	1315.93 ± 1801.33	0.0001	913 ± 2260.06	<0.001	970.24 ± 1452.94	<0.0001
	Salivary strip (μm)	20.00 ± 53.36	435.00 ± 332.24	<0.0001	435.00 ± 335.17	<0.0001	435.0 ± 297.93	<0.0001

[a] Data present by median ± SD and *P* value comes from Wilcoxon Signed Rank test.

first step in thrombus formation [50]. *Potassium nitrate (KNO_3)* ingestion significantly rises the plasma NO_3^- and NO_2^- concentration reflecting the entero-salivary circulation and oral bacterial reduction of NO_3^- to NO_2^-. Based on these findings, Kapil et al. reported that the BP changes were attributed to the endogenous conversion of NO_3^- to NO_2^- to NO but not to K^+.

Ascorbic acid (Vitamin C) is a water soluble vitamin and a potent antioxidant rich in citrus fruits. An intake of vitamin C somewhat

higher than normal may help protect the levels of NO by reducing the oxidation of the NO back to NO_3^- and NO_2^- [10]. A pooled analysis of 44 clinical trials demonstrated a significant positive effect of vitamin C supplementation on endothelial function (SMD: 0.50, 95% CI: 0.34, 0.66, $P < 0.001$) [51]. Additionally, the analysis stratified by health outcomes reported improved endothelial function in atherosclerotic (SMD: 0.84, 95% CI: 0.41, 1.26, $P < 0.001$), diabetic (SMD: 0.52, 95% CI: 0.21, 0.82, $P < 0.001$) and heart failure patients (SMD: 0.48, 95% CI: 0.08, 0.88, $P < 0.02$) with vitamin C supplementation. *Folic acid* is essential for amino acid metabolism and plays a vital role in cellular homeostasis, and DNA synthesis. A recent study suggests that folic acid and its active metabolite 5-methyl tetrahydrofolate increase NOS coupling and NO production to restore vascular reactivity, and depress cardio metabolic and cardiovascular risk markers [9].

Pomegranate extract reduces the blood pressure mediated by angiotensin II by inhibiting the angiotensin converting enzyme (ACE) [20]. ACE inhibition prevents the conversion of angiotensin I to angiotensin II which is a potent vasoconstrictor [52]. Inhibition of ACE not only increases the synthesis of NO but also increase the production endothelial-derived relaxing factors via elevation of intracellular calcium [23]. Furthermore, ACE inhibition upregulates the eNOS protein expression which further improves the endothelial function and vascular integrity. Whereas, *methylcobalamin* provides protection in oxidative stress associated pathologies like atherosclerosis, IR injury, by acting as an intracellular superoxide O_2^- scavenger and reduces inflammation [11]. The main component of *GCE* is chlorogenic acid and is metabolized in to caffeic acid, quinic acid, ferulic acid after its absorption from small intestine which improves vasoreactivity by acting on NO derived from vascular endothelium and also has an antioxidant property [21]. Kozuma et al. [21] have shown that the daily ingestion of 93–185 mg of GCE significantly reduces the both SBP ($p < 0.05$) and DBP ($p < 0.01$) in hypertensive individuals.

Importantly, unlike the chronic administration of prescription organic NO donor anti-hypertension drugs, dietary NO_3^- does not appear to lead to the development of tolerance, hypotension, and endothelial dysfunction [53]. Taking these observations together, enriching the diet with NO_3^-, natural antioxidants and vitamins represents a valuable, safe and effective intervention to provide a sustained lowering of BP and improvement in vascular health in the population as a whole there by endothelial function, including hypertensive individuals and those at risk of becoming hypertensive.

5. Limitations

The current study has several limitations. First, this relatively small sample size and short-term follow-up study did not have enough power to show the significant differences in DBP in between the groups (active vs placebo). However, we were able to show a significant reduction of DBP with in the active group. Second, patients were under different therapies for high BP and medications that improve endothelial function. Some patients used varying medications and different doses. Because of our small sample size, a separate analysis by different hypertensive medications was not performed. Third, we did not assessed any association among the salivary and plasma NO_3^- and NO_2^- concentrations. A much larger study randomizing patients to individual components of the therapy would be needed.

6. Conclusion

This study indicates that both SBP and DBP were significantly reduced with daily intake of plant based bioequivalent inorganic NO_3^- supplement (Berkeley Life – Chicago, IL) which is rich in vitamins, natural antioxidants and polyphenol food extracts to facilitate NO bioavailability. Reduction in BP and improvement in endothelial function with NO_3^- supplement was likely attributed to nitric oxide. Plant based bioequivalent NO_3^- supplement has the potential to be used to complement anti-hypertensive therapies, including plant-based dietary approaches, for BP control by improving endothelial function.

Conflict of interest and funding disclosure

Matthew J. Budoff, M.D., FACC discloses work for the National Institutes of Health and General Electric Healthcare; All other authors have no conflicts of interest. Study was funded by Berkeley Life, Illinois, USA.

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MJB, SG and WS conceived of and designed the study; LC, DB, BTC, CS, SH, KS and FF collected the patient information and generated the clinical data; AK, LC, DB, BTC, SKR, WS, SG and MJB analyzed and/or interpreted the data; and LC, DB, BTC, AK, WS, SG and MJB drafted or revised the manuscript. All authors read and approved the final version of the manuscript.

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