Evidence for a vasomotor cyclo-oxygenase dependent mechanism of sensitization at the cutaneous level.

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SUMMARY:

AIMS: Current-induced vasodilation (CIV) is an axon-reflex response observed during monopolar current application such as iontophoresis. Cyclo-oxygenase derivates (COD) participate in CIV and act as sensitizing agents at the anodal level. Mechanisms involved during cathodal current application (CCA) are partially unknown. In a randomized double blinded crossover trial, we tested in sixteen healthy subjects (i) the influence of the inter-stimulation interval (I-I) by comparing CIV following all-at-once 10 s CCA against 2x5 s CCA with intervals ranging [15 s-16 min], (ii) the participation of COD in CIV using 1g aspirin or placebo intake.

METHODS: Measurements were repeated 2 h and 14 days after treatment. Laser Doppler flowmetry assessed cutaneous blood flow, reported in multiple of baseline.

RESULTS: Before treatment, peak vasodilation 10 min after the last current application (CVC_{stim2}) increased compared to baseline whatever the I-I. Increase in CVC_{stim2} from baseline was greater for the 4 min (9.4 (5.3, 10.9) times; median (1rst percentile, 3rd percentile)) and higher I-Is compared to all-at-once delivery (3.0 (2.1, 4.3) times, $p<0.05$). The response was similar after placebo but aspirin abolished this vasodilation (increase by 1.2 (1.1, 1.3) times for all-at-once delivery and by 1.5 (1.3, 1.7) ± 0.3 times for 4 min interval, 2 h after aspirin intake) that was recovered after 14 days.

CONCLUSIONS: This confirms the participation of COD in CIV with CCA and their sensitizing action. This model can represent an attractive way to study the axon-reflex and sensitizing function of COD in humans.

What is already known about this subject:

-Monopolar current applications (segmented or not) at the cutaneous level can elicit vasodilatation.

-Cyclo-oxygenase derivates (propably prostaglandins) participate in this phenomenon and, during segmented current applications, can have a sensitizing effect on the nervous afferent stimulated.

-This effect is well known at the anodal level but must be better described for cathodal current.

What this study adds:

-Participation of cyclo-oxygenase derivates is confirmed for cathodal segmented current application.

-A minimal interval must be respected between two current applications to observe the sensitizing effect of cyclo-oxygenase derivates.

-This model is usefull to study the sensitizing function of cyclo-oxygenase derivates in humans.

INTRODUCTION:

Monopolar current application as those performed during iontophoresis can elicit a current-induced vasodilation (CIV) at the skin microcirculation level [1, 2]. This phenomenon is considered an adverse effect of the technique since CIV can possibly make confused the interpretation of the effect of the drug applied through iontophoresis [2]. This response is assumed to result from an axon reflex because it is abolished under local anesthesia or after chronic capsaicin desensitization [3]. Among the different mechanisms involved in CIV, Gohin *et al*. (2011) showed that prostacyclin (PGI2), a potent vasodilator produced by the vascular endothelium participates in CIV in healthy rat skin [4]. Furthermore, it has been revealed that for the same amount of electrical charge, segmented current application leads to a greater CIV than all-at-once delivery [5]. Despite the fact that this phenomenon has been observed both at the anode and at the cathode, it has been poorly described at this latter level. However, some observations confirmed that this mechanism of sensitization is aspirin-sensitive suggesting the involvement of cyclo-oxygenase derivates (COD) (probably prostaglandins) [5, 6].

It has been previously reported that the recovery of CIV after aspirin intake and following segmented cathodal or anodal current delivery was long lasting, since sensitization is still abolished or dramatically reduced two days after aspirin intake [5, 6]. Such results suggest that COD are a major factor in the development of CIV promoting vasodilation either directly or through a mechanism of sensitization. We consider that this mechanism likely represents an interesting physiological model of the role of COD as neural sensitizing agent and deserves that we focus on it.

It has been previously observed, for anodal segmented current application and an interstimulation interval shorter than 10 min, a sensitization mechanism based on COD [5].

Yet, whereas Durand *et al.* (2002) observed an influence of the length of the interstimulation interval on the amplitude of sensitization at the anode, Tartas *et al*. (2005) did not report any change in CIV amplitude whatever the interstimulation interval (10, 20 or 40 min) at the cathode [6]. The reason for this discrepancy remains unclear but could rely on a long lasting effect of the mediators of sensitization.

Consequently, we screened whether a minimal interval (inferior to 10 min) during cathodal segmented current application was required to lead to amplification of CIV compared to all-at-once delivery. We crossed these measurements with aspirin or placebo intake to confirm the involvement of COD in CIV in the responses observed before and at 2 hours and 14 days after a single oral dose of 1g aspirin or placebo.

METHODS:

Population

Sixteen healthy subjects participated in the study. The local ethics committee approved the study. All volunteers provided written, informed consent prior to participation. This study was carried out in accordance with the Declaration of Helsinki. It is registered to the American National Institutes of Health database under reference No: NCT 01572961. None of the investigators involved in the clinical and microvascular assessments were aware of the subjects' treatment allocation at any time during the study. The study design is shown in Figure 1. Before each microvascular assessment, subjects were asked to refrain from consuming caffeine in the four hours preceding the test. After baseline microvascular assessment (i.e. standard response), subjects were randomly assigned to either one single-dose of Aspirin (Aspégic adulte 1g; Sanofi-Aventis®) dissolved in 125 ml of orange juice or

orange juice alone. Two hours and 14 days after the allocation, microvascular assessments were repeated and a global wash out period of 14 days was respected before the protocol was repeated [7].

Subjects' preparation

Participants were placed supine in a quiet room with the ambient temperature set at 23±1°C, and left at rest for 15 min before each experiment for temperature and cardiovascular adaptations [8].

Assessment of skin microcirculation:

For microvascular recordings, we studied successively skin blood flow on the volar aspect of both forearms using laser Doppler flowmetry (LDF). On each forearm, different probes were used. "Active electrodes" were specifically designed to allow for simultaneous blood flow recording and current application, and are referred as the "active electrodes", (probe 481-1, Perimed, Sweden) (Figure 2). The "active" electrodes have a circular chamber of ~1 cm^2 allowing for the positioning of the specially designed disposable sponge for iontophoresis. At the center of the sponge, skin LDF was measured through a multifiber laser probe (780 nm, 1 mW maximal emission, and bandwidth for Doppler shift 20–20,000 Hz). A third electrode (PF408, Perimed, Jarfalla, Sweden) was used as a reference to confirm the absence of response to the current application at an adjacent unstimulated site, and to account for eventual systemic variations. The electrodes were placed at different points on the forearm, each measured area being at a minimal distance of 4 cm from each of the others.

All the probes were connected to laser Doppler flowmeters (Periflux PF 4001, and

Periflux PF 5000, Perimed, Sweden). The "active" electrodes were also connected to current suppliers (Periiont micropharmacology System, PF 382 Perimed, Jarfalla, Sweden). For those electrodes, a disposable adhesive electrode (Kendall) was positioned 5 cm away from the laser probes, forming the anode and closing the current system [9] (Figure 2).

The changes of skin blood flow in response to cathodal current application of 0.1 mA, through deionized water, were recorded on each forearm and expressed in perfusion unit (PU) or multiple of baseline. The current application was delivered on each active probe either all-at-once (10 s) or in two consecutive 5-s applications with different intersimulation intervals. Four different intervals were tested: 15 s (1/4 min), 30 s (1/2 min), 1 min, 2 min, 4 min, 8 min and 16 min. The procedure is described on Figure 3.

Cardiovascular and temperature recordings

Blood pressure was continuously recorded from the left middle finger (Finapres, Ohmeda, Madison, WI, USA) with mean arterial pressure (MAP) obtained from the continuous blood pressure signal. The MAP signal was recorded at a sampling rate of 18 Hz using an analog to digital converter (MP100, Biopac sys, Goleta, USA) and analyzed offline using the Acknowledge® software V3.5.4 (Biopac sys, Goleta, USA).

Local skin temperature was measured using a surface thermocouple probe positioned 5 cm from any of the other electrodes [8]. The thermocouple was connected to an electronic thermometer (BAT-12, Physitemp Instruments, Inc., Clifton, NJ, USA).

Data and statistical analyses

Results are presented as mean ± SD, and for CVC, mediane (1^{rst} percentile, 3^{rd} percentile). Cutaneous temperature was expressed in °C.

Results for raw data were expressed in CVC or in multiple of baseline CVC values. CVC was calculated as the ratio of cutaneous blood flow to mean arterial pressure (mmHg) and expressed in PU/mmHg [10].

The values recorded before placebo or aspirin intake have been considered as the standard response.

For further analysis, the following points were determined for each subject and measurement:

- mean reference CVC (average of the CVC values during the first min of the reference period, CVC_{ref}),
- CVC_{stim2} corresponding to the CVC values 10 min after the end of the second current application for the segmented current applications or 10 min after the 10s current application during all-at-once delivery.

Parametric or non parametric tests were performed according to the distribution of values using SPSS (IBM SPSS statistics V15.0, Chicago, USA). Differences in CVC_{ref} and CVC_{stim2} were analyzed via the test of Wilcoxon using. Increases in CVC_{stim2} from baseline in function of the delivery mode and the interstimulation interval were compared via repeated measures ANOVA. When a significant difference was found, Tukey post hoc analysis was performed. For each statistical analysis, a two-tailed p value ≤ 0.05 was considered significant.

RESULTS:

Subjects were 22 ± 2 years old (10 males, 6 females). Mean weight and height were 68.1 ± 9.4 kg and 172.8 ± 9.5 cm, respectively. At the beginning of the protocol, their blood characteristics were the following: glycemia: 4.6 ± 0.5 mmol/L, LDL cholesterol: 2.7 ± 0.6 mmol/L, HDL cholesterol: 1.5 ± 0.3 mmol/L, creatinemia: 73.3 ± 9.2 µmol/L and number of platelets: 247.6 ± 49.8 (giga/L).

There was no significant change in cutaneous temperature during the measurement, whatever the day considered. Mean skin temperature ranged between 33.8 ± 1.5 to 34.6 ± 1.1 °C.

Table 1 presents the CVC values (CVC_{ref} and CVC_{stim2}) recorded in standard conditions, i.e. before any kind of treatment, and the CVC values observed after placebo or aspirin intake depending on the delivery mode of current application (10 s or 2 x 5 s with various intervals).

In standard condition, i.e. before treatment, CVC increased significantly from 0.08 (0.06, 0.11) to 0.22 (0.13, 0.42) PU/mmHg following an all-at-once 10 s continuous current application. When the current application was segmented (i.e. 2x5 s with different intervals), CVC also increased significantly from baseline whatever the interval considered. However, CVC_{stim2} was significantly greater when the total current charge was segmented compared to all-at-once delivery when the interval between the two 5 s current application was at least of 4 min. For example, CVC increased by 3.1 (2.5, 5.8) times for a 2x5 s current application with an interval of 1 min (non significant (NS) vs. all-at-once delivery). On the contrary, it increased by 9.4 (5.3, 10.9) times with an interval of 4 min ($p<0.05$ vs. all-at-once delivery). We did not observe any change in CVC_{stim2} amplitude when the interstimulation interval ranged between 4 and 16 min (p=NS).

Placebo treatment did not affect significantly the responses observed in standard conditions and the values recorded 14 days after placebo intake were not different from the standard response (Table 1).

Intake of 1g aspirin resulted in the disappearance of CIV following the all-at-once 10 s current application (Table 1). Two hours after intake, CVC_{ref} was 0.09 (0.06, 0.012) PU/mmHg and CVC_{stim2} was 0.11 (0.07, 0.15) PU/mmHg (NS vs. CVC_{ref}). Aspirin also abolished the CIV induced by segmented current application whatever the interval considered and there was no difference in CVC_{stim2} between the continuous 10 s current application and the segmented current application. For example, CVC increased by 1.2 (1.1, 1.3) times for all at once 10 s current application and by 1.5 (1.3, 1.7) times for a segmented current application with a 4 min interval.

Fourteen days after aspirin intake, values recorded were not different from those recorded before the treatment. Again, after current application (all at once or segmented), CVC increased significantly from baseline (Table 1). In addition, increase in CVC_{stim2} was significantly greater when the total current charge was segmented compared to all-at-once delivery when the interstimulation interval between was 4 min and more. CVC increased by 3.9 (3.4, 9.7) times for a 2 x 5 s current application with an interval of 2 min (NS vs. all at once); but it increased by 8.0 (5.9, 10.9) ($p<0.05$ vs. all at once) with an interval of 4 min.

We did not observe any change in CVC all along the different microvascular assessments at the reference probe (laser probe 3 on the right forearm and laser probe 4 on the left forearm).

DISCUSSION:

This study confirms that segmented application of cathodal low intensity (0.10 mA) current leads to a significant and greater vasodilation in healthy human skin compared to the one observed when the current total charge is applied all-at-once. This suggests that the first current application can activate a sensitization mechanism that is abolished by aspirin. The magnitude of this sensitization mechanism relies on the length of the interstimulation interval and a minimum of 4 minutes is necessary to observe a significant sensitization mechanism. Due to its sensitivity to aspirin, this mechanism likely relies on the release of prostaglandins.

CIV is probably based on an axon reflex [2, 11] but the mechanisms participating in CIV with all-at-once or segmented cathodal current application are not perfectly known [3]. CIV in response to anodal or cathodal current application (whatever the delivery mode) is abolished by aspirin suggesting the involvement of COD (prostaglandins?) in the vasodilator mechanism [5, 6]. Gohin *et al.* confirmed the participation of PGI2 in the CIV in response to a single prolonged (240 s) current application in rat skin [4]. PGE2 and PGI2 are powerful vasodilators on their own but prostaglandins are also able to sensitize the response of afferent unmyelinated fibres in animal [12, 13].

We previously hypothesized the participation of COD in the neural sensitization to anodal current application [5]. This would explain the amplified CIV observed for an amount of electrical charge delivered segmented compared to all-at-once [5]. This hypothesis was comforted latter at the cathodal level by the observation of Tartas *et al.* [6]. Durand *et al.* in supplementary material brought initially the evidence that this mechanism of sensitization was long lasting since still occurring 90 min after the first current application but yet its intensity was declining with time [5]. Using segmented current application (two sequences of anodal current, 0.1mA, 1 min with interstimulation interval of 5, 10, 20, 40, 60 or 90 min), we observed that the maximal amplification was observed for the 5 min interval at the anodal

level and progressively diminished with time. We hypothesized that the initial anodal current application had lead to the release of prostaglandins acting as sensitizing agent on the neural fibres involved in CIV leading to a large CIV when current application is repeated and that the progressive catabolism of prostaglandins would explain the blunting of this sensitizing mechanism [5]. At the cathodal level, the CIV is usually more important than the CIV at the anodal level for the same intensity and duration of current application [3]. Consequently, Tartas *et al*. used shorter segmented current application (two sequences of cathodal current, 0.10 mA, 10 s with interstimulation interval of 10, 20 or 40 min) to investigate the presence or not of the mechanism of sensitization at the cathodal level [6]. This mechanism was observed again with an amplitude in CIV, for a given total electric charge, superior when the current application was segmented compared to all-at-once. However, the authors did not report any difference in CIV amplitude whatever the interval considered. Considering the fact that cathodal current applications elicit more CIV than anodal current, this observation could be explained by an important and long lasting release of prostaglandins during the first current application leading to an important and long lasting mechanism of sensitization. However, some questions remain concerning this mechanism.

In the aim to better characterize this mechanism of sensitization at the cathodal level, we used half of the amount of electrical charge used by Tartas *et al*. (i.e. in our study two sequences of cathodal current, 0.10 mA, 5 s) and proposed shorter interstimulation interval (from 2 min to 16 min) [6]. The hypothesis here was that a minimal interval was necessary to have the mediators of sensitization released. We observed that after the second current application, a significant amplification of CIV, compared to CIV measured after a single all-at-once 10 s cathodal current application, appears for an intersimulation interval of 4 min. This amplification was abolished 2 hours after aspirin intake whatever the interstimulation interval considered. This result confirms what has been described for segmented anodal

current application. Indeed, we previously reported a disappearance of CIV following 2 x 1min current applications with a 10 min interstimulation interval 2 hours after aspirin intake [5]. COD like prostaglandins are known to act as sensitizing agent and to be aspirin sensitive. It is likely that the same kind of COD participate in the mechanism of sensitization at the anode and at the cathode. Consequently we hypothesized that a single 5 s cathodal current application is sufficient to induce a significant release of COD acting as sensitizing agents when a cathodal current application is repeated. A minimal delay between two monopolar current applications has to be respected to observe the sensitizing effect of COD leading to amplification of CIV (4 min in our experimental conditions). The exact nature of these COD remains to be determined.

In conclusion, several tests exist to investigate cutaneous vasomotor function. For example, post occlusive reactive hyperemia stimulates both the endothelial and the non-endothelial pathway [14, 15]. Sodium nitroprusside and acetylcholine iontophoresis investigate the non-endothelial and the endothelial pathways, respectively [9, 10, 16, 17]. Gohin et al. proposed to use CIV to investigate the vascular function of prostacyclin in rats [4]. We confirm the involvement of COD in CIV and the existence of a sensitizing mechanism leading to amplification of CIV when cathodal current application is repeated. The same observation has been previously made for segmented anodal current application. This suggests that the study of CIV in response to segmented anodal or cathodal current application, when the appropriate charge is applied and a minimal interstimulation interval respected, can be an attractive way to study the neural sensitizing function of microcirculation in humans.

Limits

This study has been conducted in young and healthy subjects. We cannot exclude a change in the amplitude and mechanisms in other populations such as elderly people, diabetic subjects or patients suffering from cardiovascular disease. Furthermore, to guarantee the same cardiovascular state for determination of CVC, all measurements (i.e. the tests of the different interstimulation intervals) have been performed during the same experimental session on the two arms. It could be consequently hypothesized that the different current applications performed could interfere one over another on a same arm and that the responses could differ between the two arms. However, sites of current applications on the forearms were randomized and the current was locally applied. There is no report of a difference in CIV in function of the arm (left or right), there was no change in CVC at the reference probe and we clearly observed an influence of the interstimulation interval on the apparition of CIV. All these elements lead us to the conclusion that there was no interference between the different current applications.

LIST OF SYMBOLS AND ABBREVIATIONS

CIV: Current Induced Vasodilation

CVC: Cutaneous Vascular Conductance

COD: Cyclo-Oxygenase Derivates

PGE2: Prostaglandin E2

PGI2: Prostaglandin I2

NS: Non Significant

AUTHORS CONTRIBUTIONS

Conceived and designed the experiments: SD, GM, PA. Performed the experiments: LG, GM Analyzed the data: SD, GM. Wrote the paper: SD, GM. Statistical analysis: SD, GM. Final approval of the article: SD, GM, AH, LG, GL, PA.

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STATEMENT OF CONFLICTS OF INTEREST

"All authors have completed the Unified Competing Interest form at http://www.icmje.org/coi_disclosure.pdf (available on request from the corresponding author) and declare: no support from any organisation for the submitted work; no financial relationships with any organisations that might have an interest in the submitted work in the previous 3 years; no other relationships or activities that could appear to have influenced the submitted work ."

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TABLE 1

Amplitude of CVC observed at rest (CVC_{ref}) 10 minutes after the end of the last current application (CVC_{stim2}) of a segmented 2 x 5 s cathodal current application, in the standard conditions and after placebo or aspirin intake. CVC is expressed in Perfusion Unit. * means p <0.05 vs. corresponding CVC_{ref}.

Type of stimulation (0.10 mA)	Standard Conditions		Placebo				Aspirin			
			H2		D14		H2		D14	
	CVC_{ref}	CVC_{stim2}	CVC_{ref}	CVC_{stim2}	CVC_{ref}	CVC_{stim2}	CVC_{ref}	CVC_{stim2}	CVC_{ref}	CVC_{stim2}
10s	0.08 (0.06,0.11)	0.22 (0.13,0.42)*	0.08 (0.07,0.10)	0.20 (0.11,0.52)*	0.09 (0.06,0.11)	0.35 (0.09,0.66)*	0.09 (0.06,0.12)	0.11 (0.07,0.15)	0.08 (0.07,0.13)	0.27 (0.16,0.41)*
2 x 5 s (Interval 0.25 min)	0.08 (0.07,0.11)	0.47 (0.22,0.67)*	0.08 (0.07,0.10)	0.18 (0.14,0.34)*	0.07 (0.06,0.11)	0.27 (0.16,0.61)*	0.09 (0.08,0.11)	0.10 (0.09,0.12)	0.07 (0.06,0.09)	0.27 (0.15,0.47)*
2 x 5 s (Interval 0.5 min)	0.07 (0.05,0.12)	0.18 (0.10,0.80)*	0.11 (0.07,0.17)	0.30 (0.19,0.78)*	0.10 (0.08,0.11)	0.55 (0.28,1.15)*	0.11 (0.09,0.14)	0.14 (0.11,0.18)	0.08 (0.07,0.11)	0.32 (0.21,0.46)*
2 x 5 s (Interval 1 min)	0.12 (0.08,0.16)	0.47 (0.29,0.66)*	0.14 (0.12,0.16)	0.41 (0.21,0.60)*	0.12 (0.09,0.16)	0.87 (0.42,1.22)*	0.15 (0.12,0.21)	0.19 (0.15,0.26)	0.13 (0.08,0.19)	0.40 (0.33,0.79)*
2 x 5 s (Interval 2 min)	0.08 (0.06,0.10)	0.52 (0.32,0.75)*	0.11 (0.08,0.12)	0.58 (0.21,0.87)*	0.12 (0.08,0.13)	0.93 (0.47,1.35)*	0.11 (0.09,0.14)	0.17 (0.10,0.19)	0.08 (0.07,0.11)	0.41 (0.24,0.66)*
2 x 5 s (Interval 4 min)	0.13 (0.09,0.18)	1.17 (0.77,2.10)*	0.22 (0.15,0.30)	1.09 (0.94,2.22)*	0.15 (0.12,0.19)	1.23 (1.01,1.72)*	0.19 (0.14,0.24)	0.23 (0.20,0.38)	0.15 (0.10,0.18)	1.34 (0.75,1.57)*
2 x 5 s (Interval 8 min)	0.12 (0.09,0.15)	1.17 (0.91,1.53)*	0.12 (0.10,0.17)	0.96 (0.46,1.15)*	0.12 (0.09,0.18)	1.52 (0.93,1.92)*	0.14 (0.10,0.17)	0.24 (0.16,0.29)	0.11 (0.09,0.15)	1.00 (0.69,1.14)*
2 x 5 s (Interval 16 min)	0.13 (0.10,0.20)	1.28 (0.91,1.49)*	0.17 (0.10,0.27)	1.08 (0.54,1.68)*	0.16 (0.11,0.19)	1.29 (1.04,1.75)*	0.21 (0.14,0.26)	0.33 (0.20,0.44)	0.16 (0.10,0.21)	1.31 (1.10,1.51)*

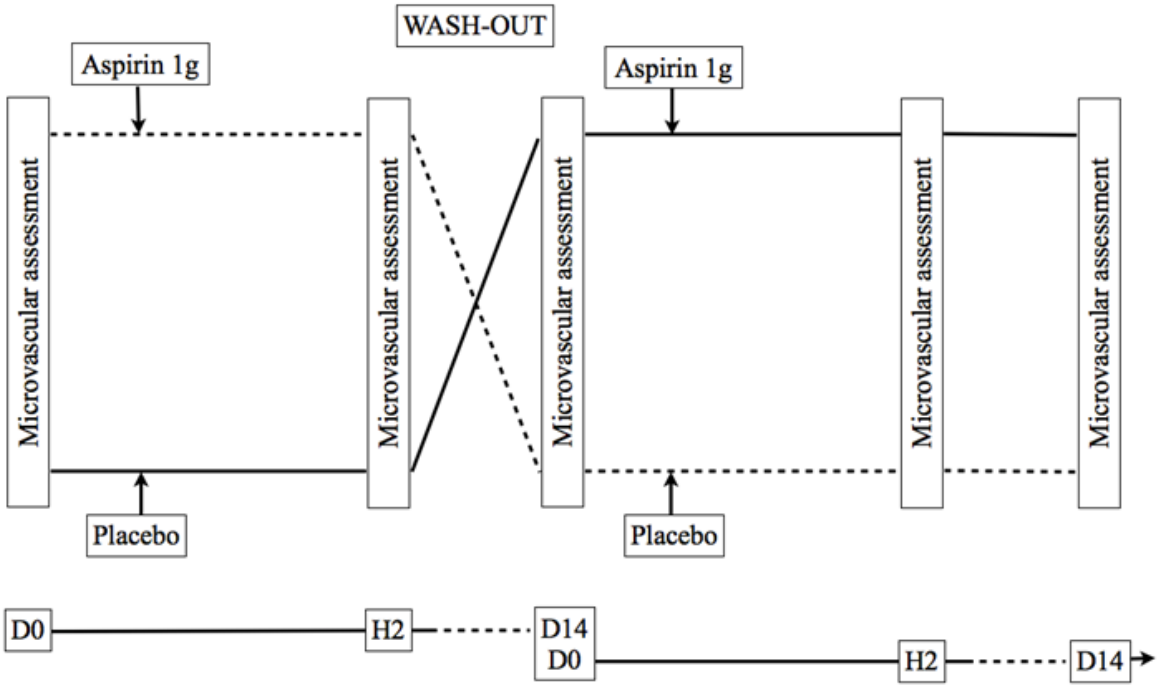

Fig. 1: Design of the study. D0 means day of treatment. H2 means time of measurement 2 hours after treatment, D14 means time of measurement 14 days after treatment.

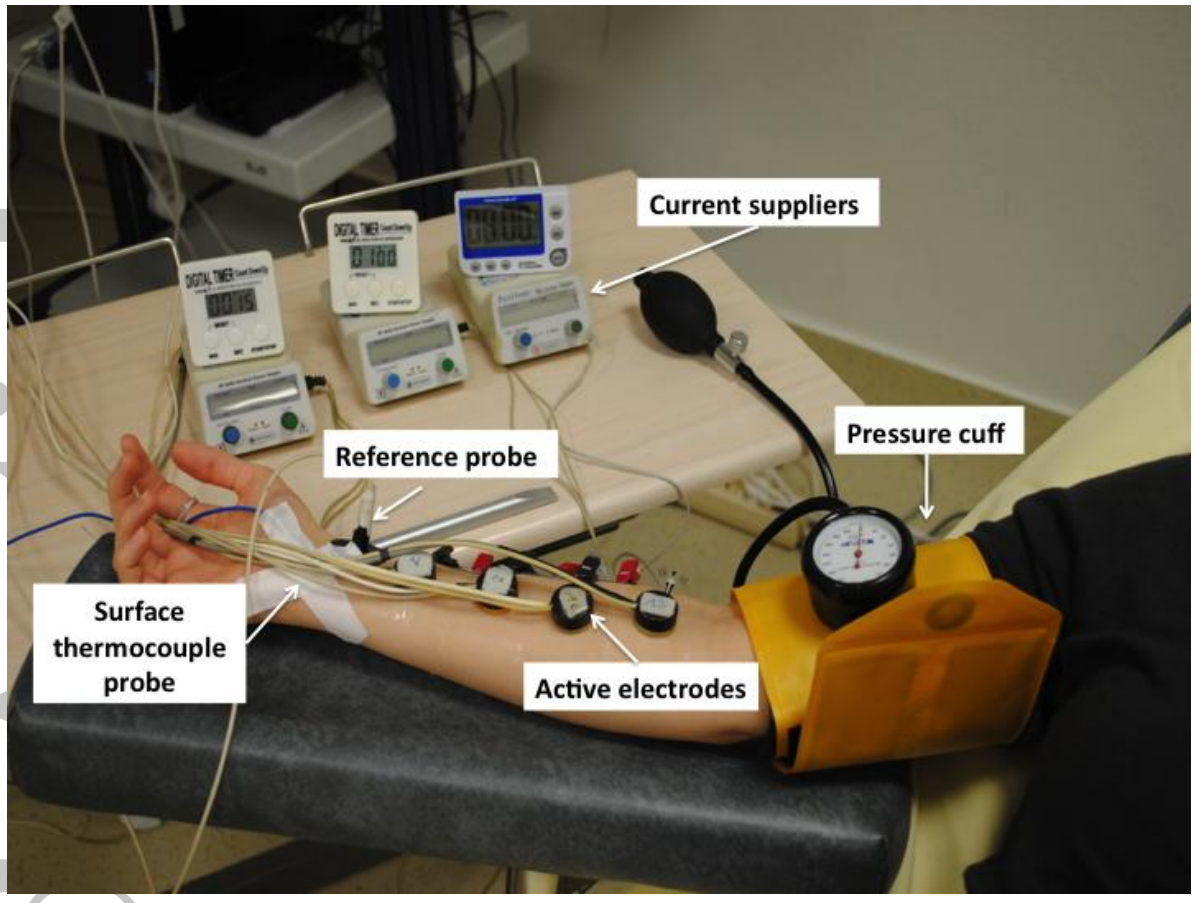

Fig. 2: Experimental setup (for clarity, only one arm is presented)

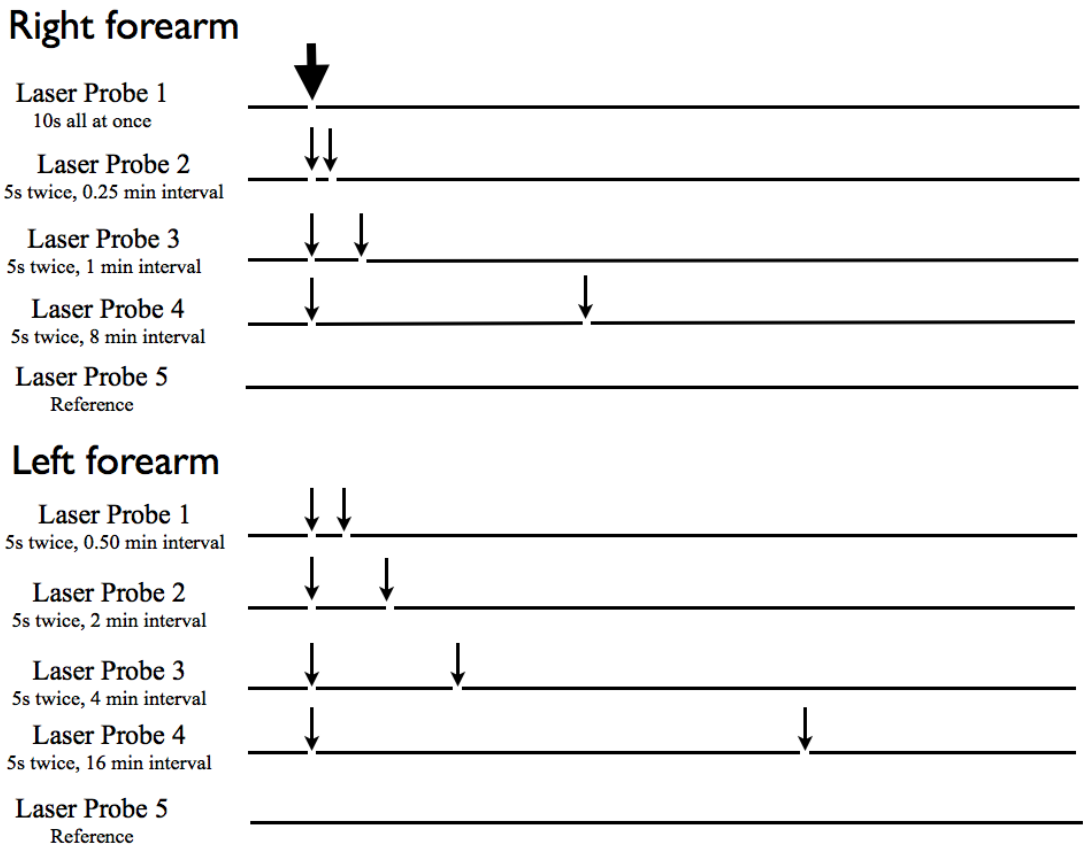

Fig. 3: Protocol