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Acute interaction between oral glucose (75 g as Lucozade) and inorganic nitrate: decreased insulin clearance, but lack of blood pressure-lowering

Short running title: Acute glucose and nitrate interaction on insulin and blood pressure

Christopher N Floyd*, Satnam Lidder*, Joanne Hunt, Sami A Omar, Karen McNeill, Andrew J Webb

**The first two authors contributed equally to this article*

The authors confirm that the Principle Investigator for this paper is Dr AJ Webb and that he had direct clinical responsibility for patients.

Affiliations of all authors:

1. King's College London British Heart Foundation Centre, School of Cardiovascular Medicine and Sciences, Department of Clinical Pharmacology, London, UK
2. Biomedical Research Centre, Clinical Research Facility, Guy's and St Thomas' NHS Foundation Trust, London, UK

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Address for correspondence: Dr. Andrew J Webb: andrew.1.webb@kcl.ac.uk, +442071887188 ext 84700 (Senior Lecturer in Cardiovascular Clinical Pharmacology, King's College London, Department of Clinical Pharmacology, St. Thomas' Hospital London SE1 7EH)

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ABSTRACT

Introduction: Dietary inorganic nitrate (NO_3^-) lowers peripheral blood pressure (BP) in healthy volunteers, but lacks such effect in individuals with, or at risk of, type two diabetes mellitus. Whilst this is commonly assumed to be a consequence of chronic hyperglycaemia/hyperinsulinaemia, we hypothesised that acute physiological elevations in plasma [glucose]/[insulin] blunt the haemodynamic responses to NO_3^-; a pertinent question for carbohydrate-rich Western diets.

Methods: We conducted an acute, randomised, placebo-controlled, double-blind, crossover study on the haemodynamic and metabolic effects of potassium nitrate (8 or 24 mmol KNO_3) versus potassium chloride (KCl; placebo) administered 1 h prior to an oral glucose tolerance test in 33 healthy volunteers.

Results: Compared to placebo, there were no significant differences in systolic or diastolic BP (*P*=0.27 and *P*=0.30 on ANOVA, respectively) with KNO_3, nor in pulse wave velocity or central systolic BP (*P*=0.99 and *P*=0.54 on ANOVA, respectively). Whilst there were significant elevations from baseline for plasma [glucose] and [C-peptide], no differences between interventions were observed. A significant increase in plasma [insulin] was observed with KNO_3 versus KCl (*n*=33; *P*=0.014 on ANOVA) with the effect driven by the high-dose cohort (24mmol, *n*=13; *P*<0.001 on ANOVA; at T=0.75 h mean difference 210.4 pmol/L (95% CI 28.5 to 392.3), *P*=0.012).

Conclusions: In healthy adults, acute physiological elevations of plasma [glucose] and [insulin] result in a lack of BP-lowering with dietary nitrate. The increase in plasma [insulin] without a corresponding change in [C-peptide] or [glucose] suggests that high-dose NO_3^- decreases insulin clearance; a likely mechanism is via NO-dependent inhibition of insulin-degrading enzyme.

What is already known about this subject:

- Inorganic nitrate lowers blood pressure and pulse wave velocity in healthy individuals.
- These effects are absent in those with, or at risk of, type two diabetes mellitus.
- This is assumed to be a consequence of chronic hyperglycaemia/hyperinsulinaemia.

What this study adds:

- Acute physiological elevations of plasma [glucose] and [insulin] result in a lack of BP-lowering with dietary nitrate.
- High-dose inorganic nitrate reduced insulin clearance, probably via NO-dependent inhibition of insulin-degrading enzyme.

INTRODUCTION

The role of dietary inorganic nitrate (NO_3^-) as an alternative source of nitric oxide (NO) via the enterosalivary nitrate-nitrite-NO pathway is recognised as a physiological mediator of blood pressure (BP), endothelial function and platelet aggregation (1-3). In both healthy individuals and those with chronic cardiovascular conditions, NO_3^- supplementation has been shown to increase exercise capacity (4-8). This beneficial effect is thought to arise from the action of NO on skeletal muscle where it modulates excitation-contraction coupling, mitochondrial respiration, autoregulation of blood flow, and glucose homeostasis (9). However, individuals with, or at risk of, type two diabetes mellitus (T2DM) fail to exhibit a reduction in peripheral BP or pulse wave velocity (PWV) in response to NO_3^- supplementation (10-12). There are a number of mechanisms that might contribute to this lack of effect including dysfunctional NO synthesis, increased NO scavenging and altered redox balance (13). To what extent this is a consequence of acute or chronic hyperglycaemia/hyperinsulinaemia is unknown.

Carbohydrate (CHO) ingestion also has established benefits on exercise performance (14). However, the effects of concurrent NO_3^- and CHO intake on cardiovascular haemodynamics and glucose homeostasis (both important determinants of exercise capacity) have not been studied in detail.

Type two diabetes mellitus is a condition associated with excess CHO intake (15), although the aetiology of the condition is more complex (16). It has been observed that in both healthy individuals and those with T2DM, plasma [nitrate] and [nitrite] fall acutely in response to an oral glucose tolerance test (OGTT) (17, 18), likely reflecting an increase in NO consumption. However, there is a lack of agreement with regards to basal plasma [nitrate] and [nitrite], with conflicting results reported (17, 19). This lack of agreement regarding basal concentrations may be the result of the use of the Griess reactions which measures combined plasma [nitrate/nitrite] and is not sufficiently sensitive to measure physiological plasma [nitrite].

Systemic inhibition of NO synthesis results in a deterioration in glucose tolerance in non-diabetic individuals in response to an OGTT, accompanied by an elevation in BP (20, 21). However, the effects of NO_3^- supplementation on glucose homeostasis are less clear. In healthy individuals, NO_3^- supplementation appears to result in lower plasma [glucose] post-exercise (22, 23), but without changing homeostatic responses to glucose at rest (24-26). In those with, or at risk of T2DM, studies are heterogeneous in their design and report either an improvement or null effect of nitrate on insulin sensitivity following glucose administration (26-29).

In studies investigating the haemodynamic effects of NO_3^- supplementation in individuals with T2DM there is greater consistency, as neither peripheral BP nor exercise tolerance are improved (10-12); although we have demonstrated a lowering of central SBP with 6 months'

dietary nitrate [24], with a decrease in left ventricular volumes (30). This lack of effect in those with impaired glucose tolerance may be due to impaired insulin-mediated vasodilation (31-33), but whether this is a consequence of acute or chronic hyperglycaemia/hyperinsulinaemia has not been established.

The purpose of this study was to determine whether there is an interaction between NO_3^- and glucose on BP and glucose homeostasis in healthy individuals. We hypothesised that acute physiological elevations in plasma [glucose] and [insulin] would blunt the haemodynamic responses to NO_3^-. This study was therefore conducted to address two complimentary questions; (i) is the BP response to NO_3^- supplementation affected by concurrent glucose ingestion? and (ii) is the metabolic response to an OGTT affected by NO_3^- supplementation?

METHODS

Participants

Participants were healthy, normotensive volunteers aged 18 to 45 years. All participants had a body mass index (BMI) 18 to 35 kg/m^2, no current or recent illness and were not taking systemic medication other than the oral contraceptive pill. A negative urine dipstick result for nitrite was required on the morning of each visit.

The study was approved by the South East London Research Ethics Committee (10/H0802/52). Written informed consent was obtained from all participants.

Study protocol

We conducted an acute, randomised, placebo-controlled, double-blind, crossover study of potassium nitrate (KNO_3) versus potassium chloride (KCl; placebo) (both Martindale Pharma) followed by an OGTT performed 1 h later. The study consisted of two independent cohorts based on the dose of KNO_3/KCl ingested: (i) a 'high-dose' cohort received 24 mmol, and (ii) a 'low-dose' cohort received 8 mmol. Each study visit lasted 4 h and was separated by a minimum of 7 days. The order of allocation to KNO_3 or KCl for each participant was performed using a random, computer-generated order produced by an independent researcher.

Participants were asked to fast overnight (>12 hours) and to avoid nitrate-rich foods, strenuous exercise, smoking and the use of mouthwash for 24 h before the study. To minimise any dietary confounders, participants were asked to consume the same meals for the day prior to each arm of the study.

On the day of the study and following an hour's equilibration period during which baseline measurements were taken (see below), participants were randomised to receive KNO_3 versus KCl at Time -1 h. Both were administered with low-nitrate water (300 ml; Buxton Water) and an antacid (10-20 mL repeated if necessary; Gaviscon, GSK) to minimise gastrointestinal discomfort from the potassium supplement. A standard OGTT (75g glucose as Lucozade, GSK) was performed at Time 0 h. A schematic of the events is presented in **Figure 1**.

Measurements

Blood pressure and heart rate (HR) readings were taken in triplicate every 15 min using an oscillometric BP monitor (Omron 705CP, UK) according to guidelines. The average of the second and third readings were used for analysis to diminish the impact of any alerting response. Central systolic blood pressure (cSBP), pulse wave velocity (PWV) and

augmentation index (AIx) were measured (Time -1 h and Time 2 h) using Finometer (Finopress Medical Systems, Netherlands) and Vicorder (SMT Medical, Germany) devices according to manufacturers' instructions.

Blood samples were taken from a cannula in the antecubital vein at time intervals shown in **Figure 1**. An initial 2 mL of blood was discarded, before 6 mL of blood was collected and transferred into chilled lithium heparin blood collection tubes. Blood samples were immediately centrifuged at 4500 rpm for 5 min at 4°C (Hettich Mikro 220R, Germany). Plasma was stored in duplicate in 1 mL aliquots at -80°C prior to analysis.

Plasma concentrations of glucose, insulin and C-peptide were measured using standardised clinical assays (Viapath, St Thomas' Hospital). Nitrate and nitrite concentrations in urine and plasma were measured by ozone-based chemiluminescence as previously described (1, 34). The coefficient of variation was <10% for both nitrite and nitrate quantification. Exhaled NO (eNO) was measured using a NObreath monitor (Bedfont Scientific, UK), according to the manufacturer's instructions.

Insulin sensitivity during each study arm was calculated via the Matsuda index, where a higher value represents greater insulin sensitivity (35).

Data and Statistical Analyses

All data were analysed with GraphPad Prism software (v7.03), and are expressed as mean±SEM unless otherwise stated. Repeated-measures two-way ANOVA with Sidak's post-test was used for comparison of the data between the two interventions. Repeated-measures one-way ANOVA with Dunn's post-test was used for comparison with baseline. Correlation was assessed using Pearson's correlation. Where data were non-parametric, appropriate equivalent statistical tests were used. $P<0.05$ was considered statistically significant.

Nomenclature of Targets and Ligands

Key protein targets and ligands in this article are hyperlinked to corresponding entries in http://www.guidetopharmacology.org, the common portal for data from the IUPHAR/BPS Guide to PHARMACOLOGY (36), and are permanently archived in the Concise Guide to PHARMACOLOGY 2017/18 (37, 38).

RESULTS

Thirty-three participants completed both visits of the study, of which 13 received high-dose (24 mmol) and 20 received low-dose (8 mmol) KNO_3/KCl. Mild gastrointestinal discomfort lasting <15 min was reported by 42.4% (14/33) of participants following dosing, with no significant difference between dose or intervention. Demographic data for participants are summarised in **Table 1**.

Nitrate metabolism

The metabolism of ingested NO_3^- was confirmed by a significant time-dependent increase in plasma and urinary [nitrate] and [nitrite] and eNO following KNO_3 compared to KCl (**Figure 2**). In the high-dose cohort, plasma [nitrite] was significantly increased for KNO_3 versus KCl at both the time of the OGTT (Time 0 h, 399±104 versus 81±16 nmol/L; P<0.01) and at peak plasma [glucose] (Time 1 h, 721±95 versus 60±13 nmol/L; P<0.001); see Figure 2.

Haemodynamic response

Haemodynamic parameters pre-intervention (Time -2 h to -1 h) were similar for KNO_3 versus KCl interventions (Table 2). There were no significant differences in BP or HR for KNO_3 versus KCl throughout the study (Time -2 h to +2 h; SBP P=0.27; DBP P=0.30; PP P=0.74; HR P=0.12) (**Figure 3**). Similarly, there were no significant differences in PWV, cSBP or AIx pre- and post-OGTT (Time -1 h versus +2 h; all P>0.05; **Figure 4**).

Subgroup analyses of high-dose (24 mmol) and low-dose (8 mmol) cohorts also revealed similar haemodynamic parameters at baseline (*data not shown*). However, in contrast to the main analysis, significant differences in HR were observed between interventions within each cohort. In the high-dose cohort, HR was reduced with KNO_3 versus KCl (mean 67.29±0.55 versus 68.36±0.56 mmHg; P=0.01) (**Figure 5**).

For the low-dose cohort, the opposite effect was observed with a significantly higher HR with KNO_3 versus KCl (mean 65.89±0.62 versus 64.40±0.40 mmHg; P<0.01) (**Figure 6**). There were no significance differences in ΔPWV, ΔcSBP or ΔAIx for the interventions within either cohort (all P>0.05; *data not shown*).

Glucose Homeostasis

There were no significant differences between interventions for plasma [glucose] (P=0.58) or [C-peptide] (P=0.84), but significantly higher plasma [insulin] was observed for KNO_3 versus KCl (P=0.01) (**Figure 7**). Insulin sensitivity, as represented by the Matsuda index, was not significantly different for KNO_3 versus KCl (mean 4.26±0.48 versus 4.37±0.48; P=0.59).

Subgroup analyses of high-dose (24 mmol) and low-dose (8 mmol) cohorts revealed that the significant difference in plasma [insulin] was driven by the high-dose cohort (P<0.001 on ANOVA; at t=0.75 h mean difference 210.4 pmol/L (95% CI 28.5 to 392.3), P=0.012) (**Figure 8**). There was no significant difference in the Matsuda index for KNO_3 versus KCl with either 24 mmol (mean 5.42±0.86 versus 5.60±0.75; P=0.77) or 8 mmol (mean 3.50±0.51 versus 3.57±0.57; P=0.31).

high- or low-dose. No significant correlation was observed between ΔSBP/DBP and Δ[insulin]/[glucose] at timepoint 1 h and 2 h (*data not shown*).

DISCUSSION

This acute, crossover study investigated the effects of concurrent inorganic nitrate and glucose ingestion on blood pressure and glucose homeostasis in healthy individuals. The principal findings of this study were as follows: (i) physiological elevation of plasma [glucose] and [insulin] resulted in a lack of BP-lowering with inorganic nitrate, despite elevated plasma [nitrite], and (ii) the increase in plasma [insulin] without a corresponding change in [C-peptide] or [glucose] suggests that high-dose NO_3^- decreases insulin clearance.

A dose-response relationship has previously been demonstrated between NO_3^- ingestion (as beetroot juice or nitrate capsules) and peripheral BP reduction (1, 39, 40). Doses as low as 5.1 mmol have been shown to cause significant SBP reductions (39, 40), with higher doses (up to 22 mmol as beetroot juice, and 24 mmol as potassium nitrate, as used here) resulting in SBP/DBP reductions of 10.4/8.0 mmHg, and 9.4/6.0 mmHg, respectively (1, 39). Reductions in arterial stiffness have also occurred with both acute and chronic dosing (41, 42).Whilst several studies in healthy individuals failed to show a peripheral BP decrease with NO_3^- supplementation, this is the first study with a neutral effect for ≥12 mmol/d NO_3^-. There is a strong correlation between PWV and PP, and so the lack of change in PWV is consistent with the peripheral measurements (43). Based on our previous work in those with, or at risk of, T2DM we would have expected to observe a reduction in cSBP following NO_3^- ingestion through a selective dilatory effect on medium-sized conduit vessels (12, 44). However, nitrate had no effect on cSBP with an acute glucose load.

The lack of effect on both peripheral and central haemodynamics suggest that normal, physiological responses to glucose are sufficient to prevent the BP-lowering effects of NO_3^- supplementation. The observed differences in HR between interventions were small and, as the magnitude of change was opposite to that expected for the two doses, their biological validity is uncertain. The lack of BP-lowering is consistent with other studies that have demonstrated inhibition of NO-dependent flow mediated dilatation of conduit and small resistance arteries following acute physiological elevations in plasma [glucose] and [insulin] (45-47). Furthermore, in a study of overweight men Joris *et al* reported that co-ingestion of beetroot juice (approximately 8 mmol NO_3^-) counteracted the decrease in FMD associated with the intake of a mixed meal, without differences in PWV or peripheral BP between groups (48). Whilst our study was not designed to disentangle the relative contributions from glucose and insulin, we hypothesise that lack of effect was modulated by elevated plasma glucose given that insulin-mediated vasodilatation within skeletal muscle is NO-dependent (31). The elevated exhaled NO demonstrated an increase in systemic NO availability following nitrate supplementation, and that the lack of BP-lowering was therefore unlikely due to interruption of the nitrate-nitrite-NO pathway.

In agreement with previous studies, NO_3^- supplementation did not lower resting plasma [glucose] or improve insulin sensitivity as assessed by the Matsuda index (24-26). However, in the high-dose cohort we did observe an increase in plasma [insulin] without a

corresponding increase in [C-peptide], thus suggesting decreased insulin clearance. A change in plasma [insulin] without a corresponding change in [glucose] is consistent with the multifaceted mechanisms responsible for glucose homeostasis (49, 50). Dietary nitrate has been demonstrated to enhance glucose uptake in skeletal muscle independent of insulin via translocation of glucose transporter 4 (GLUT4) (51). It is therefore possible that high-dose dietary nitrate facilitated glucose uptake via insulin-independent mechanisms, thus reducing insulin clearance at the same site. Our finding is also consistent with a previous study which showed that systemic inhibition of nitric oxide synthase (NOS) with N^G-monomethyl-L-arginine (L-NMMA) in healthy volunteers increased insulin clearance without an effect on peripheral insulin sensitivity (21). The mechanism of increased insulin clearance following NOS inhibition was attributed to activation of the specific protease hepatic insulin-degrading enzyme (IDE), which is largely responsible for whole-body insulin clearance (52). IDE is dose-dependently inhibited by NO *in vitro* and provides a plausible mechanism for our observation of decreased insulin clearance. Furthermore, as NO mediates glucose uptake by skeletal muscle *in vitro* through insulin-independent mechanisms, decreased insulin clearance may also occur peripherally following NO_3^- (53, 54).

This study differs from those previously conducted with regards to the nitrate dose, glucose load and relative timing of ingestion. Our use of high-dose nitrate, a full OGTT and coordination of peak plasma [glucose] with elevated [nitrite], optimised any interaction and may explain why other studies did not observe changes in plasma [insulin]. Furthermore, we opted to deliver NO_3^- via capsules rather than beetroot juice, to avoid additional uncontrolled CHO ingestion (37.5 g sugar per 500 mL; James White Drinks Ltd). It is a limitation of this study that although Lucozade is routinely used to administer OGTTs in clinical practice, we cannot exclude confounders mediated by other ingredients. However, the ingredients of Lucozade are similar to those in many other sports drinks and so the potential impact on exercise may represent a 'class effect'. Thus, the lack of an effect of concomitant administration of glucose with nitrate on BP suggests the possibility that glucose might also negate the beneficial effects of nitrate on exercise performance.

In summary, our findings describe decreased insulin clearance as a previously unidentified consequence of NO_3^- supplementation and provide further information regarding how diet can acutely modulate blood pressure. Further investigation is required into the potentially antagonistic interaction between glucose and NO_3^-.

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Tables with Legends

Table 1. Demographic data for participants. Data expressed as mean±SD. [BMI: body mass index; SBP: systolic blood pressure; DBP: diastolic blood pressure; HR: heart rate]

	All participants	Subgroups	
		24 mmol	8 mmol
Number of participants (*n*)	33	13	20
Gender (*n* male)	15	6	9
Age (years)	27.1±6.5	27.8±7.2	26.5±6.0
Weight (kg)	70.1±13.9	69.4±9.9	70.5±16.1
Height (m)	1.7±0.1	1.7±0.1	1.7±0.1
BMI (kg/m^2)	23.3±2.9	23.7±3.2	23.1±2.8
SBP (mmHg)	113.4±10.1	115.0±11.1	112.4±9.6
DBP (mmHg)	71.2±5.8	72.0±5.3	70.7±6.2
HR (bpm)	67.9±9.4	69.4±10.3	67.1±8.0
Fasting glucose (mmol/L)	4.7±0.6	4.7±0.4	4.7±0.7
Fasting insulin (pmol/L)	44.1±22.2	39.9±16.2	46.9±25.3

Table 2. Baseline haemodynamic parameters. Time -2 h to -1 h. Data expressed as mean±SD.
[SBP: systolic blood pressure; DBP: diastolic blood pressure; PP: pulse pressure; HR: heart rate]

	KNO_3	KCl
SBP (mmHg)	113.1±10.0	113.2±10.9
DBP (mmHg)	70.8±6.7	71.1±6.4
PP (mmHg)	42.3±7.3	41.2±7.6
HR (bpm)	66.0±7.2	65.7±6.1

Figure Legends

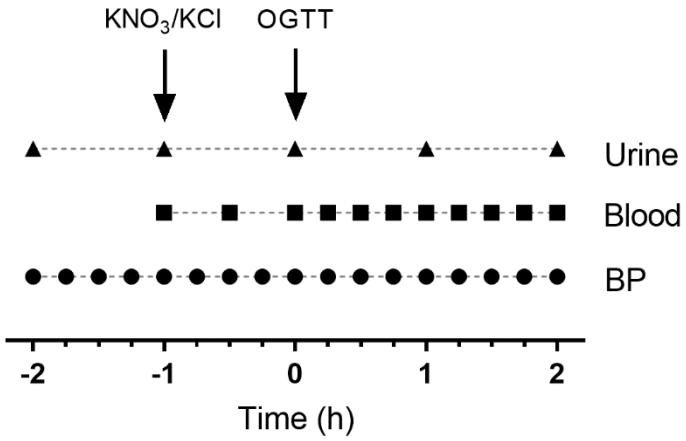

Figure 1: Schematic of events. After acclimatisation (-2 h to -1 h), participants received KNO_3 or KCl tablets (Time -1 h) followed by an oral glucose tolerance test (OGTT; 75 mg glucose) at Time 0 h. Blood pressure (BP) measurement, blood tests and urine collection occurred as indicated.

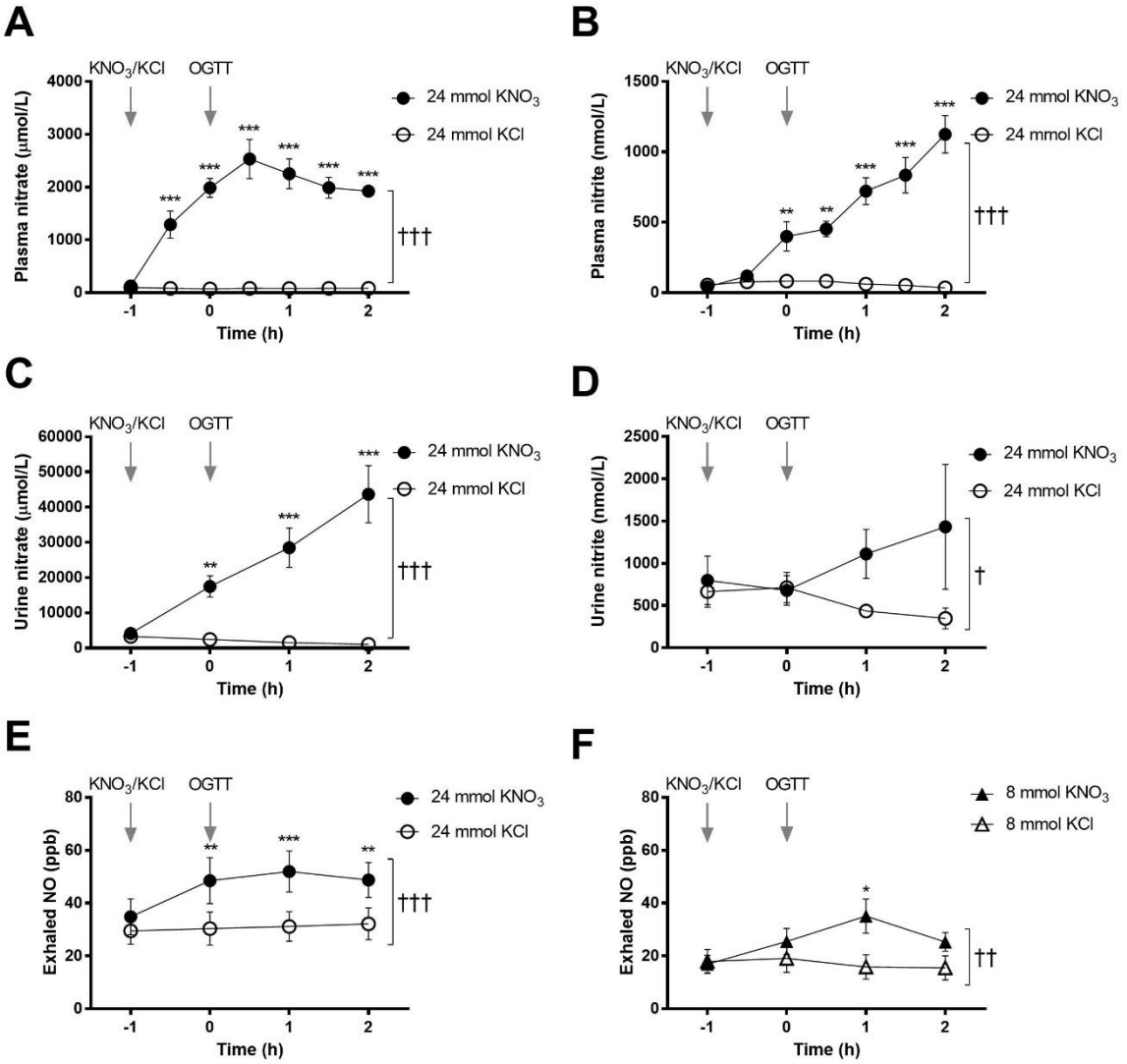

Figure 2: Effect of 24 mmol KNO_3 versus KCl (*n*=13) on: (A) plasma [nitrate], (B) plasma [nitrite], (C) urine [nitrate], (D) urine [nitrite], and (E) exhaled nitric oxide (NO). Effect of 8 mmol KNO_3 versus KCl (*n*=20) on (F) exhaled NO. Data expressed as mean±SEM. Significance shown as: †*P*<0.05, ††*P*<0.01, †††*P*<0.001 on ANOVA, followed by **P*<0.05, ***P*<0.01, ****P*<0.001, Sidak's post-test of KNO_3 versus KCl.
[OGTT: oral glucose tolerance test]

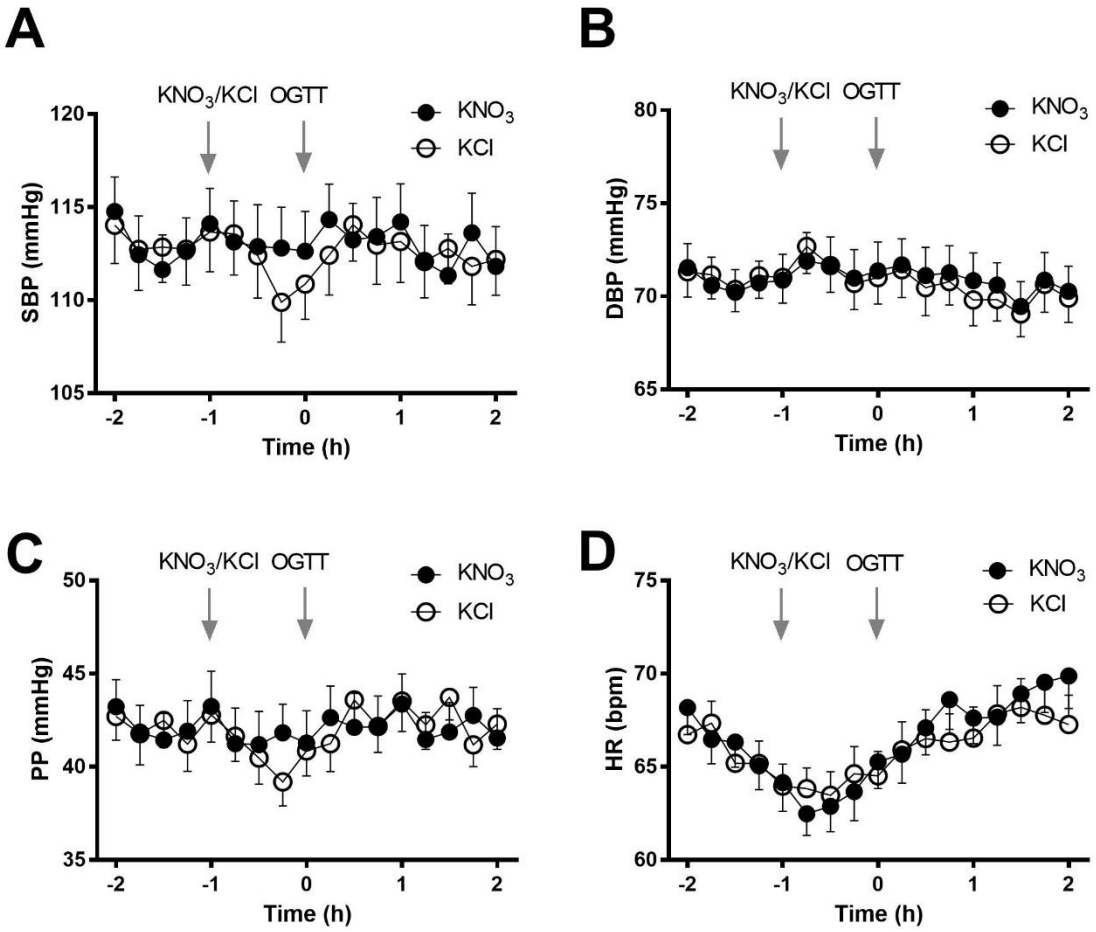

Figure 3: Effect of KNO_3 versus KCl (*n*=33) on (A) systolic blood pressure (SBP), (B) diastolic blood pressure (DBP), (C) pulse pressure (PP), and (D) heart rate (HR). Data expressed as mean±SEM. [OGTT: oral glucose tolerance test]

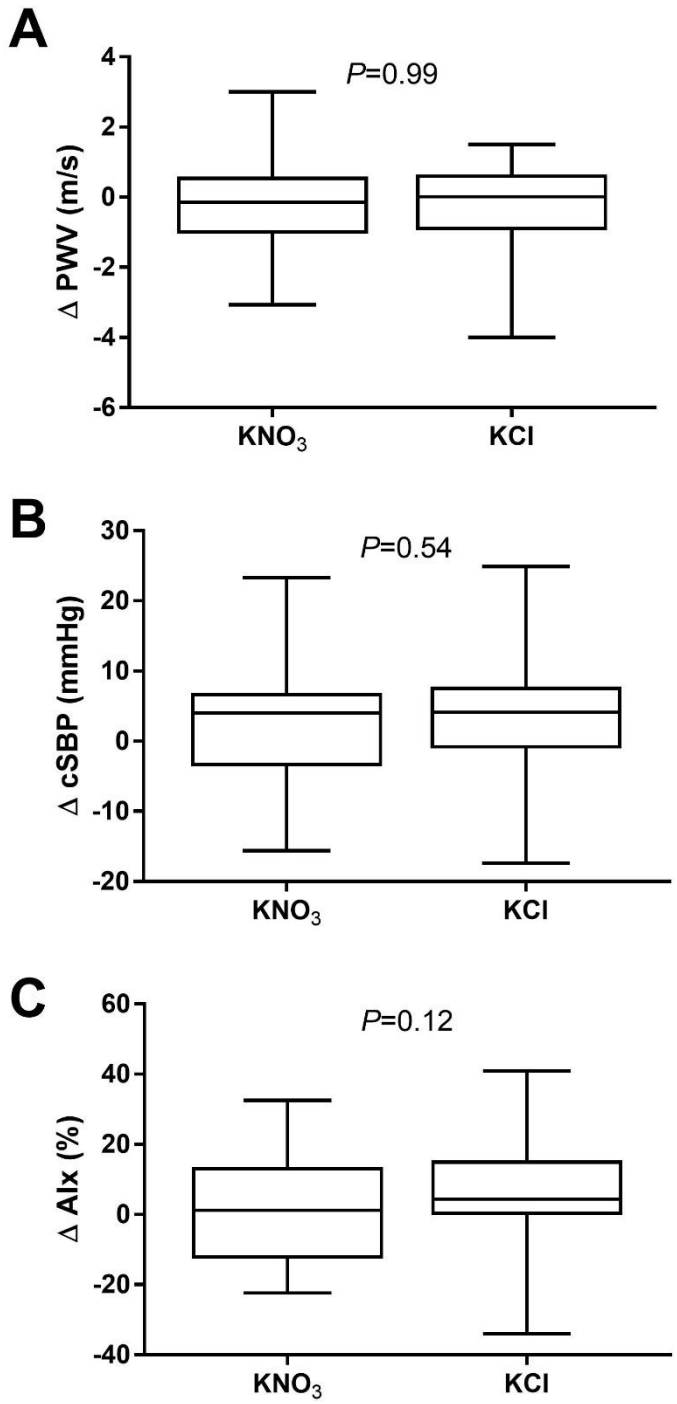

Figure 4. Effect of KNO_3 versus KCl (*n*=29) on (A) pulse wave velocity (PWV), (B) central systolic blood pressure (cSBP), and (C) augmentation index (AIx). Plots show range, median and 25 to 75th percentiles.

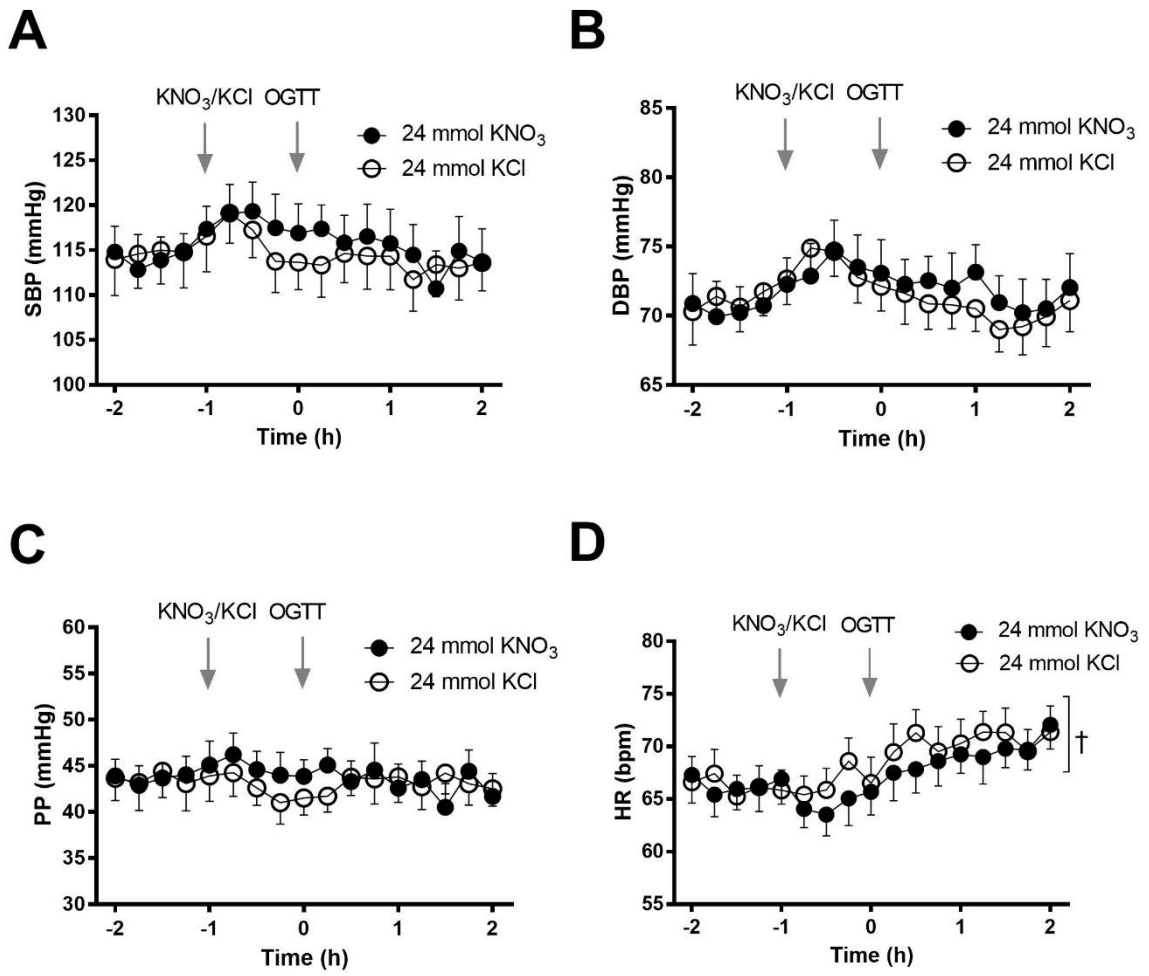

Figure 5: Effect of 24 mmol KNO_3 versus KCl (*n*=13) on (A) systolic blood pressure (SBP), (B) diastolic blood pressure (DBP), (C) pulse pressure (PP), and (D) heart rate (HR). Data expressed as mean±SEM. Significance shown as: †*P*<0.05 on ANOVA.
[OGTT: oral glucose tolerance test]

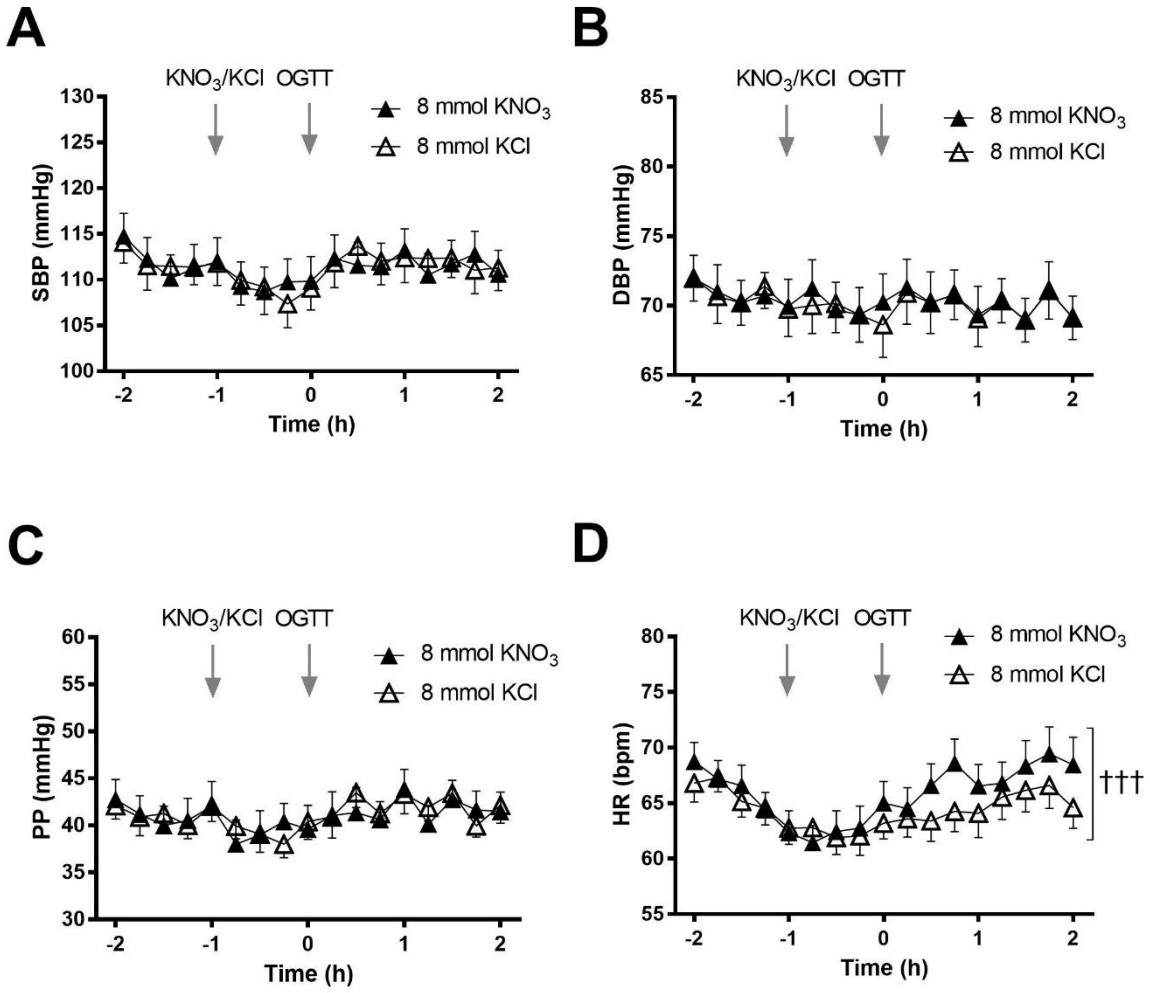

Figure 6: Effect of 8 mmol KNO_3 versus KCl (*n*=20) on (A) systolic blood pressure (SBP), (B) diastolic blood pressure (DBP), (C) pulse pressure (PP), and (D) heart rate (HR). Data expressed as mean±SEM. Significance shown as: †††*P*<0.001 on ANOVA.
[OGTT: oral glucose tolerance test]

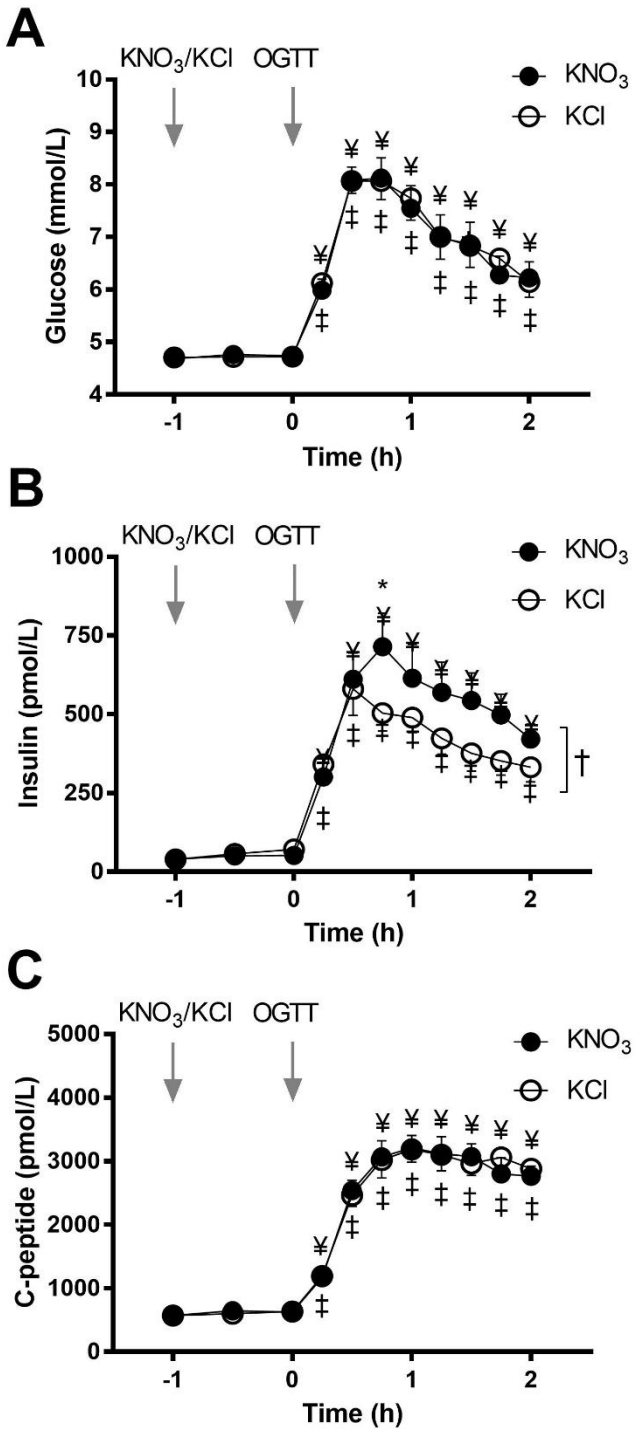

Figure 7: Effect of KNO_3 versus KCl (*n*=33) on (A) plasma [glucose], (B) plasma [insulin], and (C) plasma [C-peptide]. Data expressed as mean±SEM. Significance shown as: †*P*<0.05 on ANOVA, followed by **P*<0.05, Sidak's post-test of KNO_3 versus KCl. ¥ *P*<0.01 on ANOVA for KNO_3 versus baseline (-1 h), with Dunn's post-test. ‡ *P*<0.01 on ANOVA for KCl versus baseline, with Dunn's post-test.
[OGTT: oral glucose tolerance test]

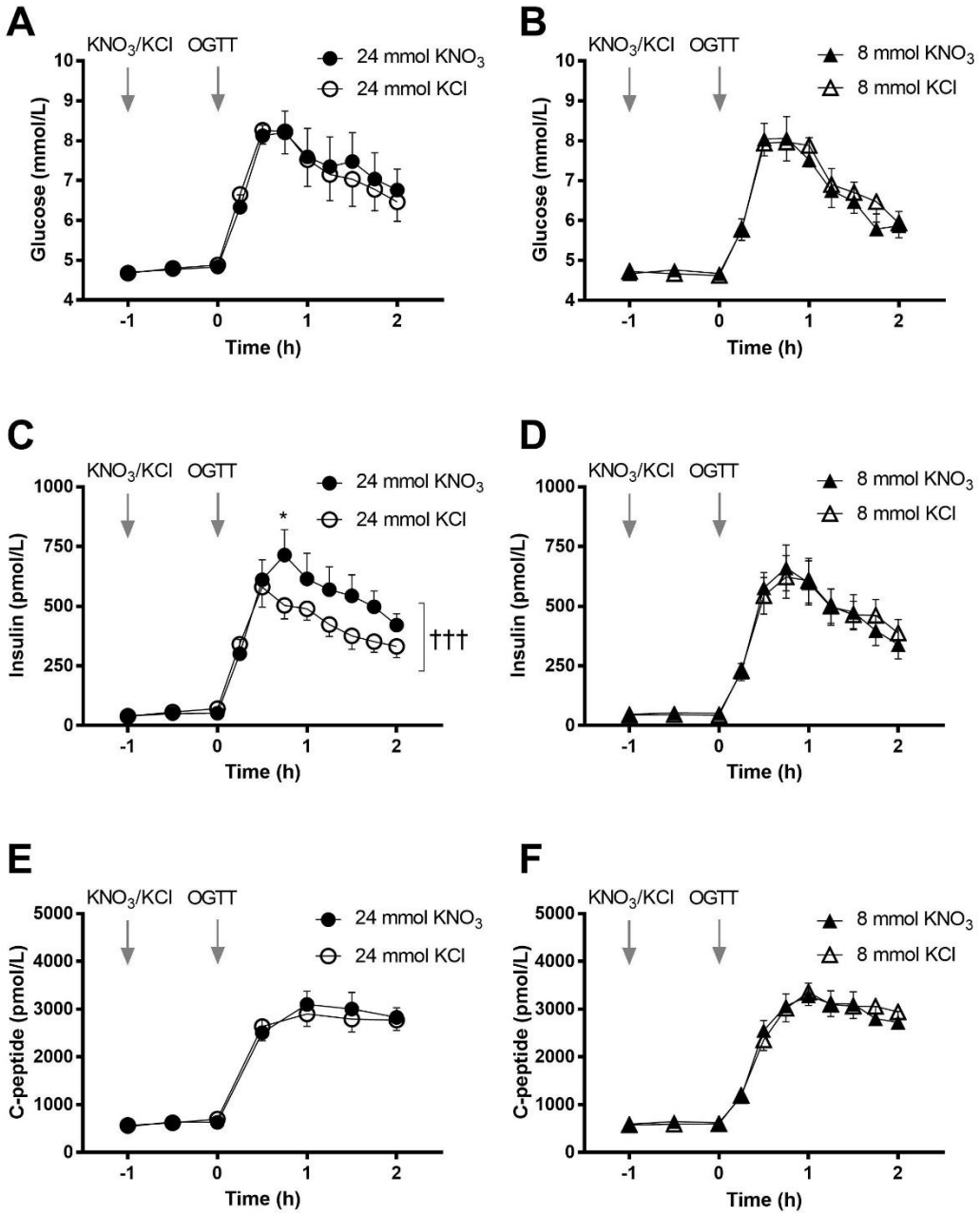

Figure 8: Effect of KNO_3 versus KCl (24 mmol, *n*=13; A, C and E; 8 mmol, *n*=20; B, D, F) on: (A) and (B) plasma [glucose], (C) and (D) plasma [insulin], and (E) and (F) plasma [C-peptide]. Data expressed as mean±SEM. Significance shown as: †††*P*<0.001 on ANOVA, followed by **P*<0.05, Sidak's post-test of KNO_3 versus KCl.
[OGTT: oral glucose tolerance test]

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