TITLE: Ischemic preconditioning prevents impact of prolonged sitting on glucose tolerance and markers of cardiovascular health, but not cerebrovascular responses

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Running title: Ischemic preconditioning effect during prolonged sitting.

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Abstract

Prolonged, uninterrupted sitting is demonstrated to acutely impair glucose homeostasis, but also leads detrimental cardiovascular health effects. We examined whether ischaemic preconditioning (IPC) prevents the impact of prolonged sitting-induced glucose intolerance, and measured related influencing factors such as (para)sympathetic nerve activity (assessed by heart rate variability (HRV)) and blood pressure during 2h prolonged sitting. In this randomized, controlled cross-over study, 15 healthy participants (80% men) with a mean age of 21±1 years (means±SD) and body mass index of 25.0±2.4 kg m^2 performed IPC (IPC; 4×5-min 220-mmHg unilateral occlusion at the thigh muscle) or a sham intervention (Sham; 4×5-min 20-mmHg), followed by 2h sitting. After IPC or Sham intervention, fingertip blood glucose was measured before and after 30, 60, 90, and 120 min of 75 g of glucose ingestions. Blood glucose responses during an oral glucose tolerance test was significantly attenuated, resulting in a lower area under the curve when sitting was preceded by a bout of IPC than Sham (P<0.05). IPC increased high-frequency oscillations and decreased the ratio of low-frequency to high-frequency oscillations at 120 min in HRV (P<0.05). Moreover, a lower blood pressure was observed with IPC compared to Sham (P<0.05). Prolonged sitting or IPC did not affect cerebrovascular responses (P>0.05). Collectively, these results indicate that the application of IPC prior to prolonged, uninterrupted sitting bout, was associated with a better glucose tolerance and prevented impairment in (para)sympathetic nerve activity and blood pressure in healthy young men and women.

Key words: *cardiovascular risk, heart rate variability, metabolic health, sedentary behavior, sympathetic nerve activity*

Introduction

Accumulating evidence indicates that increased amounts of sedentary behavior elevates risk for all-cause mortality, metabolic disorders, and cardiovascular disease (1). To better understand this relation, studies have explored the acute, short-term effect of uninterrupted sitting on outcomes related to metabolic and cardio-/cerebrovascular health. For example, prolonged sitting has been associated with a greater area under the curve post-oral glucose tolerance test or post-prandial glucose levels, indicative of impaired glucose tolerance (7). Prolonged sitting is also associated with elevated blood pressure (BP) (5), impaired endothelial function (25, 27) and lower cerebral perfusion (4). Possibly through repeated elevations in physiological stimuli (e.g., glucose uptake, blood pressure, shear stress), physical activity breaks *during* a period of sitting minimize risks associated with glucose tolerance (7), blood pressure (5), and endothelial function (25). Few studies examined if effects of sedentary behavior can also be prevented by strategies applied *prior to* sitting, without affecting the physiological stimuli during sitting.

Repeated bouts of ischemia followed by reperfusion, known as ischemic preconditioning (IPC) seems to have a capacity to prevent or attenuate ischemia-induced vascular function in peripheral arteries (9). These protective effects of IPC may also be present in cerebral arteries (21), although some studies report mixed findings (3, 20). Related to the metabolic pathway, a previous study in animals found that IPC alters AMP-activated protein kinase activity in the mitochondria, potentially contributing to improved regulation of glucose

metabolism (17). This raises the question whether IPC prior to sitting prevents harmful effects of prolonged sedentary behavior in humans. This may provide better insight into the impact of prolonged sitting and how (and when) to prevent its impact on health outcomes.

Accordingly, we sought to investigate whether IPC may attenuate or prevent the metabolic (7) and cerebrovascular (4) effects associated with prolonged sitting. We hypothesized that, based on the assumption that prolonged sitting causes impaired glucose tolerance (7), IPC would attenuate prolonged sitting-induced an impairment of glucose tolerance. To test this hypothesis, we performed 2h- oral glucose tolerance test for evaluation of glucose tolerance that has been used in previous studies (7). To better understand a potential impact of IPC on glucose homeostasis, we also explored measures of cardiovascular health (cerebral blood flow, blood pressure and sympathetic nervous activity).

Methods

Participants

All procedures were approved by the ethical committee of the Mount Fuji Research Institute in Japan and were performed in accordance with the guidelines of the Declaration of Helsinki (ECMFRI-01-2017). After a detailed explanation of all study procedures, including the possible risks and benefits of participation, each participant gave written consent. Fifteen healthy inactive participants (80% men) with a mean age of 21±1 years (means±SD) and body mass

index of 25.0±2.4 kg m^2 were enrolled. They were free from any cardiovascular or cerebrovascular diseases, and were not taking any medications. Participants did not engage in regular physically active sports. Before the main study, performed on a different day, participants were familiarized with the measurement techniques (i.e., thigh-cuff occlusion and deflation for IPC, measurement of blood flow in the ICA, and fingertip blood sampling). Women had regular menstrual cycles and were studied during days 1–5 of the menstrual cycle (27). Women did not take hormonal contraceptives. Participants were requested to abstain from caffeinated beverages for 12h and from strenuous exercise and alcohol for a minimum of 24h before any experimental sessions. Participants were instructed to avoid the consumption of foods high in nitrate, as these foods may affect vasculature responses. Therefore, subjects were provided with a list of foods rich in nitrate, and were instructed to maintain their normal dietary intake for the duration of the study (15). All studies were performed in an environmental chamber (TBR-4, 5SA2GX, Tabai Espec Co, Ltd., Tokyo, Japan) set at an ambient temperature of 24°C and at relative humidity of 40%.

Experimental protocol

Each participant visited the laboratory twice to undergo experimental procedures. After 10 min of a supine baseline measurement, four cycles of 5min alternating unilateral cuff inflation of the thigh muscle to 220 mmHg (IPC) or to 20 mmHg (Sham) were performed at

supine position, followed by 2h of quiet sitting period (**Figure 1**). The reason why we set 2h of sitting period is based on presence of the first IPC effect, namely, "*early phase*" (23). Between the 2h sitting period and IPC or Sham intervention, participants ingested 75 g of glucose for the oral glucose tolerance test. Throughout the sitting protocol, participants' feet were placed on a non-slip mat keeping the feet in place and avoid muscle contraction. Study personnel monitored the participants to ensure they remained seated and did not fidget as muscle contraction affect glucose metabolism (11). Participants were allowed to read a book or watch a video; however, they were not allowed to move arms and hands excessively, such as typing, writing, or using a tablet game, and the manipulations of laptop. Moreover, participants were asked not to choose a type of serious, horror or comedy medium because of potential psychological stress (28) and/or positive emotional states (22), which may affect vascular function. Each protocol trial (i.e., Sham or IPC condition) was separated by at least 48–72h to avoid carry-over effects of IPC (18). Participants were randomized to Sham or IPC. The protocol of the present study is shown in **Figure 1**.

Measurements

Blood glucose

Consistent with the guidelines of the American Diabetes Association, after a 12h overnight fast, participants ingested 75 g of glucose (2). Fingertip blood samples were obtained about 5 min

prior to glucose ingestion and then 30, 60, 90, and 120 min post ingestion (2). Glucose levels were measured using a hand held blood glucose analyzer (Glutest Neo Alpha; Sanwa Kagaku Kenkyusho, Nagoya, Japan).

Cardiorespiratory variables

Systolic and diastolic arterial blood pressure (SBP and DBP), heart rate (HR), and partial pressure of the end tidal carbon dioxide output ($P_{ET}CO_2$) were measured for 5 min at ~ 10, 55-60, and 115–120 min into the 2h sitting period. SBP and DBP were measured using an automated blood pressure monitoring system (HEM907, Omron, Tokyo, Japan) at least twice, with a 1min interval between replicates. If the difference between the measurements of either SBP or DBP was > 5 mmHg, the measurements were repeated. The average BP values of the pair of measurements were taken as the BP values, excluding those that were > 5mmHg values (14). HR was measured using a portable HR monitor (Check-My-Heart, TRYTECH Co., Ltd., Tokyo, Japan), which has been used in previous studies (16). To assess heart rate variability (HRV) further, the recordings of electrocardiogram (ECG) signal were transferred to a computer, and the data for each 5 min ECG signal were analyzed automatically by an attached HRV analysis software. Both HR and HRV were measured simultaneously using the same device (Check-My-Heart, TRYTECH Co., Ltd., Tokyo, Japan) in the sitting position. Participants were asked to breathe normally and not to change normal breathing patterns at testing (i.e., ~ 10, 55-

60, and 115–120 min) and during both conditions (Sham and IPC). Time domain HRV was calculated by the standard deviation of the normal-to-normal intervals (SDNN) and the root-mean-square of successive differences in R-R interval (RMSSD). SDNN is considered an estimate of overall HRV, and RMSSD is an index of short-term components of HRV, which is mainly mediated by parasympathetic nerve activity (13). In the frequency domain, the extent of very-low-frequency oscillations (0.0033–0.04 Hz), low-frequency oscillations (LF: 0.04–0.15 Hz), and high-frequency oscillations (HF: 0.15–0.4 Hz) was quantified using a fast Fourier transformation(13, 16). HF power and LF/HF are considered to predominantly represent parasympathetic and sympathetic tone (13, 16). $P_{ET}CO_2$ and breathing frequency were measured using a pocket CO_2 monitor (WEC-7301; Capno puti, Nihon Kohden, Tokyo, Japan).

Internal carotid artery

Right ICA measurements were performed 1.0–1.5 cm distal to the carotid bifurcation with a Doppler ultrasound set at 10.0 MHz and a linear transducer (Logic-e; GE Healthcare, Tokyo, Japan). For the measurement, ICA blood flow was averaged over 2 min during the last 5 min of the 10-min supine resting period, ~10 min, 55-60 min, 115-120 min into the sitting period. To calculate the average ICA blood flow, we analyzed the mean vessel diameter (D_{mean}) and flow velocity as described in a previous study (15). Briefly, after obtaining a clear image of the vessel using the brightness mode, the mean vessel diameter was calculated as: mean diameter

= (systolic diameter × 1/3) + (diastolic diameter × 2/3). The time-averaged mean flow velocity obtained using the pulse wave mode was defined as the mean blood flow velocity (V_{mean}; in centimeters per second). Blood flow was calculated by multiplying the cross-sectional area × 60 (in milliliters per minute). Throughout the measurement, care was taken to ensure that the probe position was stable, the insonation angle did not vary (<60° in all cases) and the sample volume was positioned in the center of the vessel and adjusted to cover the width of the vessel diameter. Using a commercial video capture device (AmCap, Microsoft, WA, USA), recordings of the ICA were performed for 2 min at each time point. The videos were analyzed offline using custom–designed edge detection and wall–tracking software (ver. 2.0.1 No. S – 13037, Takei Kiki Kogyo, Japan) (19).

Data Analysis

The incremental area under the curve (AUC; 0-120 min) of blood glucose responses was calculated from values measured at baseline, using the trapezoidal method. Mean arterial pressure (MAP) was calculated as [(SBP−DBP)/3+DBP]. Cerebrovascular conductance was calculated as ICA flow/MAP.

Statistical Analysis

Prior to the experiments, we estimated sample size with a type I error rate of 0.05 and 80%

power, indicating that 15 participants were required to detect a change in the glucose AUC with effect size of 0.8. Values are expressed as mean±SD. Statistical analysis was performed using GraphPad Prism 7 commercial software (MDF Co., Ltd, Tokyo, Japan). Paired t-tests were used to compare the AUC between IPC and Sham conditions. Two-way repeated-measures ANOVAs (time × condition [IPC or Sham]) with *Bonferroni* post-hoc tests were used for comparisons of blood glucose responses, cardiorespiratory, and ICA variables during 2h siting period. Normality of the data was examined using Bartlett and Levene test. If equal variance failed, logarithmic transformation data were used for further analysis (only HF).

Results

Blood glucose

Figure 2 shows blood glucose responses (0-120 min) in both conditions, presented as the blood glucose at the various points (**Figure 2A**) and as the AUC (**Figure 2B**). Blood glucose increased from 0 to 30 min, and almost linearly decreased until 120 min in both conditions ($P<0.001$). We found a significant main effect of condition ($P=0.007$). As a result, the AUC in the IPC condition was significantly lower than the Sham (resulting in a lower area under the curve when sitting was preceded by a bout of IPC than Sham (4864±1714 with IPC vs. 5915±2628 mg/dl/min with Sham, $P=0.044$). This result remained present when the statistical outlier (dashed square) was deleted ($P=0.037$).

Cardiorespiratory responses

The values of all cardiorespiratory variables measured during 2h sitting periods in all participants are shown in **Table 1**. The $P_{ET}CO_2$ and HR gradually decreased or increased, respectively (both $P<0.05$), but these effects were not affected by IPC. MAP significantly increased during Sham ($P<0.05$), whilst this effect on MAP was not altered with IPC (**Table 1**). During prolonged sitting (Sham), SDNN, RMSDD, and HF gradually decreased with significant differences between those at 10 min and 120 min ($P=0.022$, 0.001, and 0.001, respectively), whilst these variables were not altered when preceded by IPC. After IPC, SDNN and HF were significantly higher than Sham ($P=0.044$ at 60 min in the SDNN, $P=0.029$ at 60 min and $P=0.022$ at 120 min in the log HF). LF/HF gradually increased across time with prolonged sitting ($P=0.009$), whilst LF/HF remained unchanged when sitting was preceded with IPC. During prolonged sitting, the LF/HF at 120 min was significantly higher than in the IPC condition ($P=0.041$).

Cerebrovascular responses

IPC did not affect metrics in cerebrovascular responses (**Table 2**). ICA diameter and blood velocity slightly decreased or increased with the rime course changes ($P<0.05$, respectively).

Discussion

The major findings of the present study were three-fold. First, the characteristic bi-phasic increase in blood glucose during an oral glucose tolerance test was significantly attenuated when sitting was preceded by a bout of IPC. Second, the increases in MAP and HF with 2h sitting, were significantly abolished when preceded with IPC. Finally, prolonged sitting or IPC did not alter cerebrovascular responses. These observations suggest that IPC is able to prevent some of the detrimental effects of prolonged sitting on metabolism and vascular effects, which may help to shed some light into the detrimental effects of sedentary behavior.

This study revealed that a lower AUC for glucose is also observed when sitting was preceded with IPC. A possible explanation for the observation that IPC prevents impaired glucose homeostasis during prolonged may relate to the effects of IPC on blood flow and/or AMPK, subsequently altering glucose homeostasis. For example, several studies found that IPC leads to local (9) and central (30) increases in resting blood flow, possibly through upregulation of NO (10, 23). A larger blood flow likely increases glucose uptake (8), potentially contributing to changes in glucose homeostasis. Alternatively, previous work in animals found IPC to increase AMPK activity (17). Since activation of AMPK activity results in increases in GLUT-4 translocation, leading to an increase in glucose uptake in tissues (12) such effects of IPC may ultimately alter glucose homeostasis. Taken together, IPC may indirectly affect glucose

237 homeostasis through its effects of mild vasodilation and activation of AMPK, although also
238 other pathways should be considered and can be topic of research for future work.

239 An alternative mechanism explaining the improved glucose tolerance with IPC relates
240 to activation of hypoxia-induced factors, especially since previous work in animals has linked
241 hypoxia-induced factors to glucose homeostasis (26). As the IPC protocol consists of repeated
242 bouts of ischemia followed by reperfusion, IPC causes intermittent hypoxia. Although
243 similarity is present between intermittent hypoxia and ischemia (with IPC), caution is warranted
244 to extrapolate these findings to IPC. Although the exact mechanisms remain unclear, our study
245 reveals that IPC improves glucose homeostasis when applied prior to prolonged uninterrupted
246 sitting.

247 Another intriguing finding from the present study was that IPC was associated with an
248 attenuated decrease in SDNN and HF, and an increase in LF/HF, compared to the Sham-
249 condition of prolonged sitting. These results suggest that IPC may affect cardiac autonomic
250 nervous activity, a finding that is consistent with a previous study (9). Based on the close
251 relation between sympathetic nervous activity and blood pressure, the effects of IPC on
252 modulations of cardiac autonomic nervous activity may explain the lower blood pressure
253 observed in the IPC-trial. This could be of particular interest to prevent the rise in blood pressure
254 typically observed during prolonged sitting.

255 Although positive effects of IPC on peripheral blood vessels have been demonstrated

(9), our results suggest that IPC does not affect ICA during prolonged sitting. In two very recent studies, IPC was also found not to alter cerebral blood flow when measured at the MCA during a short period of sitting (3) or at the ICA in the supine position (20). Our data, supported by these recent studies, therefore suggests that IPC unlikely alters blood flow in centrally located arteries, a finding that contrasts with peripheral arteries. A possible mechanism to account for benefits of IPC on peripheral vasculature may be associated with hormonal factors, such as adenosine, bradykinin, and nitric oxide (10). Although we did not assess directly these hormones, a previous study demonstrated endothelial cells from cerebral and peripheral vessels exhibit different vascular regulation (24). Heterogeneity in the pathways contributing to blood flow regulation between these arteries may contribute to our observations.

Methodological considerations

Several limitations should be considered when interpreting our results. First, sample size and statistical power in the results of the blood glucose AUC were lower than expected, thus increasing the chance for false-negative results (type II error). A second limitation is that we did not measure other relevant parameters involved in glucose control, such as insulin (6), which would have provided a more in-depth analysis. Third, we adopted cardiac autonomic nervous activity variables, whilst direct measurement of sympathetic nerve activity using microneurography is preferred. Finally, recruited participants in the present study were healthy,

Japanese young men and women. Thus, it is uncertain whether our results can be translated to other more clinically relevant populations such as elderly and patients with diabetes.

Clinical relevance

Previous work revealed that physical activity breaks are effective to prevent detrimental health effects on metabolic and cardiovascular parameter associated with prolonged sitting. However, in some clinical settings, this behavior is challenging. In these conditions, IPC can be applied in wheelchair-bound individuals to prevent effects of prolonged sedentary behavior or prior to prolonged sitting that cannot be interrupted. Nonetheless, we do not foresee IPC as an intervention for the general population to prevent effects of sitting. At least, our observations highlight that interventions (e.g. IPC, exercise) (29) can be applied prior to prolonged periods of sitting to prevent associated health effects.

In summary, the present results suggest, for the first time in humans, that IPC may affect glucose tolerance as evaluated by the oral glucose tolerance test and suppress prolonged sitting-induced increases in MAP. In contrast, IPC did not alter ICA flow responses during prolonged sitting. These findings suggest that IPC could potentially prevent the detrimental effects of prolonged sitting-induced glucose intolerance.

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Author contributions: M.H. and D.H.J.T conceived the design and concept of this study. M. H. preformed the experiment and analyzed data. M.H. and D.H.J.T. interpreted the results. M.H. drafted the first manuscript. M.H. and D.H.J.T. revised and approved the final manuscript.

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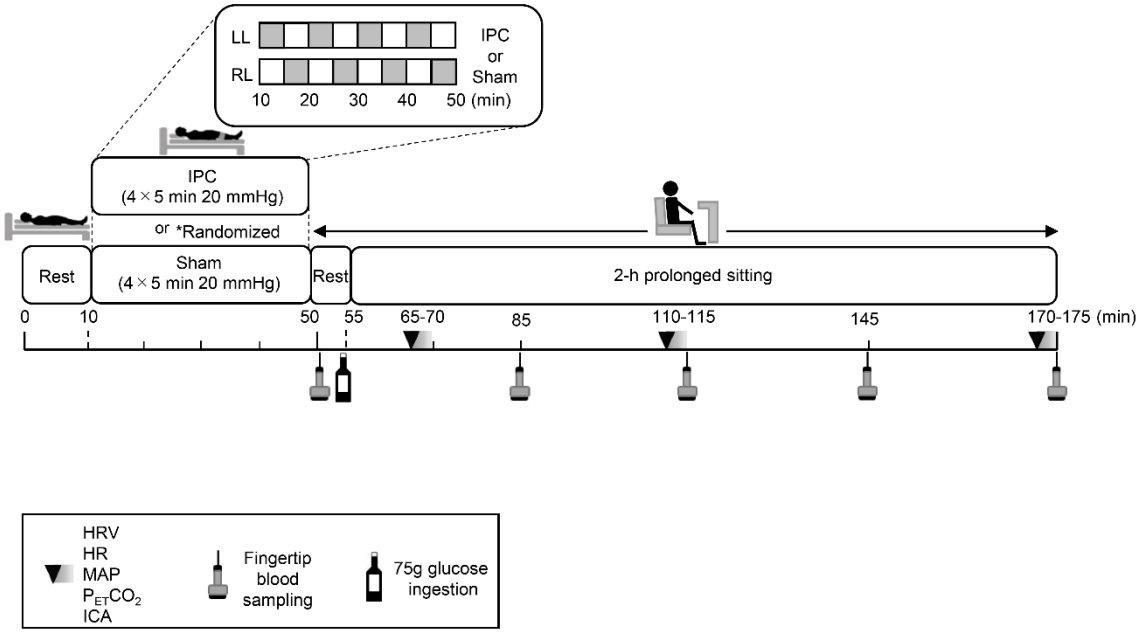
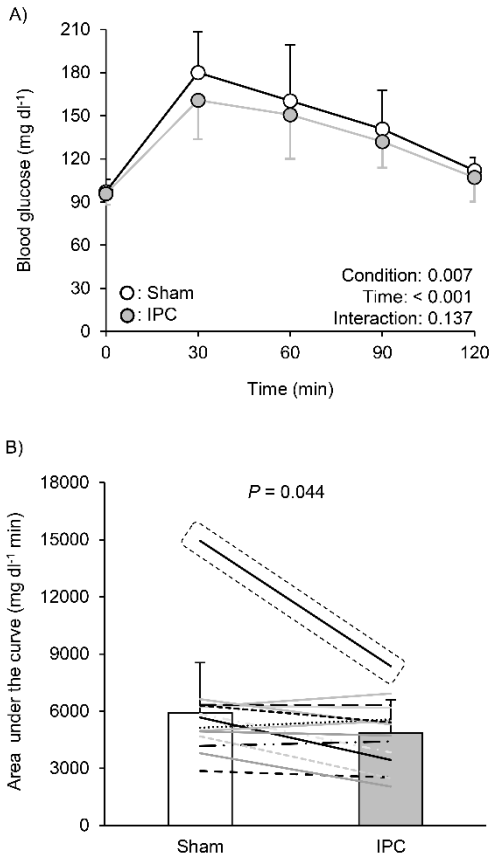


Figure 1. Protocol of the study. IPC, ischemic preconditioning (220 mmHg) 4 × 5-min bilateral thigh cuff occlusion; Sham, reduced cuff pressure of 20 mmHg; LL, left leg; RL, right leg; HRV; heart rate variability, HR, heart rate; MAP, mean arterial pressure; $P_{ET}CO_2$, partial pressure of end tidal carbon dioxide output; ICA, internal carotid artery.



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410 **Figure 2.** Blood glucose responses during 2h oral glucose tolerance test. White and gray circles
411 indicate Sham and IPC conditions, respectively. Values are mean±standard deviation. (panel A).
412 Area under the curve of blood glucose during 2h oral glucose tolerance test between Sham and
413 IPC. Bars indicate mean values with standard deviation. Each line indicates an individual value.
414 When the outlier (top solid line) is removed (n=14), the *P*-value was 0.037.