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Effect of Trehalose on Arterial Stiffness and Oxidative Stress in Human Vascular Endothelium

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ABSTRACT:

EFFECT OF TREHALOSE ON THE HUMAN ENDOTHELIUM

BACKGROUND: Cardiovascular diseases (CVD) are the most common cause of death worldwide; the majority is associated with the dysfunction of arteries, characterized by impaired endothelial function and arterial stiffness. Oxidative stress is a primary mechanism underlying vascular dysfunction and CVD. Trehalose, a naturally occurring disaccharide, has been shown to have antioxidant effects in different cell and animal models but the effect of trehalose in humans is unknown.
OBJECTIVE: The aim of this study is to evaluate the effect of trehalose on oxidative stress levels in biopsied human endothelial cells and its effect on arterial stiffness in elderly adults.
METHODS: To answer these questions, we developed a novel method to directly measure oxidative stress in endothelial cells harvested from a superficial forearm vein from 7 healthy donors aged 23±2 years. In a subset, whole cell reactive oxygen species (ROS) and mitochondrial superoxide levels were measured in endothelial cells treated with trehalose (2 hour incubation; 100mM). β-stiffness index and carotid artery compliance were evaluated in elderly adults (n=10; aged 65±7) in a 12-week randomized, double-blind, dose response study with oral trehalose supplementation (placebo, 50g or 100g daily).
RESULTS: Trehalose significantly reduced both whole cell ROS and mitochondrial superoxide levels in endothelial cells compared to the control condition (-48%, $p<0.001$ and -26%, $p<0.01$, respectively). Moreover, trehalose supplementation tends to improve β-stiffness index and carotid artery compliance compared to the placebo group.
CONCLUSION: Trehalose can be a promising novel therapeutic strategy in the treatment of CVD. However, the presented results on arterial stiffness are preliminary and more research is needed to further elucidate the effect of trehalose on endothelial dysfunction and arterial stiffness.

WHAT IS KNOWN: Cardiovascular diseases (CVD) are the most common cause of death worldwide. An important factor causing vascular dysfunction resulting in CVD is oxidative stress. Trehalose has been shown to have antioxidant effects in different cell and animal models.

WHAT IS NEW: Trehalose reduces oxidative stress in human endothelial cells and trehalose supplementations tends to improve arterial stiffness in elderly. Therefore it can be a promising novel therapeutic strategy in the treatment of CVD.

KEYWORDS: Trehalose, Endothelium, Oxidative Stress, Arterial Stiffness, Ageing

Introduction

Cardiovascular diseases (CVD) are the most common cause of death worldwide, representing 30% of all global deaths 1. The great majority of CVD deaths are associated with dysfunction and disorders of arteries 2. Although epidemiological studies have discovered many risk factors, such as diabetes, sedentary lifestyle and hypertension, advancing age is the major risk factor for CVD 3. Ageing adversely affects arteries and causes vascular endothelial dysfunction (characterized in part by impaired endothelium-dependent dilation (EDD)) 3-6, which results in reduced compliance and increased arterial stiffness. With age, conduit and resistance arteries develop impaired EDD due to reduced bioavailability of vascular-protective nitric oxide (NO) 7. One of the mechanisms causing this reduction in NO bioavailability is oxidative stress 8.
Oxidative stress can be defined as increased bioactivity of reactive oxygen species (ROS) relative to antioxidant defenses 9. Oxidative stress reduces NO bioavailability via excessive production of superoxide, produced in the mitochondria or by oxidant enzymes such as NADPH oxidase 10. Superoxide can react with NO to form peroxynitrite. Subsequently, peroxynitrite oxidizes tetrahydrobiopterin (BH4) which is an essential cofactor for endothelial nitric oxide synthase (eNOS). The reduction in bioavailability of BH4 also leads to the "uncoupling" of eNOS, which then produces more superoxide and less NO in a vicious cycle that further reduces NO bioavailability (Figure 1) 11, 12. Therefore, normalizing oxidative stress levels in endothelial cells is a promising therapy to improve endothelial dysfunction and large elastic artery stiffening with ageing in humans.
We investigated trehalose, a naturally occurring disaccharide of glucose found in many foods such as mushrooms, honey and baker's yeast. Trehalose is hydrolyzed in the intestinal lumen by trehalase, but a small percentage is absorbed passively into circulation. Trehalose protects proteins from denaturation and acts as a chemical chaperone in vitro 13, 14. In vivo, supplementation with trehalose improves function in animal models of age-related neurological diseases, protects against diet-induced insulin resistance, decreases adipocyte inflammatory signaling and extends longevity 15-17. Although the exact mechanism of action is unknown, trehalose has been reported to induce autophagy, a cellular process of recycling damaged bio-

molecules/organelles that can suppress oxidative stress and inflammation, both in vitro and in vivo 13, 17. Recently, trehalose supplementation in old mice has been shown to restore eNOS and reduce superoxide production to levels observed in young mice 18.
In the present study we tried to illuminate the effects of trehalose on oxidative stress in the endothelial cells from human subjects using a newly developed method. Moreover, we aimed to examine the effect of trehalose on arterial stiffness in a small cohort of elderly. We hypothesize that trehalose can decrease oxidative stress in biopsied human endothelial cells and improve arterial stiffness in elderly adults.

Methods

Arterial Stifness

Study design
We conducted a 12-week randomized, double-blind, dose response study with oral trehalose supplementation. All testing took place at the Clinical and Translational Research Center (CTRC) in the Wardenburg Health Center on the University of Colorado at Boulder campus. The nature, risk and benefits of all study procedures were described to subjects and their written consent was obtained prior to participation in the study. Subjects underwent telephone and CTRC screening with those eligible randomly assigned (using a "RAND()" function in Excel) to 1 of 3 groups: 100 g maltose/350 ml water 1x/d (placebo group), 50 g trehalose + 50 g maltose/350 ml water 1x/d (low-dose group), and 100 g trehalose/350 ml water 1x/d (high-dose group). Maltose was used as a placebo and filler, because it has a similar structure, sweetness and caloric content to trehalose, but does not have effects on physiological function at similar concentrations. Subjects were advised to consume the trehalose drink over the course of the day at their own pace. The investigators involved in the acquisition and analysis of key outcomes were blinded to the trehalose intake status of subjects. CTRC nurses and professional research assistants from our laboratory were informed of the group status of all subjects throughout the study.
All measurements were made under supine, overnight fasted (water only) conditions. Subjects were asked to refrain from non-prescription medications for 48 hours; alcohol, exercise, and prescription medications for 24 hours; and caffeine for 12 hours prior to all study visits as these are factors known to modulate vascular function. Subjects on prescription medications were required to consult their physician and obtain a signed letter of consent prior to participation in the study. Measurements were made at baseline and after 12 weeks of each condition. Figure 2 shows a schematic overview of the study design.

Subjects
Men and women between the ages of 50 and 79 from all ethnic backgrounds were recruited (n=10, placebo n=3, low dose n=2, high dose n=5). Subject characteristics are expressed as mean±SD (Table 1). There are no significant differences in these characteristics between groups. All subjects signed an informed consent. Exclusion criteria were baseline brachial flow-mediated dilation (FMD, a measure of EDD) >7% Δ, body mass index (BMI) >40 kg/m2, unstable weight in the prior 3 months or unwilling to remain weight stable throughout the study, having unstable cardiovascular or metabolic disease or suffering from diabetes. Other exclusion criteria were having past or present alcohol or nicotine dependence or abuse, scoring <23 on the mini-mental state examination, having abnormal blood chemistries for renal and liver function (>1SD outside the normal range), suffering from moderate or severe peripheral artery disease (ankle-brachial index <0.7) or having insufficient health to participate in a VO2 max test. Perimenopausal women were also excluded. All procedures were approved by the University of Colorado Institutional Review Board.

Measurements of arterial stiffness
Two common indices of arterial stiffness were measured: carotid artery compliance, which is the change in cross-sectional area of a vessel per unit of pressure, and β stiffness index, which provides an index of arterial compliance adjusted for distending pressure. Carotid arterial compliance was calculated as described by Armentano et al. [(D1-D0/D0)/(2(P1-P0)) x π x (D0)2] 19 and carotid artery β-stiffness index was calculated using the formula by Harai et al. [ln(P1/P0)]/[(D1/D0)/D0], where P1=carotid systolic blood pressure (BP), P0=carotid diastolic BP, D1=carotid end-systolic diameter, and D0=carotid end diastolic diameter) 20. The common carotid artery diameters and carotid artery BP were sequentially assessed by high-resolution ultrasonography (PowerVision 6000, Toshiba, Inc.) and non-invasive carotid artery applanation tonometry with a pencil-type probe (Noninvasive Hemodynamics Workstation, Cardiovascular Engineering, INC., Norwood, MAs), respectively. AVI images were acquired for subsequent off-line analysis with image analysis software (Vascular Research Tools 5.0, Medical Imaging Applications, LLC).

Data analysis
Statistical analyses were performed with IBM SPSS (version 21). All data are reported as mean±SD. Differences were analyzed by an ANOVA with Bonferroni adjustment and considered to be statistically significant if the P-value was < 0.05.

Oxidative Stress

Current methods to assess oxidative stress in endothelial cells are limited to measuring downstream effected proteins or studying human derived cell lines. In this paper a novel method was used to directly assess oxidative stress in human endothelial cells harvested from an antecubital vein.

Subjects
Healthy men and women between the ages of 20 and 40 were included (n=7). Exclusion criteria were BMI>25 kg/m2 and having unstable cardiovascular or metabolic disease or suffering from diabetes. The average age of the volunteers was 23±2 and the average BMI 21±2. One volunteer donated twice for different experiments. All volunteers signed an informed consent. All procedures were approved by the University of Colorado Institutional Review Board.

Endothelial cell oxidative stress assessment
Up to six sterile J wires (Daig Corp, Minnetonka, Minn) were advanced and retracted one at a time through an 18-gauge catheter placed in an antecubital vein. The obtained cells were isolated, divided in two wells and allowed to adhere to poly-L-lysine–coated slides (Sigma Chemical, St. Louis, Mo).
Cells in one well were treated with trehalose (100 mM for 2 hours), cells in the other well served as a control. Oxidative stress was measured by CellROX or MitoSOX staining for assessment of whole cell ROS production and mitochondrial superoxide production, respectively. Next, the collected cells were fixed with 3.7% formaldehyde. Endothelial cells were stained for Vascular Endothelial-Cadherin (VE-CAD) and nuclei were made visible with DAPI (4',6'-diamidino-2-phenylindole hydrochloride).
For analysis, slides were viewed with a fluorescence microscope

(Eclipse Ni-U, Nikon, Melville, NY), and cell images were captured digitally by a Clara CCD digital camera (Andor Technology, Belfast UK). Endothelial cells were identified by staining for VE-CAD and nuclear integrity. Once endothelial cells with intact nuclei were identified, they were analyzed with Metamorph Software (Universal Imaging Corp, Downingtown, Pa).

Data analysis

Statistical analyses were performed with IBM SPSS (version 21). All data are reported as mean±SD. Changes were analyzed by a two-tailed T-test and considered to be statistically significant if the P-value was < 0.05.

Results

Trehalose reduces ROS levels in human endothelial cells

To investigate whether trehalose ameliorates ROS levels in endothelial cells, we collected live endothelial cells from healthy donors and treated these cells with trehalose. In order to resemble physiological conditions, we did not induce oxidative stress artificially. Whole cell ROS levels in trehalose treated cells, as determined by CellROX staining, decreased 48% compared to control cells (n=5; p<0.001). However, only in subjects with relatively high basal values (subjects 1, 2 and 4), mean intensity was significantly decreased compared to control cells (p<0.01) (Figure 3a). In subjects with low basal values (mean intensity<200) (subjects 3 and 5) trehalose did not reduce ROS levels.

Trehalose reduces mitochondrial superoxide levels in human endothelial cells

To assess in more detail the ability of trehalose to reduce oxidative stress, we performed a similar experiment and measured mitochondrial superoxide production by treating cells with MitoSOX. Mitochondrial superoxide levels decreased 26% in cells treated with trehalose compared to the control cells (n=3; p<0.01). Again, a significant difference between the trehalose and control condition was seen in subjects with elevated basal levels (subjects 6 and 8) (Figure 3b). Mitochondrial superoxide levels in the trehalose treated cells were slightly increased in subject 7 compared to the cells in the control condition.

The effect of trehalose supplementation on arterial stiffness in ageing humans

To determine effects of trehalose on the vasculature in vivo, we conducted two measurements of arterial stiffness in our subjects before and after a 12-week intervention with trehalose. Our placebo group showed an increase in stiffness during our 12-week intervention indicated by an increase in β-stiffness index and a decline in carotid artery compliance (Figure 4). Although differences between groups were not significant (placebo vs. high dose p=0.26), the low dose group showed a slightly lower increase in β-stiffness index, while the high dose showed a tendency towards decreased β-stiffness index (Figure 4c) (placebo 1.61±1.11; low dose 1.04±1.97; high dose -0.02±2.07). Both the low dose and high dose group showed a smaller decrease in carotid artery compliance (increase in local arterial stiffness) compared to the placebo group, although the differences did not reach statistical significance (placebo vs. high dose p=0.11) (Figure 4d) (placebo -0.019±0.016; low dose -0.003±0.009; high dose -0.002±0.011).

Discussion

Oxidative stress plays a major role in the development of endothelial dysfunction, associated with CVD 8. Since trehalose has been attributed with antioxidant effects, we investigated trehalose in its ability to reduce excess of oxidative stress in endothelial cells from human subjects and to improve arterial stiffness in elderly.

The key finding of this study was that trehalose decreased oxidative stress levels in biopsied human endothelial cells with elevated baseline levels. Both whole cell ROS and mitochondrial superoxide levels were reduced in cells treated with trehalose compared to the cells in control (media treated) condition. These data suggest that trehalose has antioxidant effects in endothelial cells biopsied from human subjects. Moreover, increases in large elastic artery stiffness in a 12-week period tended to be blunted following oral trehalose supplementation versus placebo. This finding suggests that trehalose may be able to reverse age-associated increases in arterial stiffness.

These positive effects may be due to the capacity of trehalose to induce autophagy as described in literature 13, 17. In cardiovascular cells, autophagy acts predominantly as a pro-survival pathway, protecting the cells from oxidative stress 21. Autophagy can involve the direct engulfment of cytoplasmic material into the lysosome (microautophagy), thereby reducing cytoplasmic ROS, or the degradation of damaged cell organelles such as mitochondria (also called mitophagy) 22. This can result in reduced mitochondrial superoxide levels. A more classic autophagy enhancer, rapamycin, has shown to have a maximal effect of inducing autophagy 2 hours after addition 23, indicating that trehalose could also have been inducing autophagy during the 2 hour incubation time used in our experiments.

Although trehalose decreases oxidative stress in subjects with elevated basal levels, trehalose does not further reduce oxidative stress in subjects with already low basal levels. The fact that trehalose does not totally deplete ROS levels is important, because normal ROS levels are required for several intracellular signalling pathways 24. Therefore, trehalose seems an antioxidant that can be safely administered to humans. It also indicates that dysfunction must be present in order for trehalose to have an effect, suggesting trehalose can be more effective treating rather than preventing CVD.

Why we measured an increase of ROS following trehalose treatment in subject 7 is unclear. Except for its ability to induce autophagy, trehalose has many other intracellular functions, for instance participation in stabilization of proteins and membrane structures 25. However, the exact mechanisms of all the different pathways trehalose is involved in are unknown. Possibly, one of the pathways can cause the finding seen in this study. It is also possible that compensatory mechanisms are increasing ROS to maintain basal signaling required for normal cellular homeostasis. Yet the small increase in oxidative stress in the endothelial cells found in subject 7 is unlikely to have any physiological relevant effects.

Reducing excess of oxidative stress seems a potential therapy in treating CVD. However, conventional antioxidants such as Vitamin E or Vitamin C have shown to be incapable of reducing endothelial dysfunction by reducing the concentration of reactive oxygen species in the vessel wall 26. A current theory states conventional antioxidants may not be targeting where they are needed most, namely in the mitochondria, or that they do not reach concentrations where they are effective at sequestering ROS signaling [22]. Subsequently, mitochondrial-targeted antioxidants have been developed and show promising results in rat models 27. Stimulating autophagy may be another way to bypass this problem. Since trehalose was also found to reduce mitochondrial superoxide in the present study, it may be a promising treatment for CVD.

A limitation in our study is that the donors of endothelial cells were all young volunteers. Addressing oxidative stress in an ageing population would have been more clinically relevant. Subsequent studies should evaluate basal differences between young and old volunteers and examine the effect of trehalose in a 12-week intervention study on oxidative stress levels. However, the fact that the effect of trehalose reducing ROS and mitochondrial superoxide can even be seen in some young healthy subjects makes it very promising. Trehalose probably has a greater effect in elderly, because oxidative stress is increased and autophagy impaired in arterial ageing 18.

Although measurements of arterial stiffness show a tendency for trehalose to blunt increases in arterial stiffness when compared to the placebo (maltose) condition, differences were not significant. It is likely that the differences in carotid compliance and β-stiffness index would have reached statistical significance with a larger sample size.

In the study on arterial stiffness we used maltose as placebo and filler in the low dose group. Although no effects of maltose on vasculature have been previously described, it can be argued that maltose has adverse effects on arterial stiffness. Both data from carotid artery compliance and β-stiffness index indicate that arterial stiffness increases in subjects taking 100g maltose daily over a 12-week period. However, others have shown a similar trend in their control group during a 12-week intervention, indicating that this trend can be explained by normal ageing 28. Moreover, the increase in arterial stiffness observed in our subjects is less than can be expected when compared to a data from Hansen et al. 29. Therefore, it is more likely the observed increase in arterial stiffness is due to normal ageing rather than an adverse effect of maltose.

In conclusion, trehalose decreases oxidative stress in human endothelial cells with elevated ROS and may blunt increases in arterial stiffness. Therefore, trehalose is a potential drug in treatment of CVD. However, data concerning the effect of trehalose on arterial stiffness in elderly are preliminary and more research is needed to further elucidate its effect.

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Tables

Table 1: Subjects characteristics

	Placebo	Low dose (50g/d)	High dose (100g/d)
Age, years	64 ± 2	65 ± 9	65 ± 9
Men/Women, n	0 / 3	1 / 1	3 / 2
Body mass, kg	57 ± 11	78 ± 11	77 ± 18
BMI, kg/m^2	22 ± 3	28 ± 2	26 ± 2
Waist:hip ratio	0.77 ± 0.06	0.91 ± 0.09	0.88 ± 0.10
VO_2 peak, ml/kg/min	28 ± 5	26 ± 2	28 ± 6
Brachial SBP, mmHg	133 ± 13	124 ± 21	128 ± 14
Brachial DBP, mmHg	75 ± 8	76 ± 10	71 ± 10
Total Cholesterol, mg/dL	217 ± 34	152 ± 1	184 ± 41
HDL, mg/dL	66 ± 12	49 ± 15	51 ± 25
LDL, mg/dL	133 ± 21	88 ± 11	114 ± 22
Triglycerides, mg/dL	90 ± 9	37 ± 42	99 ± 24
Glucose, mg/dL	91 ± 8	89 ± 6	89 ± 4
Insulin, mU/mL	8 ± 1	11 ± 6	10 ± 3

BMI=body mass index, SBP=systolic blood pressure, DBP=diastolic blood pressure, HDL=high-density lipoprotein, LDL=low-density protein

Figures

Figure 1: Proposed scheme of pathways contributing to vascular dysfunction. Ageing endothelial cells (ECs) show increased levels of oxidative stress characterized by increased superoxide production. Superoxide (O2-) reduces nitric oxide (NO) bioavailability by reacting with NO to form peroxynitrite(ONOO-). Moreover, ONOO- oxidizes BH4 causing a reduction in its availability which leads to uncoupling of endothelial nitric oxide synthase (eNOS), causing eNOS to produces less NO. Reduced NO bioavailability leads to vascular dysfunction. Trehalose may lower oxidative stress in ECs and thereby reverse vascular dysfunction.

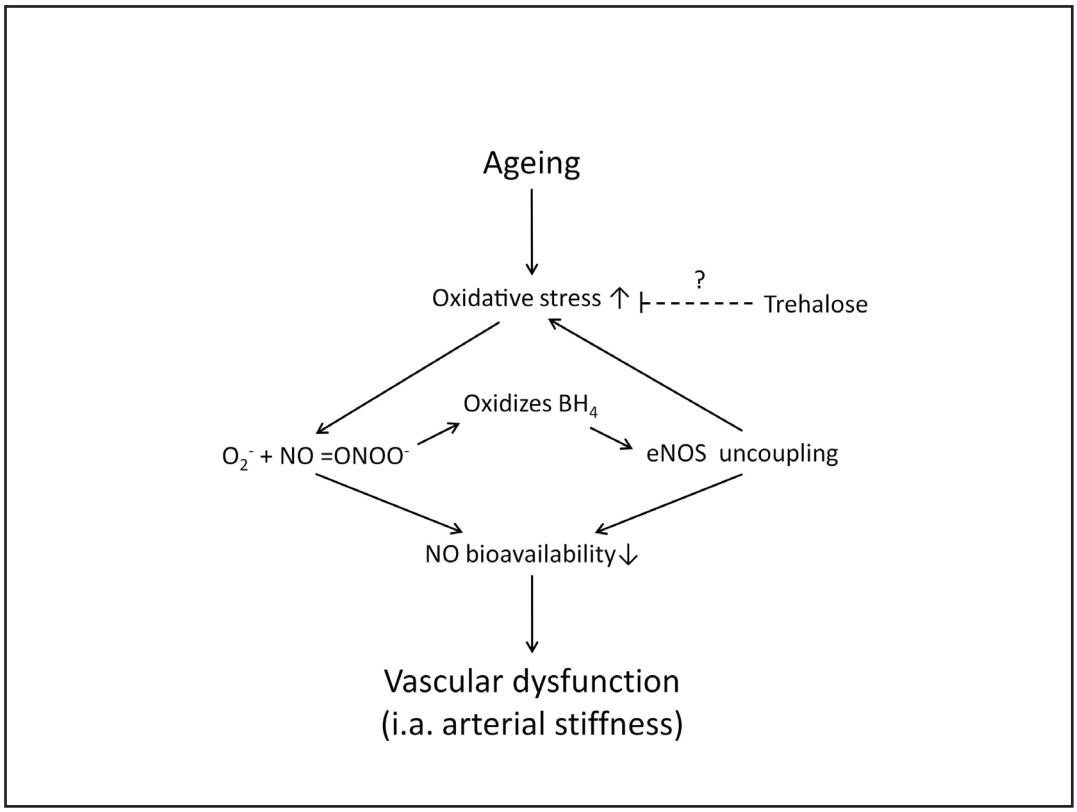

Figure 2: Schematic overview of study design. All subjects underwent screening after obtaining written consent. If inclusion criteria were met, measurements of arterial stiffness were conducted before and after a 12-week intervention of placebo, low dose or high dose study drinks.

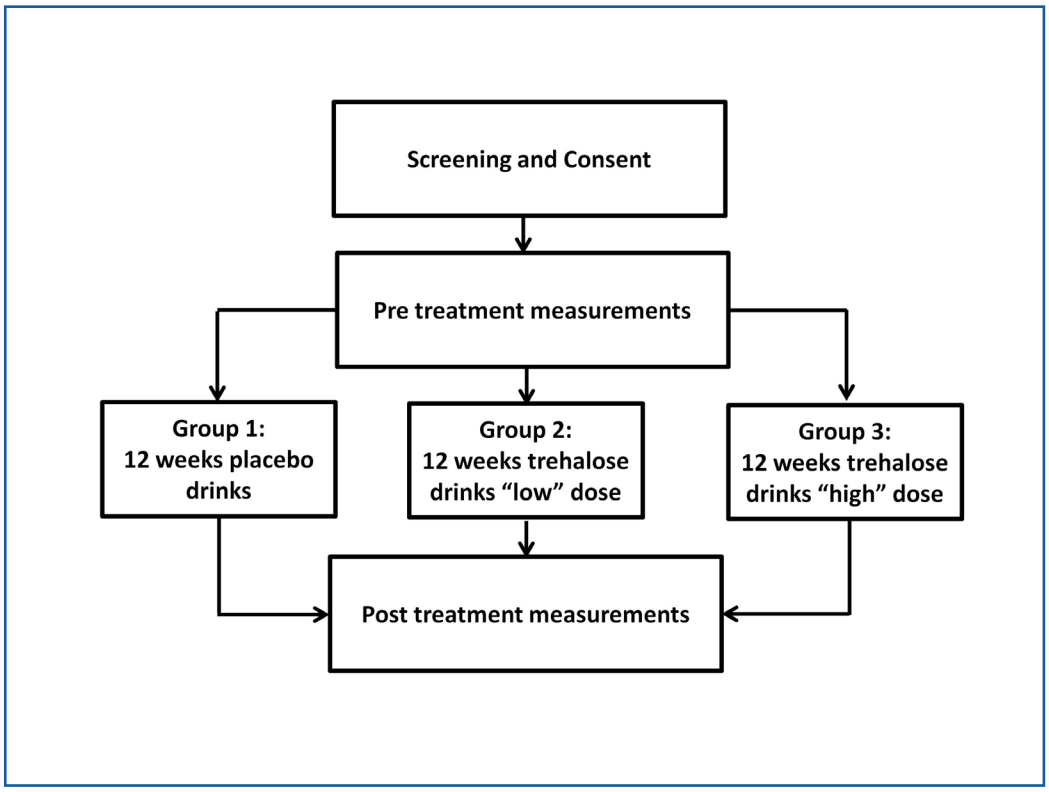

Figure 3: Schematic overview of study design. All subjects underwent screening after obtaining written consent. If inclusion criteria were met, measurements of arterial stiffness were conducted before and after a 12-week intervention of placebo, low dose or high dose study drinks.

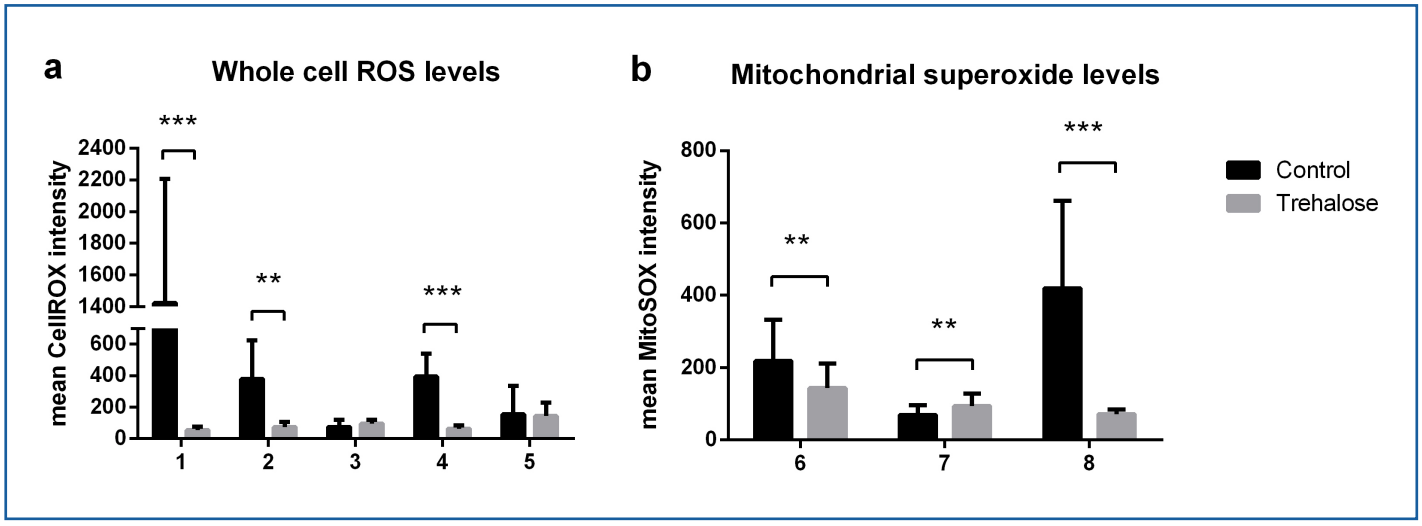

Figure 4: β-stiffness index and carotid artery compliance after a 12-week intervention with placebo (100g maltose), low dose (50g maltose/50 g trehalose) or high dose (100 g trehalose) drinks daily. (a) Individual data on β-stiffness index (b) Individual data on carotid artery compliance (c) Mean differences (post-pre) of β-stiffness index (d) Mean differences (post-pre) of carotid artery compliance.