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Caffeine, gravity and baroreceptor function: the integration of diet and cardiovascular control

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ABSTRACT

We describe a simple, cost-effective experiment to demonstrate cardiovascular integration of heart rate and blood pressure to accommodate the environmental and dietary factors of gravity and caffeine. Specific learning objectives associated with this include understanding the effects of posture on blood pressure and heart rate, coupled with the role of caffeine in modifying this response. Inclusion of ECG measurements coupled with heart rate variability analysis added a demonstration of the contribution made by the autonomic nervous system under these conditions. We clearly demonstrate that the cardiac work, estimated as rate-pressure product, necessary to undertake the transition from supine to standing is fixed for a given group of subjects. However, the individual contribution of heart rate and systolic pressure to the cardiac workload are subject to the external factors of gravity and caffeine. Such an activity also demonstrates additional benefits including unstructured teaching opportunities to augment classroom learning associated with integrative physiology and also the discussion of ethical issues with regard to human experimentation.

KEYWORDS: Caffeine, orthostasis, autonomic nervous system, blood pressure, cardiac work

ABBREVIATIONS

ANS	Autonomic Nervous System
BP	Blood Pressure
CO	Cardiac Output
DP	Diastolic Pressure
ECG	Electrocardiogram
HF	High Frequency
HR	Heart Rate
LF	Low Frequency
MAP	Mean Arterial Pressure
PNS	Parasympathetic Nervous System
SNS	Sympathetic Nervous System
SP	Systolic Pressure
SV	Stroke Volume
SVB	Sympathovagal Balance
TPR	Total Peripheral Resistance
Δ	Change

INTRODUCTION

The teaching of complex ideas in physiology to undergraduate students presents increasing challenges with larger cohorts and decreases in time available for practical activities. In addition, teaching physiology to applied science students presents additional problems as students benefit from addressing practical, study-relevant problems. These factors are all of particular importance for students studying nutrition and other medical sciences. Developing focused practical demonstrations on the integration of physiology may be a valuable mechanism to improve student learning. This practical class was devised to address many issues: firstly, to introduce the concept of integration of multiple competing stimuli, including gravity and caffeine, using low-cost equipment. Secondly, this activity was designed to remove the anxiety engendered by working in large groups, allowing the students to work in smaller groups (2-3 students) and develop independent problem-solving skills.

Manipulating the external environment to demonstrate physiological concepts traditionally requires expensive equipment; including the use of tilt-tables [1], lower-body negative pressure [8] or neck suction [18]. We demonstrate simpler and cheaper methods to manipulate the external environment in an integrated manner to investigate control of the cardiovascular system. These activities will quantify the effect of both gravity and caffeine on blood pressure (BP) and heart rate (HR), with estimates of sympathovagal balance to quantify the contribution from the autonomic NS for cardiovascular control [6].

Background

The brain is critically-sensitive to changes in oxygen tension therefore preservation of cerebral perfusion is essential. External stimuli that alter the distribution of blood can decrease the provision of blood to the brain. Key to preserving cerebral perfusion are the carotid and aortic baroreceptors forming part of the afferent arm of the autonomic nervous system (ANS) [9]. A reduction in blood pressure, is compensated through changes to heart rate (HR), stroke volume (SV; the product of which is cardiac output; CO) and peripheral resistance (TPR). However, this system is reactive rather than predictive and is subject to external stimuli [7].

Cardiac function can be modulated by the contributions from the sympathetic (SNS) and parasympathetic (PNS) branches of the ANS (Fig.1). The pressor responses of the SNS facilitate tachycardia, increases in SV and vascular tone (Fig.1), increasing both CO and BP. The PNS contributes depressor responses that affect HR predominantly, thus increases in HR may result from reduction of PNS tone.

Changes in posture alter the effect of gravity on the CVS. Moving from supine to standing initially triggers the pooling of blood in the lower extremities, decreasing venous return [16]. This reduction in ventricular filling diminishes the SV by Starling's law of the heart and decreases CO and mean arterial blood pressure (MABP; Fig.1). To restore BP and hence cerebral perfusion, heart rate is increased to augment cardiac output (CO). Second, venoconstriction then augments the venous return improving ventricular filling [13]. Hence, standing is characterised by transient tachycardia, and a modest increase in CO. Prolonged standing results in a sustained increase in sympathetic tone [10], increasing peripheral resistance maintaining elevated MABP [20]. Tachycardia is sustained to support increased CO and hence cerebral oxygenation is maintained [15].

Dietary components, such as caffeine, modulate cardiovascular responses. Caffeine consumption provides direct antagonism to the vascular effects of endogenously-released adenosine. Given that adenosine is a mediator of vasodilator nitric oxide (NO) release, the predicted effects of caffeine include vasoconstriction, increasing total peripheral resistance and therefore increased blood pressure [2]. Using the existing knowledge they had, the students were asked to devise a hypothesis to test during the activity, students anticipated:

1. Posture change (gravity) would increase both blood pressure and HR.
2. The stimulant properties of caffeine would result in a further augmentation of both blood pressure and HR in both the supine and standing positions.

Learning Objectives

After completing the activity students will be able to:

1. Describe the effects of postural change on blood pressure and heart rate.
2. Describe the effects of caffeine on both blood pressure and heart rate.
3. Explain the integration between posture and caffeine on cardiovascular control.
4. Estimate the effects of the autonomic NS on control of HR.

Activity Level

This activity has been exploited for Year 2 Nutrition students but would be equally applicable to other biomedical or physiological science students. The current intake represents 35 students/year. In our experience, these students possess a comprehension of human biology at senior/secondary school level with no experience of human experimentation. The Year 1 physiology course is designed to develop the same comprehension of biology across the entire Food Science/Nutrition course structure at the University of Leeds and regularly accepts students that have not studied biology beyond intermediate/GCSE level.

MATERIALS & METHODS

EQUIPMENT

Standard automated blood pressure cuffs that occluded the brachial artery at the height of the left ventricle were used (Omron Healthcare Inc., Bannockburn, Illinois, USA). Pulse rate was estimated using a pulse transducer (TN1012, AD Instruments, Oxford) and cardiac electrical activity was estimated using a BioAmp (AD Instruments, Oxford) to record ECG. All data was collected by a datalogger (Powerlab 4/30; AD Instruments, Oxford) and stored to computer for further analysis. Caffeine tablets (50mg caffeine per tablet; Bayer PLC, Newbury, Berks, UK) were purchased.

Time required

The measurements require a total of 90min to collect, including a 30min break for the assimilation of caffeine [3]. Typically, with efficient time utilisation, during a 3hr practical class with three students per group, all can complete the activity.

METHODS

Ethical Review

All experiments were examined and approved by the Maths and Physical Sciences and Engineering joint Faculty Research Ethics Committee (MEEC), University of Leeds (Review Number: MEEC 15-023). All students gave signed, informed, consent to undertake the experiment. Steps were taken to anonymise the data. All students were free to withdraw at any point and were under no obligation to take part. The students taking part in the study were non-smokers; criteria for exclusion from the experiments included existing cardiac and kidney disease, diabetes, hypertension and Raynaud's disease. All students participating in the practical study were well-rested, and had not eaten within at least 2hr. The students were not habitual consumers of caffeine (<2 caffeine-containing beverages/day) and had abstained from caffeine during the previous 12hr and had not undertaken vigorous exercise within the previous 24hr period.

Subject anonymity

All students taking part were allotted a subject number and the data collected written onto individual data sheets identified by this number. This data was then collated by the class tutor and amalgamated into a single dataset for which students were randomly re-allocated a number such that no individual was identifiable.

Experimental Protocol

Anthropomorphic measurements including height and body mass were recorded. Body composition was estimated using bioimpedance analysis scales (Omron Healthcare Inc., Bannockburn, Illinois, USA). All subjects were instrumented to record pulse amplitude, blood pressure and ECG. ECG was recorded using 3-lead ECG recording equipment (Bio-Amp, AD Instruments, Oxford) attached by adhesive electrodes to the inner surface of the forearms and one ankle. A pulse transducer (TN1012, AD Instruments, Oxford) was attached to the thumb of the dominant hand to record pulse pressure amplitude. All data was recorded *via* a datalogger (PowerLab 4/30, AD Instruments, Oxford) to computer for further analysis.

Active Stand Test

The Active Stand Test (AST) was performed as outlined previously [16,13]. Briefly, subjects lay in the supine position on a firm, non-slip surface in a relaxed position. Periodically ECG, pulse and blood pressure were recorded (Fig. 2) and the subjects remained in this position for 20 min. At 20 min, students were asked to stand, in a swift, continuous motion, without the use of the hands and to remain standing without moving in a relaxed position, feet approximately shoulder-width apart. Students remained standing still for a further 15 min. ECG, blood pressure and pulse amplitude were measured continuously (Fig. 2).

At the end of the protocol students consumed caffeine (150 mg/subject) with sugar-free cordial and asked to rest for 30 min [3] before the AST was repeated. Hence, each subject provided a reference control for comparison with the effects of caffeine.

Data Analysis

Using 30 s averages, heart rate (HR) was measured from raw traces, with mean arterial pressure (MAP) calculated ($MAP = [SP - DP] / 3 + DP$) and rate-pressure product (RPP) calculated as systolic pressure x heart rate. Pulse pressure was calculated as $[SP - DP]$. Heart rate variability (HRV) was estimated from ECG using proprietary software (Chart 8.0, AD Instruments, Oxford). Optimal settings for well-defined R waves were as follows: range 2 mV, high pass 0.3 Hz, low pass 50 Hz, sampling rate 1 kHz. The trace was used to calculate (beats min^{-1}) and R–R intervals (ms), as well as the relative duration of the cardiac cycle components (sampled at 1 kHz). A minimum of 250 consecutive heart beats were examined.

Data are presented as mean \pm SD; statistical analysis was carried out using a 'repeated measures, 3-factor, analysis of variance' (ANOVA) (time vs posture vs supplement) to quantify the changes occurring during the transition from supine to standing for HR, BP and RPP and measures of heart rate variability were quantified using a 2-factor ANOVA (posture vs supplement). Alpha value for ANOVA analysis was set at $\alpha = 0.05$. Tukey's *post hoc* testing was used where appropriate.

Safety considerations

Subjects were excluded for the following reasons:

1. Pre-existing cardiovascular or cardiac disease
2. Implantation of a pacemaker
3. Students taking prescribed medications
4. Approximately 5% of subjects (from our estimates) develop postural hypotension during the prolonged stand and therefore experimenters are made aware of potential indicators

RESULTS

Anthropomorphic measurements

We present representative data from one year of these student activities. During this period 20 students consented to undertake the activity, representing 80% of those attending the class. The subjects for the experiments reflected the student population studying nutrition science at the University of Leeds (age=21.6±5.7 years) and 85% of the subjects were female (Table 1). Mean height (167±2 cm) and body mass (60.8±11.3 kg) indicated that, by calculation, our subjects all presented with a normal BMI (21.6±2.6 kg m²; Table 1). By calculation, the caffeine consumption during the experiment was recorded as 2.5±0.39 mg kg⁻¹ (Table 1) and reflects the consumption of one caffeine-containing beverage. Self-reported estimates of sleep (7.4±1.1 h) duration imply that subjects undertaking the activity were well-rested (Table 1).

Postural change

Change in posture to standing had no effect on SP (NS; Fig.3a), but the transition increased DP suggesting an increase in total peripheral resistance (TPR) (p=0.0046; Fig. 3b). This was accompanied by an increase in HR ~33% and this was sustained throughout the standing period (p=0.0002; Fig.3c) suggesting an increase in cardiac work [10]. By calculation the MAP following standing was increased, and sustained throughout the stand (Fig. 3d; p=0.018) indicating increased vascular resistance and cardiac work [20]. Standing decreased pulse pressure (42.6±2.4mmHg for supine, 37.2±2.7mmHg for standing; p<0.046) indicating a possible decrease in stroke volume (SV) as a consequence of decreased ventricular filling [11]. Furthermore, the calculated rate-pressure product, an estimate of cardiac work [19], was also increased following standing (p=0.0005; Fig.4) and supporting the increased cardiac work to sustain cerebral perfusion throughout standing.

Caffeine supplementation

Caffeine supplementation had no effect on SP at rest or during the standing (Fig.3a); however during prolonged standing caffeine increased DP 10% (p=0.013; Fig.3b) implying a greater increase in TPR than standing alone. Caffeine had no effect on HR in the supine or standing position (p=0.34 for supine; p=0.25 for standing; Fig.3c). MAP was unaffected by caffeine in supine subjects however on standing caffeine increased MAP further than standing alone (p=0.046; Fig.3d) suggesting either increased cardiac work or TPR following caffeine intake, yet we note no increase in RPP following caffeine supplementation (Fig.4) implying no further increase in cardiac work, confirming increases in TPR by caffeine. For all cardiovascular parameters there was no additional effect from combining both postural change and caffeine supplementation (Table 2).

Heart rate variability (HRV)

Sympathovagal balance, estimated from ECG analysis indicates that on standing, total frequency estimates fell and were significantly different after prolonged stand ($p=0.025$; Table 3), this was accounted for by a decrease in the high frequency component achieving 25% of supine HF during prolonged standing ($p=0.0005$; Table 3) suggesting a decrease in parasympathetic contribution to cardiac activity. In consequence, standing increased LF/HF ratio ($p=0.0008$; Table 3), implying a shift in sympathovagal balance in favour of sympathetic contribution. Caffeine had no effect on estimates of total frequency output; however with no change in low frequency output ($p=0.13$; Table 3) and a decrease in high frequency contribution ($p=0.026$; Table 3) after prolonged standing indicated an increase in LF/HF ratio ($p=0.0025$; Table 3) suggesting shifts in favour sympathetic contribution to cardiac contractility and this was manifest as the increase in HR noted on standing ($p=0.0002$; Table 3). To address the role of SNS and PNS on the control of heart rate we have estimated the change in LF and HF (ΔLF and ΔHF respectively) with change in HR (ΔHR). We note a negative correlation between ΔHF and ΔHR implicating a decrease in the parasympathetic NS contribution with increasing HR (Fig.5a; $p=0.0001$).

DISCUSSION

Changes to blood pressure are governed by three factors: HR, SV and TPR [12]. This experiment offers students the opportunity to explore changes to HR and TPR in the context of environmental and dietary stressors to develop an understanding of the interplay between these factors. The experiment demonstrates clearly that the transition from supine to standing is accompanied by increases in both blood pressure and heart rate to overcome the external force, gravity. The increase in cardiac work, estimated as RPP, is a consequence of increased TPR (to ameliorate venous pooling) AND the increased work to overcome the effects of gravity. The changes to blood pressure are a consequence of increases in vascular tone measured as increased diastolic pressure [13]. The contribution from increased LF/HF ratio to increase cardiac work was sustained throughout the standing period indicative of increased sympathetic NS outflow needed to sustain cerebral perfusion [20].

Further manipulation of the vascular tone by supplementation with caffeine increased peripheral resistance following prolonged standing, estimated as increases in diastolic pressure and MAP [17,19]. Together, these data demonstrate that independent of posture, rate-pressure product (the product of systolic pressure x HR) is preserved despite the combination of caffeine and the increased contribution to cardiac work from gravity and thus demonstrates effective cardiovascular control.

Estimation of the HRV is beneficial in exploring the contribution that the autonomic NS makes to controlling cerebral perfusion. Whilst HRV only quantifies the contribution of autonomic control to cardiac performance this demonstrated that for a young adult population change in HR is sustained through decreases in depressor effects of parasympathetic tone (estimated as decreases in HF contribution) with little or no change to sympathetic tone [21]. Therefore, the endogenous rhythmogenic potential of the sino-atrial node (SAN) makes a greater contribution to determining HR. We also note that the magnitude of the reduction in depressor activity is the same for untreated or caffeine-supplemented subjects on standing, and after prolonged stand [14]. Together our results suggest that caffeine-mediated increases in blood pressure – as a consequence of adenosine antagonism – are matched with increases in basal parasympathetic tone, decreasing HR and thus preserve RPP [4]. Furthermore, we note a negative correlation between Δ HF and Δ HR with subjects sustaining the greatest decrease in HR also recorded the greatest increase in HF contribution, supporting our conclusions.

Whilst we cannot directly determine whether this is a primary effect of caffeine or a secondary consequence of increases in peripheral resistance this does demonstrate the integrative response of the cardiovascular system.

An unintended, but useful, consequence of this activity was the opportunity for unstructured teaching for students. The practical class offered opportunities to raise points of discussion on individual topics and removed the social pressures of teaching in a large group: students were more open to discussion and engaged more readily with the critical thinking questions (below). Critical to the use of practical class teaching is demonstration of the relevance to learning. For a cohort of students undertaking the activity we can demonstrate that the students found the exercise useful in understanding the topic and that the activity augmented classroom learning (table 4). The students also directly benefited from a visual demonstration of physiological concepts. We intentionally kept the individual group size small (3 students) to give opportunities for more dedicated learning and to reduce the pressures associated with large class sizes.

Critical thinking questions

1) Explain the physiological changes noted during the transition from lying to prolonged standing: Supine subjects show adequate cerebral perfusion with a low cardiac output (CO) with low vascular tone. On standing, the blood that has pooled in the extremities is more difficult to return to the myocardium as the influence of gravity 'resists' the return. The greater proportion of this blood accumulates in the capacitance vessels (veins) with a potential large volume in these distensible vessels. As a consequence, venous return, to fill the right atrium, is decreased. Therefore, the cardiac output for consecutive heartbeats is reduced, and the left ventricle outflow is also diminished.

2) What effect would you predict for caffeine? For untreated students, the transition from supine (a position of low vascular tone) to standing produces a pooling of the venous blood in the extremities below the ventricle. This initially decreases venous return triggering a tachycardia and subsequent hypertension. The increased sympathetic tone, leading to increased vascular resistance, increases venous return to the heart (by decreasing the bed volume of the capacitance vessels). Caffeine, acting as an adenosine antagonist, will increase vasoconstriction resulting in increases in TPR, potentially increasing MAP.

3) With reference to arterial and carotid baroreceptors explain the sequence of events required to control blood pressure on prolonged standing. Standing decreases venous return and hence SV decreases. This triggers an 'unloading' of the baroreceptors within the aortic arch and carotid artery. The decrease in MAP sensed by the baroreceptor as a decrease in the 'stretch' of the vessel wall. As a compensation, this triggers an increase in HR through decreases in parasympathetic tone and potentially increases in sympathetic tone. This compensation is incomplete as the volume ejected from the ventricle remains less, therefore to increase ventricular filling further, venous tone is increased partially restoring ventricular filling. This is sustained throughout the prolonged stand to overcome the redistribution of blood caused by gravity.

4) Why does the prolonged stand place significant additional stresses on the cardiovascular system? Prolonged standing increases the volume of fluid that pools in the capacitance vessels in the lower extremities. Therefore, this decreases the available blood to contribute to the circulating volume in the cardiovascular system. Critical to this effect is 'standing still'. With prolonged standing the stimulus to 'fidget' and move the legs is strong to facilitate the skeletal muscle pump in the lower extremities to augment the return of blood towards the heart.

Wider educational outcomes

1) Understanding the ethical considerations associated with human experimentation. This activity offers an opportunity to outline the need for ethical review of an experimental procedure and potential steps to anonymise data. Students are given an opportunity to examine the practical aspects for anonymising data and how subject confidentiality can be maintained. Informed consent can also be investigated, understanding the quantity and depth of relevant information that subjects will require to give 'informed consent'.

2) 'Big data'. With the combination of datasets from future years this may build into a large database that may be interrogated in the future to explore how gender, age, body composition or habitual caffeine consumption may alter baroreceptor function and autonomic NS control. Further analysis may be possible to examine the contribution that height, body mass or BMI may contribute to blood pressure or cardiac work (estimated as RPP). With a more even gender balance the effects of gender on RPP or blood pressure may also be quantified.

CONCLUDING REMARKS

We demonstrate a simple experiment exploiting cheap and readily-available equipment (automated sphygmomanometer blood pressure cuff) to investigate the effects of gravity (**Learning outcome 1**) and nutritional components (caffeine-**Learning outcome 2**) on the control of blood pressure. With expansion of the experiment to measure ECG and therefore calculate HRV the integration of the autonomic NS with control of HR and BP can also be investigated (**Learning outcomes 3 & 4**). Together these observations develop a clearer understanding of the integration of cardiovascular responses to match cerebral perfusion dependent upon environmental stimuli. The unstructured nature of the classroom setting gives opportunities for discussion of associated topics to broaden the scope of the learning activities and also develops an understanding of the ethical considerations associated with human experimentation.

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DISCLOSURES

No conflict of interests, financial or otherwise, are declared by the authors.

TABLES

Parameter	Measurement
<i>Age (years)</i>	21.6 ± 5.7
<i>Subjects (M/F)</i>	20 (M=3/F=17)
<i>Body Mass (kg)</i>	60.8 ± 11.3
<i>Height (cm)</i>	167 ± 7.7
<i>Body Mass Index (kg/m²)</i>	21.6 ± 2.6
<i>Daily caffeine consumption (drinks/day)</i>	1.95 ± 2.03
<i>Caffeine Dose (mg/kg body mass)</i>	2.50 ± 0.39
<u><i>Exercise</i></u>	
<i>Endurance (hr/week)</i>	3.3 ± 3.0
<i>Conditioning (hr/week)</i>	2.0 ± 2.0
<i>Sleep (hr/night)</i>	7.4 ± 1.1

Table 1: Anthropometric characterisation of subjects. Data represents Mean ± SD for n=20 subjects.

Measurement	Posture	Caffeine	Posture x Caffeine
Systolic Pressure	<i>p=0.163</i>	<i>p=0.047</i>	<i>p=0.99</i>
Diastolic Pressure	<i>p=0.00001</i>	<i>p=0.00001</i>	<i>p=0.98</i>
Heart Rate	<i>p=0.00001</i>	<i>p=0.0039</i>	<i>p=0.98</i>
Mean Arterial Pressure	<i>p=0.00001</i>	<i>p=0.00082</i>	<i>p=0.99</i>
Rate-pressure product	<i>p=0.00001</i>	<i>p=0.192</i>	<i>p=0.99</i>

Table 2: Summary of the effect of postural change and caffeine on cardiovascular parameters analysed by ANOVA. Data represents 2-way ANOVA analysis between subject factors (posture vs caffeine). For further details see methods.

Question	Score
Did you attend the practical class? (YES/NO)	19/2
Were you a subject for the experiment? (YES/NO)	14/7
Did the class help your understanding of physiology?	4.50 ± 0.69
Was the lecture material given beforehand helpful in understanding the class?	4.20 ± 0.62
Should the practical class remain in the Module	4.55 ± 0.60
Did a 'visual demonstration' of the physiology help your understanding?	4.53 ± 0.77
Was the smaller class size beneficial to your understanding?	4.53 ± 0.61
Should we keep the Group size small?	4.65 ± 0.59
Was the opportunity to analyse & interpret data beneficial?	4.20 ± 0.62
Would you rate the practical class as beneficial to the Module?	4.55 ± 0.51
Total number of responses	21

Table 4: Student evaluation questionnaire of the value of practical demonstrations for estimating the effects of both posture and caffeine on baroreceptor function. Student feedback was quantified by scoring each question 1-5; 1=strongly disagree; 5=strongly agree. Data represents Mean ± SD for n=21 students.

FIGURE LEGENDS

Fig.1: Diagram illustrating the baroreceptor reflex and control of blood pressure and heart rate. NTS= nucleus of tractus solitarius; NS=nervous system.

Fig.2: Schematic diagram describing the experimental protocol. For each experiment students worked in groups of 3; one as subject and the remaining students recording data and tabulating results. For further details, see methods.

Fig.3: Effects of postural change and caffeine supplementation on cardiovascular parameters. Effects of postural change and caffeine were measured on systolic blood pressure [A], diastolic blood pressure [B], heart rate [C] and calculated mean arterial pressure [D]. For further details, see methods. Data represents Mean \pm SD (for n=20 subjects). Statistical significance represented as: Effects of caffeine supplementation; * p<0.05, ** p<0.01, *** p<0.001: Effect of standing; †† p<0.01, ††† p<0.001.

Fig.4: Effect of postural change and caffeine supplementation on cardiac rate-pressure product estimates. Rate-pressure product was calculated as [systolic pressure x heart rate] for individual subjects at each point. Data represents Mean \pm SD (for n=20 subjects). Statistical significance represented as: Effect of standing; †† p<0.01, ††† p<0.001.

Fig.5: Correlation between changes in heart rate after caffeine consumption with changes to autonomic NS. Contribution of High Frequency changes [A]; contribution of Low Frequency changes [B]. For further details, see methods.

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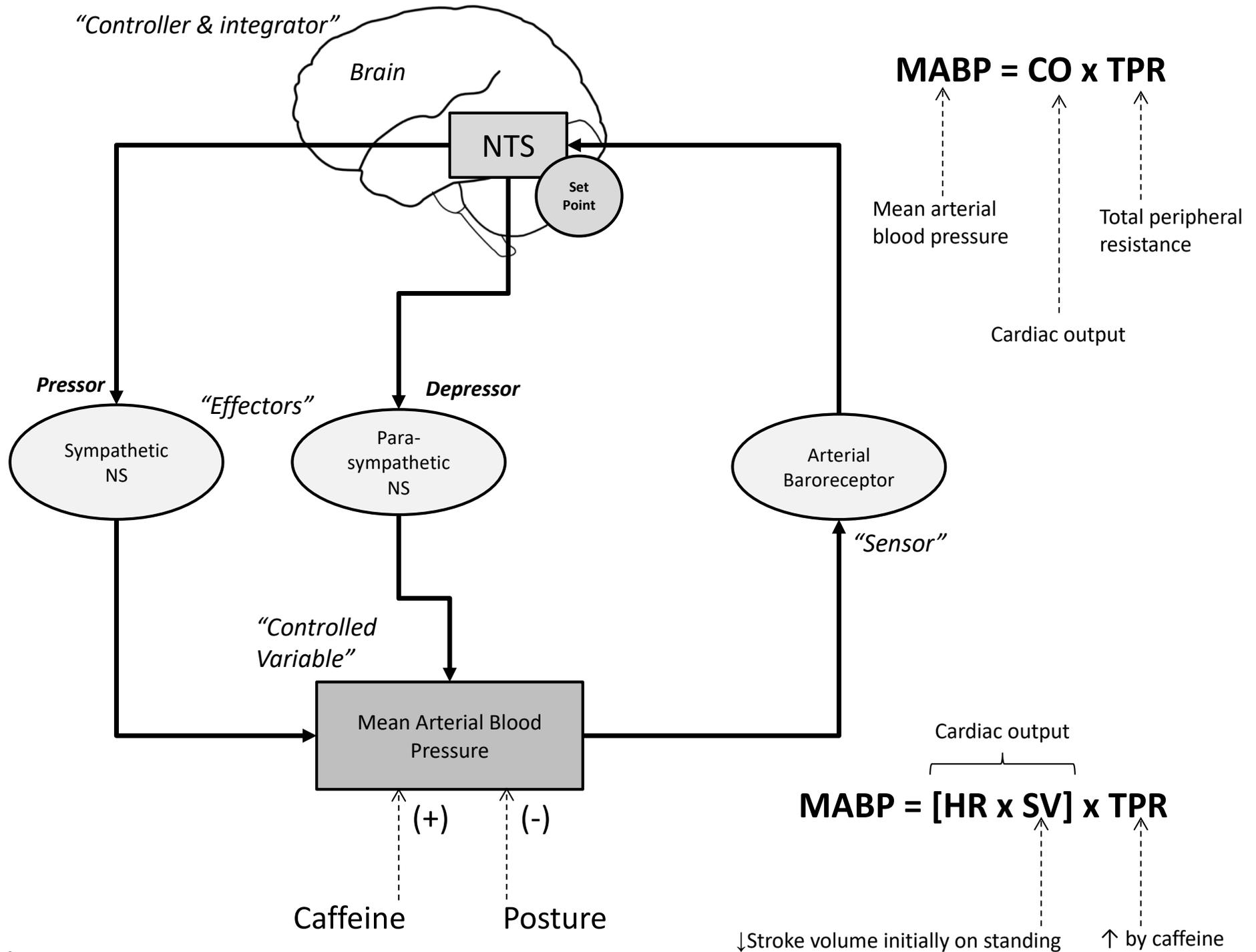


Fig.1

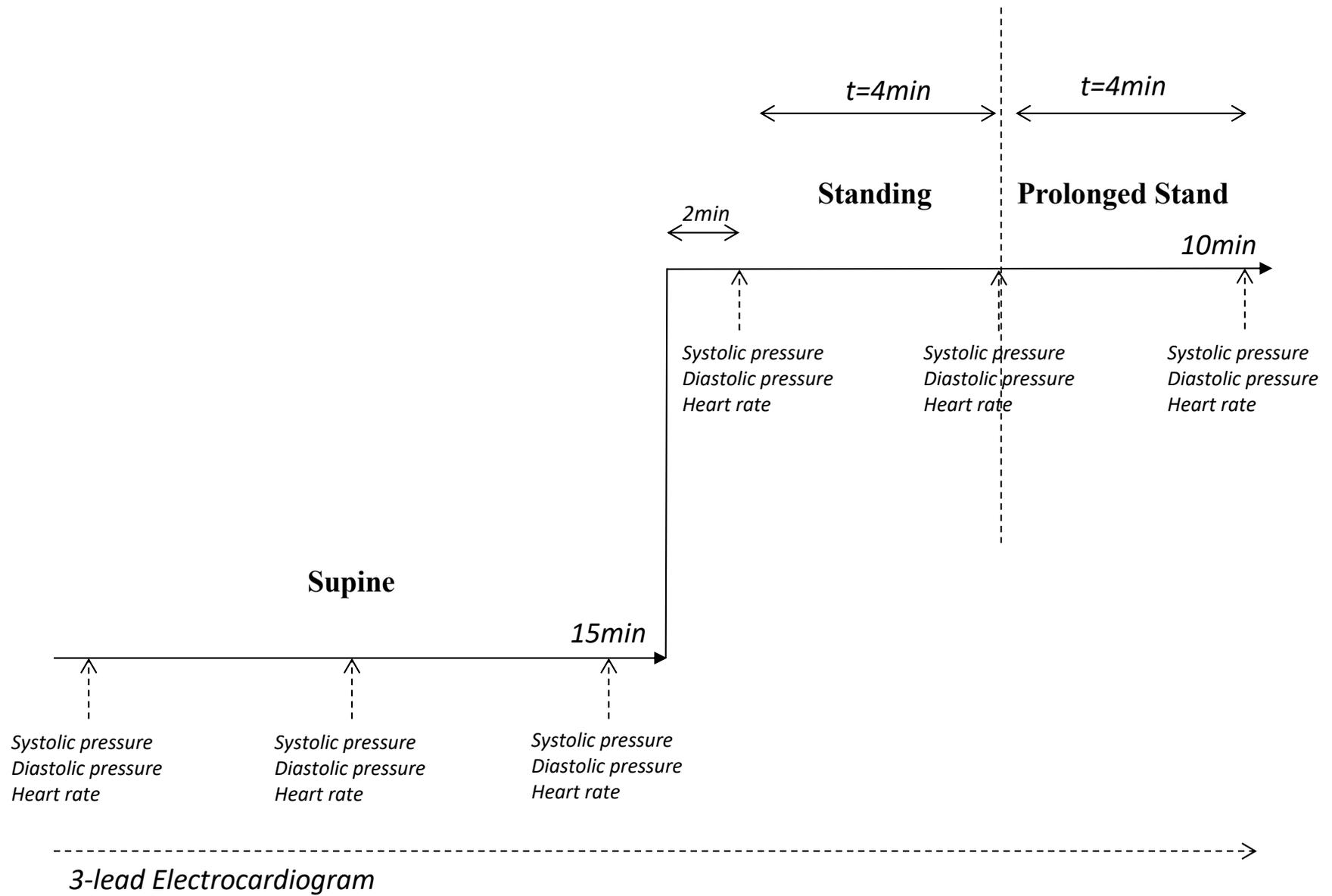


Fig.2

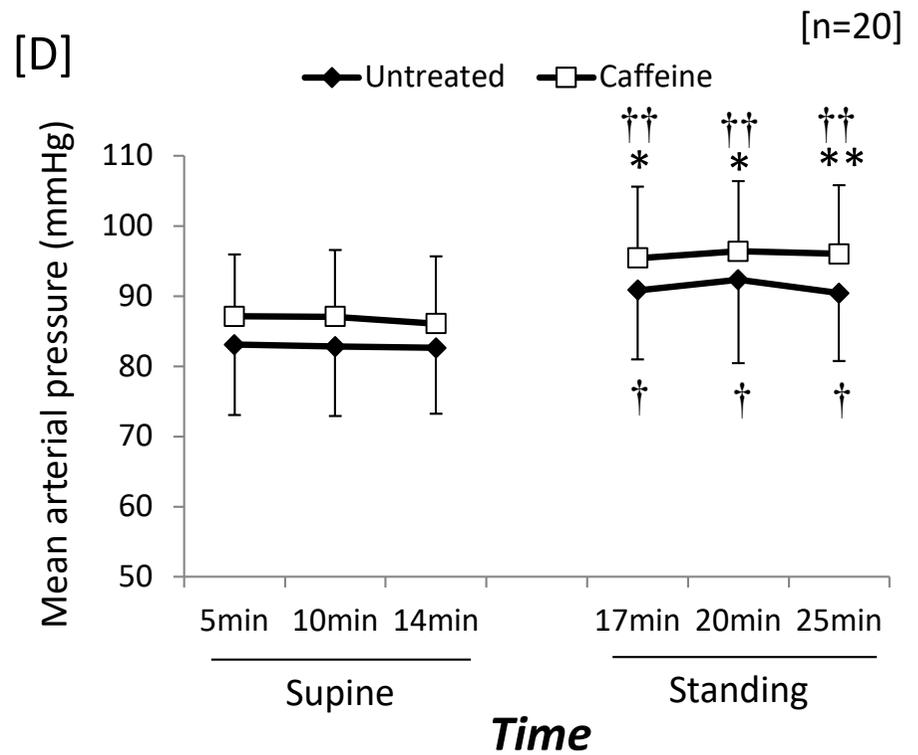
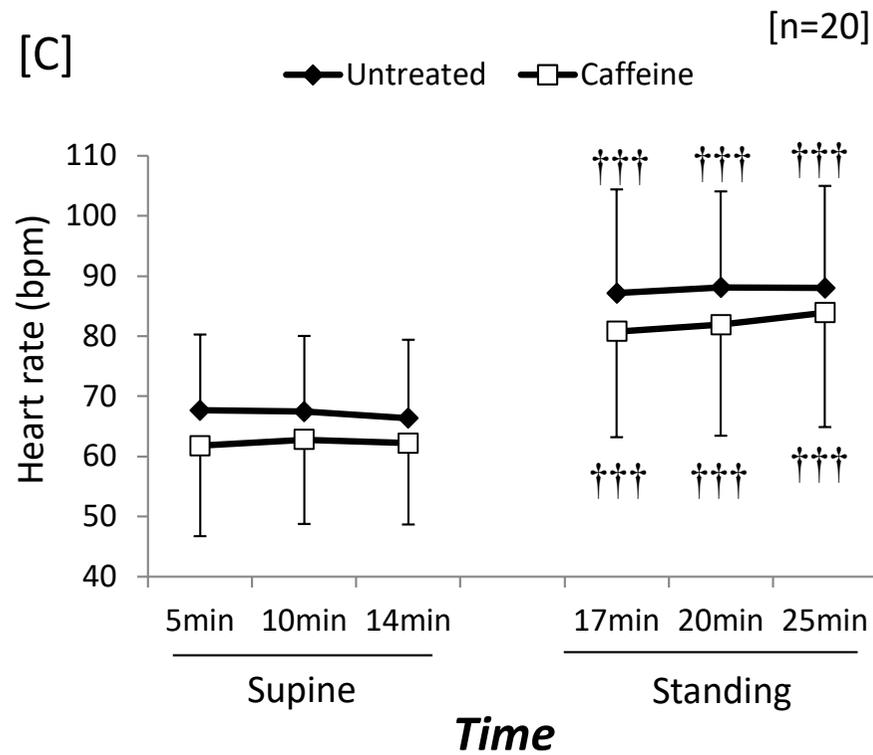
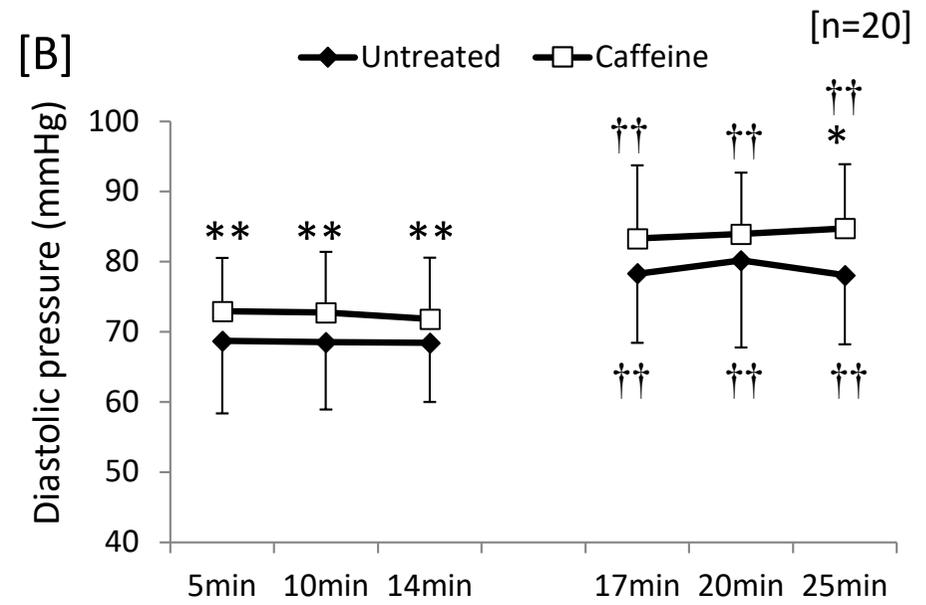
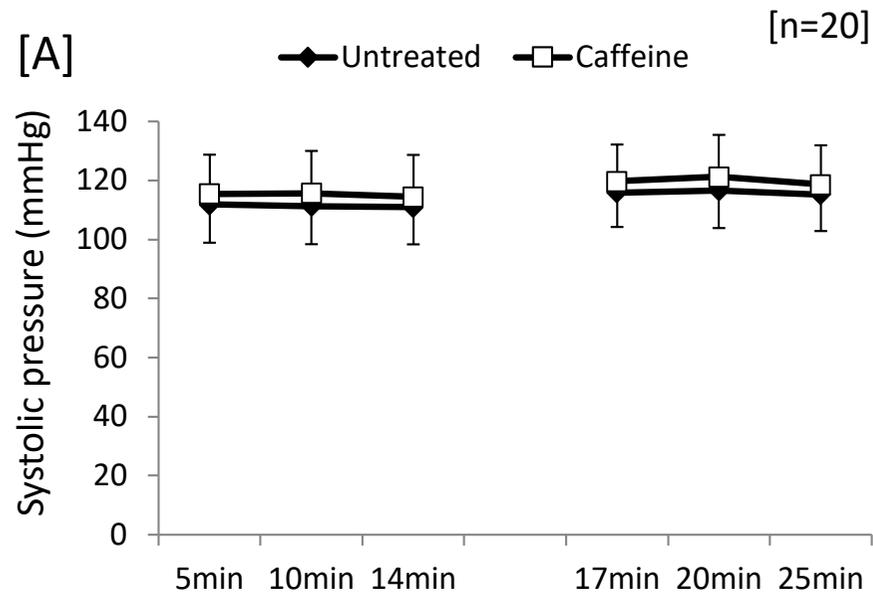


Fig.3

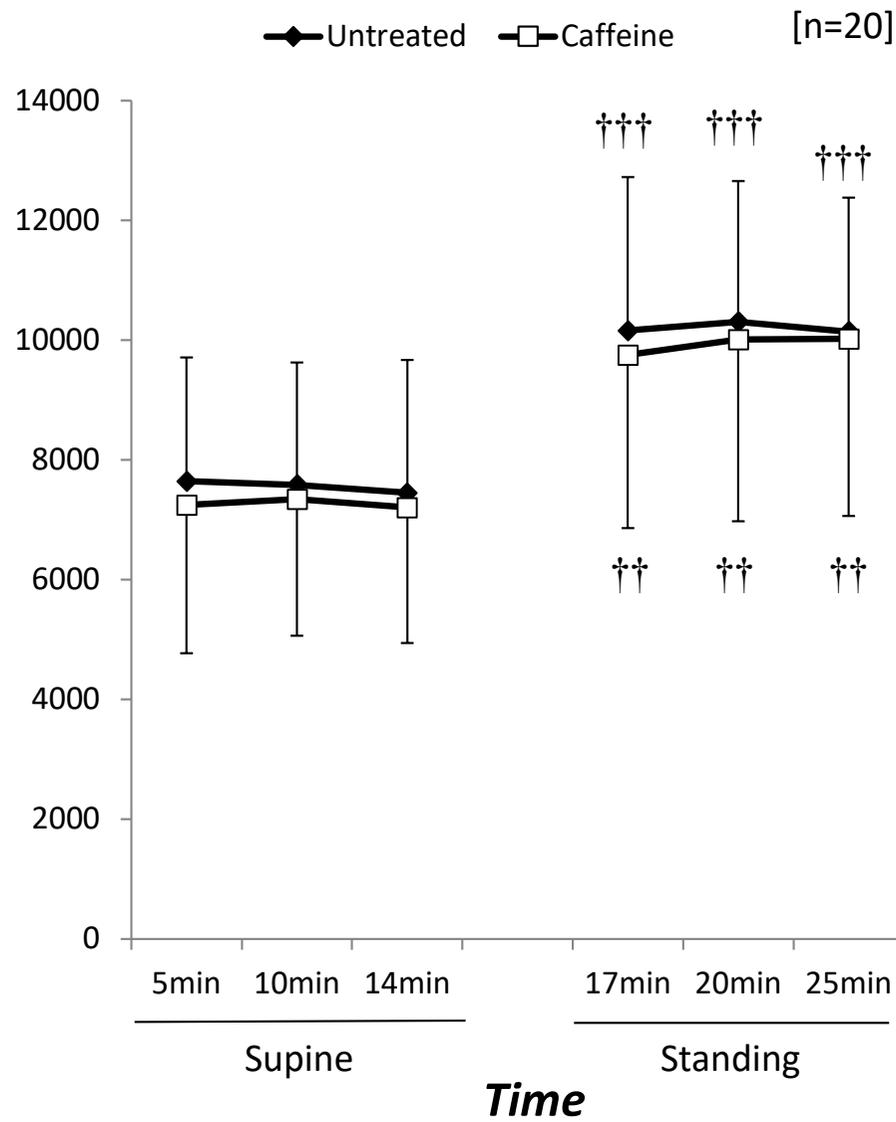
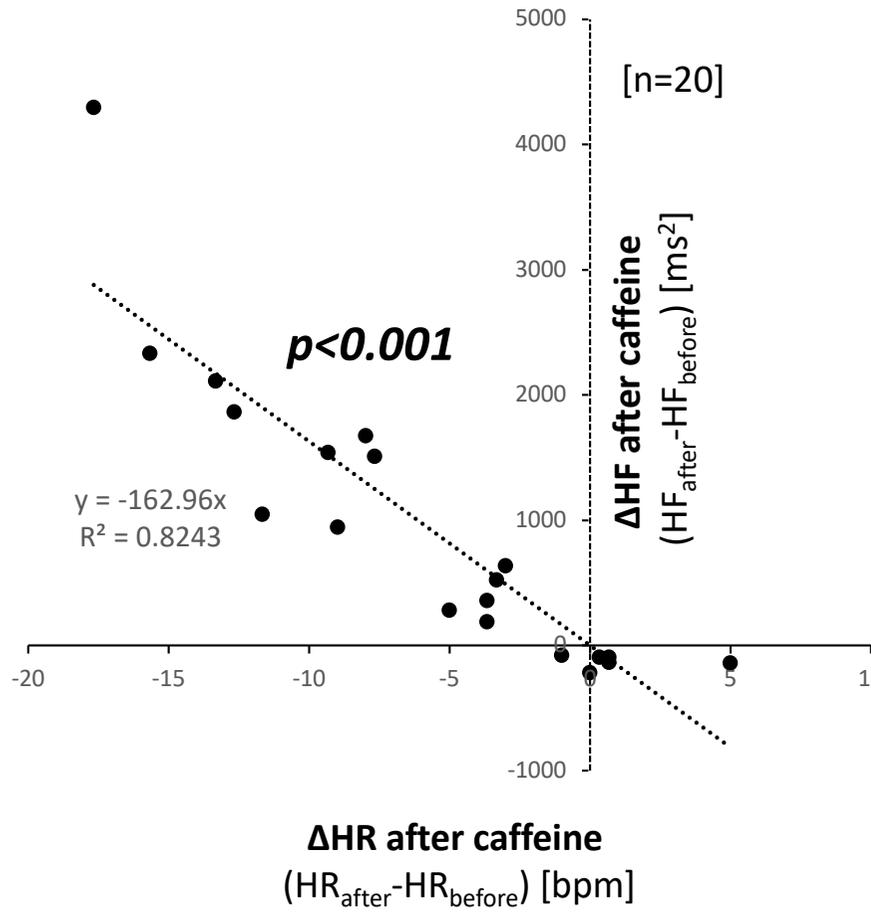


Fig.4

[A]



[B]

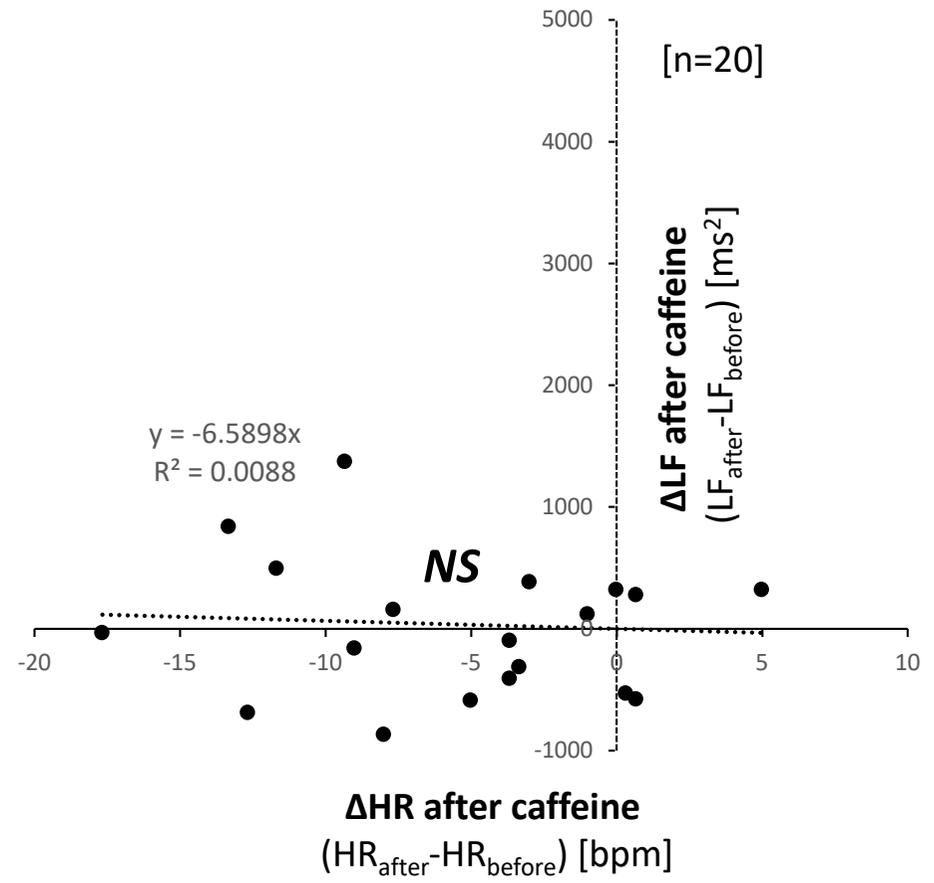


Fig.5