

Ischemic Preconditioning Negatively Affects Thrombogenic Clotting Profile in Cerebral Small Vessel Occlusion Stroke Patients

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Abstract

Background: The study evaluated the effect of an acute and a 2-week daily repetitive ischemic preconditioning (IPC) on conduit artery vascular function and thrombogenic clotting profile, in patients with a recent ischemic stroke.

Methods: Fourteen patients, aged 71 ± 8 years, with a cerebral small vessel occlusion stroke were included in a randomized, controlled, open-label cross-over study. Treatment consisted of 2 weeks of daily IPC, four 5-min rounds of upper-arm occlusion, interspersed by 5 min rest periods. Control was without treatment. Brachial artery flow-me-diated dilation (FMD) was determined at baseline and after the control and treatment periods. Before and after each period, the patients underwent an acute bout of IPC. Blood samples were obtained for thrombogenic clotting profile at baseline and after the acute IPC bout, both before and after the control and treatment periods.

Results: The period of daily IPC increased brachial artery diameter but did not influence FMD. Acutely, IPC was found to induce an increase in fractal dimension, indicating a denser clot microstructure, and a reduction in plasma levels of plasminogen activator inhibitor 1 (PAI-1). There was no effect of daily IPC on the basal thrombogenic clotting profile, or on the change in clotting profile induced by acute IPC.

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Conclusions: Collectively, the data show that acute IPC leads to a prothrombotic clotting profile, despite antiplatelet therapy. Moreover, 2 weeks of daily treatment with IPC does not influence conduit artery vascular function or thrombogenicity in stroke patients.

Keywords: Cerebral small vessel occlusion stroke; Vascular function; Preconditioning; Clotting profile

Introduction

Ischemic preconditioning (IPC) is a procedure where blood flow to a limb is repeatedly occluded for a brief period of time, with each period followed by a reperfusion phase. Just one session of the procedure has been shown to enhance both microvascular [1] and conduit artery [2, 3] function in humans and when repeated regularly over a period, IPC has been shown to improve vascular endothelial function [4-7]. Application of the IPC procedure in different clinical populations has resulted in promising effects [8-10] and a period of daily IPC may therefore be an effective strategy to improve vascular function and reduce the susceptibility to cerebral infarct in individuals at risk. Preclinical studies have initial positive results; however, these have not been fully confirmed in humans yet [11].

Cerebral small vessel occlusion (lacunar) stroke is caused by thrombosis in arterioles subcortically or deep brain regions, which may lead to significant disability [2, 3]. Lacunar stroke is prevalent in lifestyle-related diseases such as hypertension and diabetes [12] and represents approximately 20% of all cerebral strokes with a relatively high risk of a recurrent stroke [13, 14]. Home-based and easy-to-use interventions which reduce the risk of recurrent stroke are therefore warranted.

The underlying pathology of lacunar infarct includes thickening of the arterial media presence of intimal plaques [15] and endothelial dysfunction [12, 16]. The vascular endothelium prevents thrombosis by inhibiting platelet reactivity through the production of nitric oxide (NO) and prostacyclin [17]. In dysfunctional endothelium, the formation of NO and prostacyclin is reduced [18, 19] whereby the inhibition of platelet reactivity is attenuated and the risk of arterial thrombosis enhanced, in particular with additional presence of atherosclerotic plaques.

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	Age (years)	70.9 ± 8.4
	Male sex (n (%))	11 (78.6%)
	Body weight (kg)	80.2 ± 15.8
	Body mass index (kg/m ²)	25.5 ± 3.5
	Cardiovascular risk factors	
	Total cholesterol (mmol/L)	5.1 ± 0.9
	Low-density lipoproteins (mmol/L)	3.0 ± 0.9
	High-density lipoproteins (mmol/L)	1.6 ± 0.5
	Glycated hemoglobin (mmol/L)	40.8 ± 12.6

Table 1. Characteristics of 14 Stroke Patients at Baseline

Data are presented as mean ± SD. SD: standard deviation.

Intravenous thrombolysis at debut (n (%)) Endovascular thrombectomy at debut (n (%))

Prior stroke without functionally significant sequelae (n (%))

History of stroke and initial therapy

Hypertension (n (%))

History of stroke-associated comorbidities

Hypercholesterolemia (n (%)) Type 2 diabetes mellitus (n (%))

The susceptibility to arterial thrombosis is commonly assessed by measurements of plasma biomarkers such as fibrinogen and plasminogen activator inhibitor 1 (PAI-1) or by determination of platelet reactivity [20]. However, as hemostasis is a highly complex process involving many steps, such measurements provide only partial information. A new useful global hemostatic biomarker of coagulability has been developed [21, 22] which estimates the density of the microstructure of a blood clot when formed. The method involves viscoelastic measurements of whole blood to provide a measurement of the gel point or formation of the incipient blood clot. From a gel point measurement, it can be quantified how the incipient blood clot, which consists mainly of activated platelets and fibrin, is organized by calculating its corresponding fractal dimension [23]. The outcome is diagnostically useful as high values of fractal dimension indicate a denser clot structure which is more serious as it is difficult to degrade by fibrinolysis [22, 24, 25]. The influence of IPC on fractal dimension has not previously been assessed.

The present study evaluated the effect of an acute bout of IPC as well as a 2-week period of daily IPC on conduit artery vascular function and thrombogenic clotting profile in patients who had suffered from cerebral small vessel occlusion stroke. The hypothesis was that IPC would improve conduit artery function, assessed as flow-mediated dilation (FMD), and the thrombogenic clotting profile.

Materials and Methods

Participants

A total of 14 patients diagnosed with cerebral small vessel occlusion stroke were included in the study. Inclusion criteria were: >

18 years of age; clinical symptoms and corresponding computed tomography (CT)/magnetic resonance (MR) scan with lacunar infarct, defined as either: 1) lacunar stroke according to TOAST classification [26], diagnosed within the past 5 years, or 2) isolated neurologic outcomes (e.g., motoric, sensoric or language) for over 24 h; no cortical or cerebellar dysfunction, MR-verified acute lacunar supratentorial or infratentorial infarct according to STRIVE criteria [27] (< 2 cm in diameter in acute phase, 1.5cm in chronic phase); no clinically significant carotid stenosis or cardioembolic cause of infarct. Exclusion criteria were: chronic diseases not directly related to stroke, e.g. cardiac disease, cancer and immune deficiency; current treatment with peroral steroids, smoking, currently or within past 10 years, alcohol or drug abuse (Table 1). All subjects received antiplatelet treatment, clopidogrel 75 mg/day. One patient received dual anti-platelet treatment with clopidogrel 75 mg and acetylsalicylic acid 75 mg/day 3 months prior to study day. Four patients had received acetylsalicylic acid 75 mg/day for 5 days while admitted to the hospital.

5 (35.7%) 2 (14.3%)

0 (0.0%)

11 (78.6%) 12 (85.7%)

2 (14.3%)

The study was approved by the Ethics Committee of the Capital Region of Copenhagen (H-16048498) and all procedures were carried out in accordance with the Declaration of Helsinki. The study was registered at ClinicalTrials.gov (NCT03635177) prior to first inclusion.

Study design

The study adopted an open-label repeated-measures cross-over design where all participants were subjected to a 2-week treatment period of daily IPC and a 2-week control period. Participants were randomized to the order of treatment or control and each period was separated by at least a 3-week washout period. The randomization was achieved by administration of sealed opaque envelopes containing the allocation of treatment made by personnel not involved in the study.

Before and after the two intervention periods, all patients attended an experimental day that included determination of conduit artery vascular function and assessment of thrombogenic clotting profile. The determination of vascular endothelial function was accomplished by the FMD technique in the brachial artery and performed after at least 30 min rest in a supine position. Blood samples were collected at baseline and 5 min after a 4×5 min session of acute IPC. The blood samples were used for analysis of full blood count, plasma coagulation biomarkers as well as fractal dimension: viscoelastic measurement of coagulating whole blood, indicating clot microstructure.

Interventional treatment

The participants conducted IPC at home once daily for 14 days by use of an automated blood pressure cuff positioned on the upper non-dominant arm (autoRICTM, Cellaegis Devices, Toronto, Ontario, Canada). Each session consisted of four rounds of 5-min periods of occlusion, separated by 5 min of non-occluded reperfusion. All participants were carefully instructed in the use of the device prior to initiation of the treatment period, and continuous guidance was offered as well.

Due to the obvious intervention treatment, the study was conducted as open-label and the control period served as a time-control without treatment.

FMD

FMD was conducted by an experienced researcher and the procedure was conducted according to guidelines [28]. FMD was expressed as the percentage change in diameter of the brachial artery (FMD%). The change was calculated as the difference between baseline and maximal diameter measured after 5 min of blood flow occlusion. Occlusion was achieved by inflation of a pressure cuff (E20 Rapid Cuff Inflator and AG101 Cuff Inflator Air Source, Hokanson[®], Bellevue, WA, USA) placed on the arm. Analysis of the vascular 2D images was conducted in a blinded manner by use of validated software (Brachial Analyzer for Research, Version 6.8.7; Medical Imaging Applications, Iowa, USA). Brachial diameter and velocity traces were analyzed by use of automated edge detection. Obtained values were applied in the calculation of the following variables: FMD%: (Maximal diameter - Baseline diameter)/Baseline diameter) × 100. Blood flow (mL/min): $\pi r^2 \times$ Average blood velocity (cm/s) \times 60. Shear rate: $4 \times (Blood mean velocity (cm/s)/Diameter (cm)).$

Viscoelastic measurement of fresh whole blood

Rheometric determination of gel point

The viscoelastic measurement is based on rheometric analysis of the gel point [29], from which fractal dimension (d_f) of an incipient blood clot can be determined [22]. The rheology technique used to attain the gel point has been validated for use with human blood in previous studies [22, 30, 31]. In brief, blood was drawn slowly from the antecubital vein into syringes and transferred to tubes not containing any anticoagulant. Within 60 s, 7 mL of blood was placed in a double concentric measuring geometry mounted on a controlled stress rheometer (Discovery Series Hybrid Rheometers (DHR-2); TA Instruments, New Castle, USA), which was held at a constant temperature of 37 °C. The viscoelastic analysis was performed using small amplitude oscillatory shear measurements at varying frequencies (2, 0.93, 0.43 and 0.2 Hz) with an applied peak stress amplitude of 0.03 Pa sequentially with time. These sequential measurements of the four frequencies over time allow for the determination of the gel point [32].

Fractal dimension and clot microstructure

The gel point obtained by viscoelastic measurement of whole blood marks the transition of the blood from a viscoelastic liquid to a viscoelastic solid, and thereby identifies the formation of an incipient blood clot. From the gel point measurement, it can be quantified how the incipient blood clot, which consists mainly of activated platelets and fibrin, is organized by calculating its corresponding fractal dimension [23].

The relationship between fractal dimension and the microstructure and fibrin mass of an incipient blood clot has been validated by electron microscopy and computational analysis previously [30, 33, 34].

Analysis of blood samples

Metabolic and health-related biomarkers

All measures of glucose metabolism, cholesterol, triglyceride and hepatic enzymes were performed as routine clinical tests at Department of Clinical Biochemistry, Copenhagen University Hospital-Herlev Gentofte, Denmark.

Full blood count and plasma coagulation biomarkers

The full blood counts, including thrombocytes, erythrocytes and leukocytes, were measured in a fresh sample of whole blood at Department of Clinical Biochemistry, Copenhagen University Hospital-Herlev Gentofte. The plasma coagulation biomarkers were measured in aliquots of blood drawn into 3.2% sodium citrate vacutainers (Greiner Bio-One GmbH, Austria). Fibrinogen (ab108842, Abcam, Cambridge, UK), PAI-1 (ab269373, Abcam, Cambridge, UK) and D-dimer (ab260076, Abcam, Cambridge, UK) were analyzed by use of ELISA according to manufacturer's protocols.

Statistical analysis

In all studies, *a priori* sample size determination was performed for the primary outcome, FMD [35]. The required



Figure 1. (a) Change in baseline diameter, (b) peak diameter and (c) flow-mediated dilation of the brachial artery with a 2-week period of daily IPC or a control period in cerebral small vessel occlusion stroke patients. *Significant difference between control and IPC; P < 0.05. IPC: ischemic preconditioning.

sample size was calculated to be 13, based on an expected FMD mean of 10%, mean FMD difference with the intervention of 2% and a standard deviation (SD) of 2.5%. Data are presented as mean \pm SD. Statistical analyses were performed with R (version 3.4.1; R Foundation for Statistical Computing, Vienna, Austria) using the interface RStudio (version 1.1.463; RStudio Team, Boston, USA). A linear mixed-model approach was used to detect differences with the intervention. Subjects were specified as a repeated factor and identifier of random variation. For d_P, subjects and time were used as identifier of random variation. Residual and Q-Q plots confirmed homogeneity and normal distribution, respectively. *Post-hoc* analysis was used to detect all differences. The reported P-values are non-adjusted. Graphical visualization was performed in Prism (version 8.3.0; GraphPad Software, San Diego, USA).

Results

Participant characteristics

Of the 14 included patients, three were females and the mean age was 71 ± 8 years. Cardiovascular risk factors and comorbidities are presented in Table 1.

Brachial artery FMD

There was an increase in baseline diameter with IPC treatment compared with the 2-week control period (P = 0.0307; Fig. 1a). There was no change in peak diameter (P = 0.9014; Fig. 1b) or brachial artery FMD (P = 0.3467; Fig. 1c) with 2 weeks of IPC treatment compared with the 2-week control period.

Clot microstructure - fractal dimension

The 2 weeks of IPC did not influence the level of d_p compared

to the 2-week control period (P = 0.0975; Fig. 2a). Analysis of pooled data from the interventions revealed that an acute session of IPC increased d_f in the venous samples (from 1.64 ± 0.046 to 1.66 ± 0.055 , P = 0.0463; Fig. 2b).

Plasma coagulation markers and full blood count

The 2-week intervention period did not influence the full blood count (Table 2) or the concentration of D-dimer (P = 0.9970; Fig. 3a), PAI-1 (P = 0.3080; Fig. 3c), or fibrinogen (0.8423; Fig. 3e). An acute session of IPC reduced the plasma PAI-1 concentration (P = 0.0125; Fig. 3d), when pooling data from both intervention periods, whereas there was no effect on D-dimer (P = 0.103; Fig. 3b) or fibrinogen (P = 0.308; Fig. 3f). No differences were observed in any parameter of the full blood count (Table 2).

Discussion

The main finding of the present study was that, in contrast to our hypothesis, a period of daily IPC in patients who had suffered a lacunar infarct, had no beneficial influence on conduit artery vascular function, assessed by FMD, or on the thrombogenic clotting profile, assessed by fractal dimension and plasma coagulation biomarkers. Instead, an acute session of IPC resulted in an increase in fractal dimension suggesting that the procedure may increase the thrombogenicity acutely, and thereby susceptibility to stroke in this population.

In this study, we evaluated if 2 weeks of daily IPC in cerebral small vessel occlusion stroke patients would improve vascular function and in parallel improve the thrombogenic clotting profile. FMD, which indicates NO-dependent endothelial function, was used to assess conduit artery function. As NO inhibits platelet reactivity, we reasoned that improved FMD would reflect a more pronounced NO bioavailability which also would benefit the clotting profile. In contrast to our hypothesis, daily IPC did not have an effect either on FMD



Figure 2. Fractal dimension (d_f) in cerebral small vessel occlusion stroke patients (a) before and after an acute bout of IPC conducted before and after a 2-week period of daily IPC or a 2-week control period and (b) before and after an acute bout of IPC. Data combined from the acute interventions. *Significant difference between control and IPC; P < 0.05. IPC: ischemic preconditioning.

or on the clotting profile in patients with recent small vessel occlusion stroke. The lack of effect of IPC on conduit artery function contrasts that of a recent study, in which an improved brachial artery FMD response was observed in prior stroke patients after a 2-week treatment period with IPC [36]. However, there were numerous differences between the studies; as in the previous study [36], the patients had experienced either a cerebral small vessel occlusion stroke or hemorrhage and patients were included up to 8 years past the last stroke. In the current study, most of the patients had experienced a stroke within the past 6 months and only patients with previous cerebral small vessel occlusion stroke were included. Moreover, in the previous study [36], the IPC procedure was conducted on the leg

three times per week as opposed to on the arm every day. We chose arm treatment as we considered it to be practical and more feasible for the patients in real-life. Also, 1 week of daily IPC of the arm was shown to improve FMD in healthy and diabetic individuals [4, 37]. Thus, the discrepancy in disease ethiology may be a more likely cause of the disparity in results between the studies.

In contrast to our hypothesis that IPC would be beneficial for the clotting profile, we made the important observation that the treatment resulted in an immediate more pro-thrombotic clotting profile in cerebral small vessel occlusion stroke patients. This was evidenced as an increase in fractal dimension indicating a denser blood clot structure as discussed below.

Table 2.	Full Blood C	Count Before ar	nd After 2	Weeks of IPC	Treatment and Control
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			Control			
	Before	After	Before	After		
Thrombocytes (× $10^{9}/L$)	261 ± 61	249 ± 65	234 ± 47	261 ± 54		
Erythrocytes (× 10 ¹² /L)	4.47 ± 0.42	4.36 ± 0.41	4.28 ± 0.40	4.38 ± 0.49		
Hematocrit (%)	41 ± 4	40 ± 3	39 ± 3	40 ± 4		
Hemoglobin (g/dL)	8.85 ± 0.83	8.48 ± 0.88	8.28 ± 0.72	8.46 ± 0.81		
Leukocytes (× 10 ⁹ /L)	6.62 ± 1.14	6.46 ± 2.02	6.07 ± 1.60	5.94 ± 1.15		
Lymphocytes (× 10 ⁹ /L)	1.59 ± 0.44	1.51 ± 0.53	1.35 ± 0.33	1.40 ± 0.29		
Monocytes (× 10 ⁹ /L)	0.42 ± 0.12	0.44 ± 0.15	0.40 ± 0.11	0.44 ± 0.10		
Neutrophils (× $10^{9}/L$)	4.22 ± 1.18	4.07 ± 1.69	3.92 ± 1.69	3.67 ± 1.10		
Basophils (× $10^{9}/L$)	0.04 ± 0.05	0.03 ± 0.05	0.02 ± 0.04	0.02 ± 0.04		
Eosinophils (× 10 ⁹ /L)	0.25 ± 0.17	0.29 ± 0.17	0.26 ± 0.18	0.29 ± 0.24		

Data are presented as mean ± SD. IPC: ischemic preconditioning; SD: standard deviation.



Figure 3. Plasma indicators of thrombogenesis: (a, b) D-dimer, (c, d) PAI-1 and (e, f) fibrinogen, before and after an acute bout of IPC conducted before and after a 2-week period of daily IPC, or a 2-week control period, in cerebral small vessel occlusion stroke patients. *Significant difference between control and IPC; P < 0.05. IPC: ischemic preconditioning.

This observation is particularly striking considering that all patients were on antiplatelet therapy by clopidogrel. The finding of a potential pro-thrombotic effect of IPC could be important clinically and should be held up against the current lack of evidence of a beneficial effect of a period of daily treatment on stroke outcome in humans [11].

For the assessment of the thrombogenic clotting profile, we combined plasma coagulation biomarkers with a novel global biomarker of hemostasis, which involves the assessment of the viscoelastic properties of fresh whole blood. This measurement, expressed as fractal dimension, provides an indication of the integrated hemostatic property and is an immediate indication of the risk of cardiovascular events [25]. A high numerical value of fractal dimension is equivalent to a strong and dense clot with a high number of complex structured fibrin branches, which is more difficult to dissolve biologically or through pharmacotherapeutics [22, 25]. In a Welsh cohort of 149 individuals, the fractal dimension was found to be higher in stroke patients, treated either by aspirin or dual antiplatelet therapy compared to healthy control participants [38], indicating a greater propensity for the formation of dense clots in stroke patients. The current patient population, consisting of patients who had experienced a stroke within the past 6 months and who all were undergoing treatment with clopidogrel, accordingly [30] displayed fractal dimension values of between 1.59 and 1.72 at baseline, which is somewhat lower than the stroke patients in the Welsh cohort [38], probably due to a generally better lifestyle in the Danish population.

The acute session of IPC elevated the fractal dimension by, on average 0.02 units. Although numerically this appears as a small change, the functional implication is substantial; based on computational modeling of a simplified branching network, this difference can be calculated to correspond to a 20-40% increase in clot mass. In parallel with the increase in fractal dimension, there was a significant decrease in the fibrinolysis inhibitor, PAI-1, indicating improved fibrinolysis. In a previous study, it was noted that the effect of acute IPC resulted in a reduction in PAI-1 [39]. However, contrary to our findings using rotational thromboelastography, they found no change in clot mass or strength nor increase in thrombogenicity. In our study, we show a clear and marked increase in clot elasticity indicative of a prothromobotic tendency. This value may be higher than normally anticipated as all the patients were on clopidogrel treatment which has been shown previously to contribute to a looser and weakening effect on clot architecture with a corresponding reduction in $d_f[30]$. The rise in d_f is probably due to vascular endothelial damage during ischemia reperfusion; however, the finding of a difference between an elevation of d_e and reduction in PAI-1 may be an interesting mechanistic effect which requires further explanation. It is of note that the acute effect of the IPC procedure on the change in thrombogenicity was similar before and after the 2 weeks of daily treatment, suggesting that habituation to IPC had no effect on the thrombogenic response.

Study limitations

Although the current study was sufficiently powered for the primary outcome of FMD, it was a pilot study with a limited number of participants and larger studies would be required to verify the findings. However, given the observed negative influence of an acute session of IPC on thrombogenicity, it may be questioned whether a full study would be justified.

Previous studies have shown that only 1 week of daily IPC,

including in diabetic individuals, improved FMD; however, it cannot be excluded that a longer intervention period would be required for an effect to be detectable in stroke patients. In terms of thrombogenicity assessed by fractal dimension, it is unclear whether a longer period could have had an effect.

Conclusion

Two weeks of daily IPC performed on the arm in cerebral small vessel occlusion stroke patients did not improve either brachial artery FMD or basal thrombogenic clotting profile. Instead, the results show that one acute session of IPC can increase thrombogenicity in stroke patients, despite antiplatelet therapy treatment. This somewhat controversial observation suggests that caution should be exercised when subjecting stroke patients to IPC. This finding also raises the question of whether IPC to a greater extent may affect thrombogenicity in patients at risk who are not being treated by antiplatelet therapy.

Learning points

Two weeks of daily IPC increased brachial artery diameter, but did not change brachial artery FMD in patients with a recent ischemic stroke.

Acute IPC was found to increase clot microstructural density.

The novelty of this study is using a global biomarker of hemostasis to identify the density of the clot microstructure when investigating the effect of IPC on the clotting profile in ischemic stroke patients.

The findings of the present study have negative implications for the use of IPC in the treatment of ischemic stroke patients.

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None to declare.

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Conflict of Interest

The authors declare no conflict of interest associated with this study.

Informed Consent

Before enrollment, written and informed consent was obtained from all patients.

Author Contributions

YH and CK conceived the experiments. YH, CK, LC, NR, ML and PAE designed the experiments. LBN, NR, LC and LG collected the data. ML, CSH and PAE assisted with data collection. All authors interpreted the data. LBN, NR, CK and YH analyzed data and drafted the manuscript. All authors revised and approved the final version of the manuscript.

Data Availability

The data supporting the findings of this study are available from the corresponding author upon reasonable request and must be in accordance with the current guidelines of the General Data Protection Regulation (GDPR).

Abbreviations

d_f: fractal dimension; FMD: flow-mediated dilation; IPC: ischemic preconditioning; NO: nitric oxide; PAI-1: plasminogen activator inhibitor 1

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