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Compression-induced hyperaemia in the rabbit masseter muscle: a model to investigate vascular mechano-sensitivity of skeletal muscle.

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ABSTRACT

Recent evidence suggests that the mechano sensitivity of the vascular network may underlie rapid dilatory events in skeletal muscles. Previous investigations have been mostly based either on *in-vitro* or on whole-limb studies, neither preparation allowing to assess the musculo-vascular specificity under physiological conditions.

Aim of this work is to characterize the mechano-sensitivity of an exclusively-muscular vascular bed *in vivo*.

In 5 anesthetized rabbits, muscle blood flow was continuously monitored in the masseteric artery, bilaterally (n=10). Hyperaemic responses were evoked by compressive stimuli of different extent (50, 100 and 200 mmHg) and duration (0.5, 1 , 2 and 5 s) exerted by a servo-controlled motor on the masseter muscle.

Peak amplitude of the hyperaemic response ranged from 340 ± 30 % of baseline (at 50 mmHg) to 459± 57% (at 200 mmHg) ($P<0.05$), did not depend on stimulus duration and exhibited very good reliability (ICC=0.98) when reassessed at 30 min intervals. Time course of the response depended neither on applied pressure nor on duration of the stimulus.

In conclusion, for its high sensitivity and reliability this technique is adequate to characterize mechano-vascular reactivity and may prove useful in the investigation of the underlying mechanisms, with implications in the control of vascular tone and blood pressure in health and disease.

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INTRODUCTION

Recently, a number of studies have evidenced the occurrence of a rapid increase in muscle blood flow following a brief muscle compression (Kirby *et al.*, 2007; Turturici *et al.*, 2012; Kirby *et al.*, 2005; Brock *et al.*, 1998; Tschakovsky *et al.*, 1996), as previously described by Mohrman & Sparks (1974). The hyperaemia peaks a few seconds after the release of the compression, terminates within 10-20 s (Kirby *et al.*, 2007) and appears to be mediated at least in part by the active dilatation of a mechano-sensitive vascular network (Clifford *et al.*, 2006; Clifford and Tschakovsky, 2008). This characteristic of blood vessels has been implicated in the rapid hyperaemia observed at the onset of muscle exercise (Clifford and Tschakovsky, 2008; Clifford and Jasperse, 2007). In fact, intramuscular blood vessels are compressed during muscle contraction due to the associated increase in intramuscular pressure (Sadamoto *et al.*, 1983; Clifford and Tschakovsky, 2008) and the hyperaemic response to a brief compression closely mimics the response to a brief contraction (Kirby *et al.*, 2007; Mohrman and Sparks, 1974).

Investigation of the mechano-reactivity of the vascular network may support the assessment of the vascular system functionality in health and disease, which is of great clinical relevance (McCully *et al.*, 2011) In fact, recent studies investigated the hemodynamic response to voluntary or stimulated muscle contraction evidencing a dependence with age in both animal and human (Jackson *et al.*, 2010; Carlson *et al.*, 2008)

However, limitations in the experimental models limit the understanding of the exact nature of the hyperaemic response whose underlying mechanisms remain largely unknown. In fact, active muscle contraction also involves metabolic mechanisms, besides mechano-sensitive pathways, while compression of a passive limb, e.g., of the forearm by an external inflatable cuff, affects both skin and muscle tissues thus making difficult to discriminate the relative contribution of the two vascular compartments, which are known to exhibit a different control of blood flow. This is particularly relevant given that blood flow is usually recorded from large arteries, e.g., the brachial artery, supplying both muscle and skin tissues.

We recently developed an experimental model that allows to monitor blood flow from a tiny branch of the facial artery, the masseteric artery, that supplies an exclusively muscular compartment, (Roatta *et al.*, 2010). We were then able to show that the cutaneous bed of the ear artery exhibit a much lower sensitivity to mechanical stimulation than that of the masseteric artery (Turturici *et al.*, 2012). In addition we showed that mechanical stimuli of different nature exerted on the masseter muscle all produce similar hyperaemic effects (Turturici *et al.*, 2012).

In the present study we aim at characterizing the repeatability of the compression-induced hyperaemic response and its dependence on duration and intensity of the mechanical stimulus. To

this aim, a servo-controlled motor is employed to deliver pressure stimuli of adjustable intensity and duration to the masseter muscle.

METHODS

Ethical approval

The study was performed at the University of Torino in accordance with the principles of laboratory animal care. Purposes and protocols were approved by the Ethical Committee for Animal Experiments at the University of Torino.

Surgical procedure

Experiments were carried out on 5 male European rabbits (Oryctolagus cuniculus) weighing between 2.9 and 3.3 kg, anesthetized with urethane (Urethane Sigma Aldrich dose 1.2 g/kg i.v.). Full surgical anaesthesia was maintained by injecting additional doses of the drug (0.4 g/kg i.v,) through a catheter inserted in the cannulated left femoral vein. In all animals the trachea was cannulated and the head was fixed in a stereotaxic frame by screws implanted in the nasal and frontal bones.

Arterial blood pressure was monitored through a catheter inserted into the right femoral artery. A perivascular flow probe (model 0.7PSB, Transonic Systems Inc, Itaha, NY, USA) was positioned around the masseteric branch of the facial artery (Ma), bilaterally (Roatta *et al.*, 2010). To this purpose the Ma, which exclusively supplies the rostral portion of the masseter muscle, was isolated medially to the mandibular margin, immediately after its branching from the facial artery, and accommodated in the J-shaped flow probe. The experimental procedures started after stabilization of the hemodynamic variables, about 1 h after the surgical preparation was completed. At the end of the experiments the rabbits were sacrificed by a bolus i.v. injection of a lethal dose of urethane .

Vascular stimuli

Hyperaemic responses in Ma were evoked by mechanical compressions (MC) of the masseter muscle performed by means of a servo controlled motor (mod 310B Level System, Cambridge Technology,Inc). driven by a PC. The motor performs an angular movement of the rotor, thereby pushing a small bar against the cheek of the animal (Fig 1A). The bar is provided with a smooth cylindrical head (diameter: 1.3 cm) which exerts a compressions of the check at the level of the

anterior portion of the masseter muscle (Fig. 1). The motor is operated in "length control mode", which means that the length (angular displacement) performed by the motor is proportional to the analog signal supplied by the PC through an I/O board (1401, CED, UK), until a *maximum force* (torque) *level* is reached. Trapezoidal length signals were generated with the following characteristics: rising and falling time= 0.1 s, plateau: 0.5 – 5 s amplitude: 7 mm (potential displacement of the hammer head). The maximum force was set to three different possible levels: 0.9, 1.8 and 3.6 N. Thus, the head, initially positioned 1-2 mm from the skin surface, approaches and gets in contact with the skin following a trapezoidal displacement which continues until (at less than 7 mm) the preset force level is reached. From then on a constant force is exerted against the muscle until the heads returns to the original position (end of the stimulus). As it can be observed from a representative recording in Fig. 1B the motor exerts a constant force irrespective of the progressive muscle yielding, as detected by the "length" signal.

The experimental protocol was designed to test the response to MCs of different extent: Force= 0.9, 1.8 and 3.6 N (plateau duration = 1 s) and of different duration: 0.5, 1, 2 and 5 s (Force = 1.8 N). Given the surface area of the cylindrical head (1.3 cm^2), the locally exerted pressure at the different force levels is approximately 50 mmHg, 100 mmHg and 200 mmHg, respectively.

A sequence of 6 consecutive MCs, separated by 2-min intervals, was run for each stimulus configuration.

In addition a series of 3 MCs (Pressure=100 mmHg, duration= 1 s) were provided at 30-min intervals in order to test the repeatability of the response.

Responses from the left and right side were separately collected and treated as different entities.

Data acquisition and processing

Data acquisition, generation of the analog signal used to drive the servo-controlled motor and off-line processing were performed with Spike2 (CED, UK). Simple algorithms were implemented in the Spike2 script language aimed at identifying single cardiac cycles (based on systolic peak detection on the ABP signal), from which time averages of the different signals (one value per cardiac cycle) were computed. Average hyperaemic responses to MC were computed for each stimulus configuration (n=6) and were characterized in terms of *amplitude* = (peak blood flow - baseline)/ baseline, i.e. the relative blood flow increase with respect to the pre-stimulus value, *time-to-peak*, i.e., the time elapsed from the beginning to the peak of the hyperaemia and the *half-return-time*, i.e., the time required to return from the peak to the mid value between peak and baseline.

Data collected from 10 different arteries were averaged and expressed as mean ± standard deviation (SD).
The dependence of the response on pressure level and duration of the stimulus was analyzed with a one way ANOVA for the three variables: amplitude, time-to-peak and half-return-time. For all the tests, the significance of level was 0.05.
Assessment of repeatability was based on the coefficient of variation (=standard deviation / mean) and the intra-class correlation coefficient of the *amplitude* of the hyperaemic response (an average response curve was computed over each series of 3 consecutive MCs).

RESULTS

Masseteric blood flow (MaBf) ranged between 0.2 and 0.4 ml/min, (average: 0.3 ± 0.1 ml/min) in resting conditions. From the representative example of Fig 1 B, it can be observed that the hyperaemia sharply develops immediately after the release of the compression and fully develops in 1-2 seconds. This figure also shows that this extent of compression (100 mmHg) did not completely occlude MaBF, and that some increase in blood flow already develops before the end of the stimulus. Arterial blood pressure was not affected by the stimuli.

Dependence of the hyperaemic responses on applied pressure
In general increasing the applied pressure increased the amplitude of the hyperaemic response as shown by the original recordings of Fig 2 A and by the bar diagram of Fig. 2B. The amplitude of the response was significantly dependent on the pressure level ($p<0.05$): 340 ± 30 % at 50 mmHg, 371± 145% at 100 mmHg, and 459± 57% at 200 mmHg. Conversely, neither the time-to-peak ($p=0.91$) nor the half-return time ($p=0.95$) exhibited a dependence on the applied force (Fig. 2C) and were thus averaged over the three force levels, resulting in: time-to-peak= 1.1 ± 0.3 s, and half-return time = 10.1 ± 0.8 s.

Dependence of the hyperaemic responses on the duration of the stimulus
The effect of changing the stimulus duration was investigated by eliciting MCs lasting from 0.5 to 5 s with a constant pressure of 100 mmHg. A representative example of the corresponding hyperaemic responses is reported in fig 3A while average effects are shown in fig 3B and fig 3C. Neither variable exhibited a dependence on duration of the stimulus: peak amplitude ($p=0.27$) time to peak ($p=0.11$) and half-return time ($p=0.32$) were thus averaged over the three durations

resulting in: peak amplitude = 480 ± 169% , time to peak= 1.2 ± 0.3 s , and half-return time = 9.9 ± 0.9 s.

Reproducibility of the hyperaemic response

Reproducibility of the hyperaemic response was tested by comparing the response to MC stimuli (applied pressure = 100 mmHg; duration = 1 s) collected 3 times at 30-min intervals. The amplitude of response did not depend on time ($p>0.05$). The average coefficient of variation of individual measurements was 4.0 ± 1.3%. and the intra-class correlation coefficient was 0.98

DISCUSSION

In the present study we present a new technique for the investigation of the rapid mechano-sensitive regulatory mechanisms of the vascular network in skeletal muscles. The experimental model is characterized by i) *in-vivo* measurement of arterial blood flow exclusively supplying skeletal muscle tissue with high time resolution (Roatta *et al.*, 2010) ii) delivery of controlled, constant-pressure, compressive stimuli to the relevant muscle by means of a servo-controlled motor. The results indicate that the amplitude of the hyperaemic response 1) is independent on the duration of the compression (in the tested range of 0.5-5 s); 2) slightly increases with increasing applied pressure (range 50-200 mmHg); 3) can be reliably reproduced over the tested time span of 60 min (ICC=0.98).

Previous investigations on the compression-induced hyperaemia were performed by applying an external pressure to the whole isolated muscle by means of a "pressure chamber" (Mohrman and Sparks, 1974) or to the whole limb by means of an external cuff (Brock *et al.*, 1998; Tschakovsky *et al.*, 1996; Kirby *et al.*, 2007). At difference from previous studies, the external pressure is here performed by a localized unilateral compression exerted on the relevant muscle, as recently suggested (Turturici *et al.*, 2012). By this manoeuvre, the masseter muscle remains compressed in-between the bar head and the mandibular bone (*fossa masseterica*). A limitation of the present approach is that the applied pressure does not equally affect the whole muscle but is likely to undergo some attenuation in the area surrounding the application point. However the results proved that this approach can effectively evoke reproducible hyperaemic responses that qualitatively reflect the same reactivity observed in isolated vessels (Clifford *et al.*, 2006), in isolated muscles (Mohrman and Sparks, 1974), as well as in intact human limbs (Kirby *et al.*, 2007). In addition, from the quantitative point of view the present model appears to be particularly sensitive to mechanical stimuli, as compared to previous studies. In fact, external

pressures as high as 600 mmHg were employed to investigate the responsiveness of isolated vessels (Clifford *et al.*, 2006) and relatively low hyperaemic responses were observed both on the isolated muscle, peak amplitude = 50 % of baseline (Mohrman and Sparks, 1974) and in human studies, peak amplitude = 100-150 % (Brock *et al.*, 1998; Kirby *et al.*, 2007), as compared to peak amplitude of 340-460 % observed in the present study at comparable pressure levels. The reason for this difference is likely to be attributed to differences in the experimental models. In particular, i) a higher reactivity is expected from *in-vivo* as compared to *in-vitro* models as the diffusion of biochemical signals elicited by the surgery is known to impair vessels' responsiveness (Mellander, 1989) and ii) muscle tissue may present a higher vascular reactivity to mechanical stimuli as compared to cutaneous and adipose tissues, which would make relative blood flow changes in a specific muscular artery (as the masseteric artery) larger than in a mixed artery (as the brachial a.). Preliminary observations of low mechano-reactivity of cutaneous as compared to muscular vascular beds (Turturici *et al.*, 2012) support the latter hypothesis.

With regard to the relationship between the parameters of the compressive stimulus (pressure and duration) and the magnitude of muscle vasodilator response, we observed that a 4-fold increase in pressure (from 50 to 200 mmHg) produced only a 30% increase in the response amplitude, while a 10-fold increase (from 0.5 to 5 s) in the duration did not induce significant changes.

In the literature only Kirby and collaborators (2007) investigated the dependence of the hyperaemic response on the intensity and duration of the compressive stimulus. In agreement with the present study the authors evidenced a significant dependence of the amplitude of response on the applied pressure. However, they also observed increased hyperaemic responses to sustained (6 s), as compared to short (1 s), compressions at 100 or 200 mmHg (Kirby *et al.*, 2007). Further investigations possibly extended to compressions of longer duration may be necessary to clarify how the duration of the stimulus affects the amplitude of the response.

Finally, it has been shown that the compression-induced hyperaemia is very stable over time: repeated testing over 90 min exhibited very high reliability (intraclass correlation coefficient: 0.98), which makes this model suited to test the effect of different experimental interventions on mechano-vascular reactivity.

The mechanisms underlying the compression-induced hyperaemia are not completely understood. It is now accepted that the propelling action of the "muscle pump", connected to depletion of venous compartments and the ensuing increase in perfusion pressure, can only partly account for the increase in blood flow, while an active mechano-sensitive dilatation is considered to be necessarily involved (Clifford and Tschakovsky, 2008). However, the actual mechano-sensitive structures have not yet been identified. They possibly include the smooth-muscle mechano-

sensitive ion channels already involved in the myogenic response but the involvement of the endothelium and the extracellular matrix have also been hypothesized (Davis, 2012; Hill *et al.*, 2009; Clifford, 2011).

Improving the understanding of this rapid dilatory mechanism is of primary importance given its powerful influence on the myogenic tone, with consequent potential implication in the maintenance of arterial blood pressure and, more generally, in the pathophysiology of vascular diseases (Ren *et al.*, 2010; McCully *et al.*, 2011; Lorenzi *et al.*, 2010; Hill *et al.*, 2009).

In conclusion, the results showed that the vascular bed of the masseteric artery presents a prominent sensitivity to mechanical stimulation, which can be conveniently investigated by applying a controlled external compression. The choice of the extent and duration of the compression is not critical in order to evoke a marked hyperaemic response which is stable and highly reproducible over time.

Therefore, the present technique provides a good model to investigate this relatively unknown vascular characteristic and its modifications in response to different physiological conditions and pharmacological agents.

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FIGURE LEGENDS

FIG 1: Schematic view of the experimental model (A) and representative example of the hyperaemic response to a single compression (B).
A) Compressions of the cheek are exerted by a servo-controlled motor by means of a bar with cylindrical head (diameter =1.3 cm). B) From top to bottom: masseteric artery blood flow (MaBF), displacement of the bar (Length) and exerted force (Force). Note that the 1.8-N exerted force is equivalent to a locally applied pressure of about 100 mmHg. Development of the hyperaemic response starts immediately at the release of the compressive stimulus

FIG 2). Dependence of the hyperaemic response on applied pressure.
A) Original records showing hyperaemic responses to compressive stimuli of different extent (1-s duration), in the same rabbit. B and C) Average response analyzed in terms of peak amplitude (B), half return time (HRT) and time-to-peak (TTP) (C). MaBF= Masseteric artery blood flow. Peak amplitude is normalized with respect to control (pre-stimulus) value. Bars represent mean ± SD. (n= 10 arteries, collected from 5 animals). *=$P<0.05$

FIG 3, Dependence of the hyperaemic response on stimulus duration.
A) Original records showing hyperaemic responses to compressive stimuli of different duration (applied pressure = 100 mmHg), in the same rabbit . B and C) Average response analyzed in terms of peak amplitude (B), half return time and time-to-peak (C). Abbreviations as in Fig. 2. Peak amplitude is normalized with respect to control (pre-stimulus) value. Bars represent mean ± SD. (n= 10 arteries, collected from 5 animals).

Figure 1

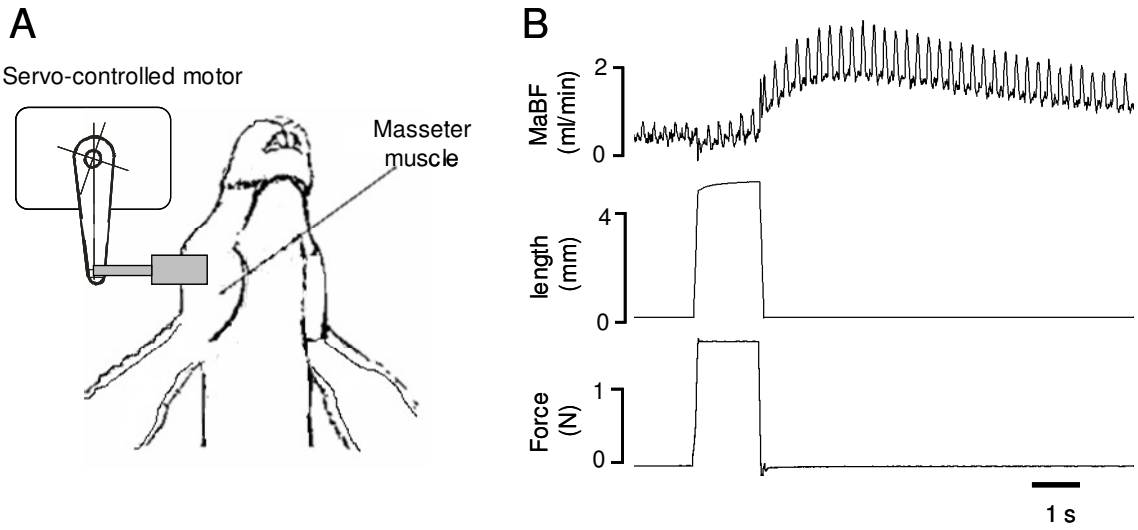

A
Servo-controlled motor
Masseter muscle
B
MaBF (ml/min)
2
0
length (mm)
4
0
Force (N)
1
0
1 s

Figure 2

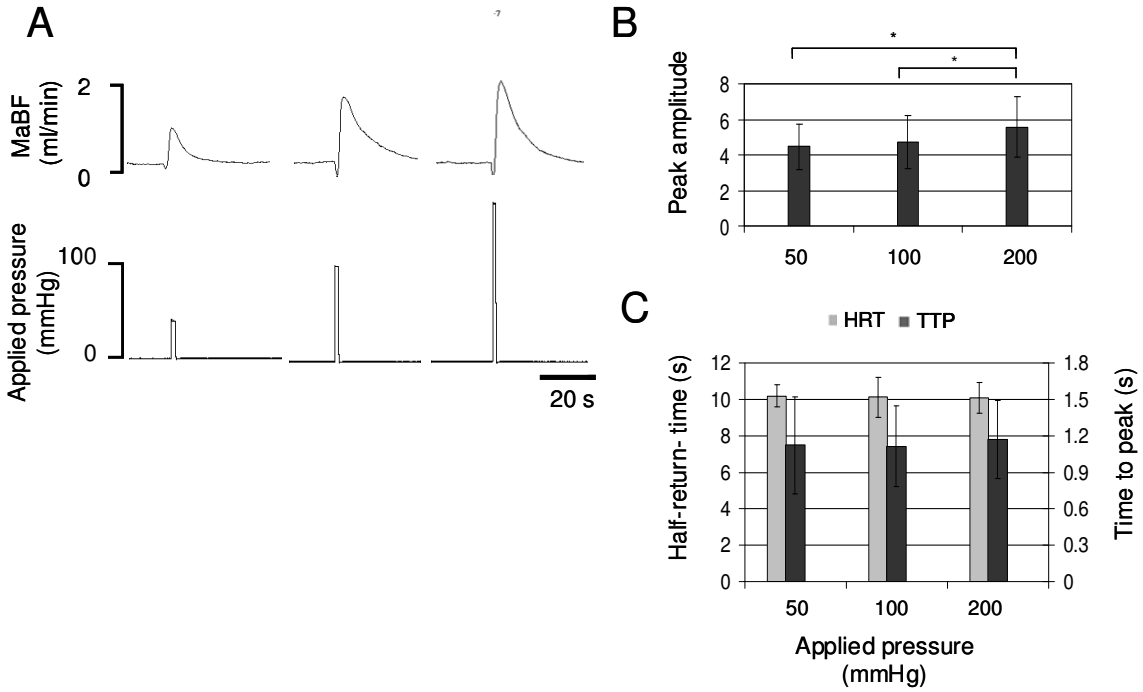
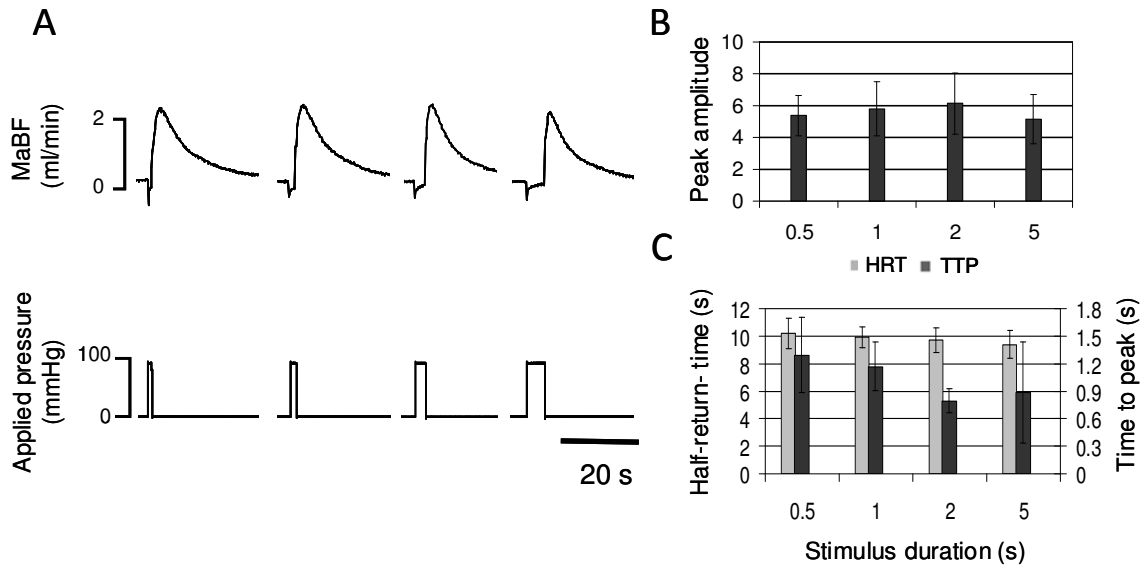

A
MaBF (ml/min)
2
0
Applied pressure (mmHg)
100
0
20 s
B
Peak amplitude
8
6
4
2
0
50
100
200
*
*
C
HRT
TTP
Half-return- time (s)
12
10
8
6
4
2
0
Time to peak (s)
1.8
1.5
1.2
0.9
0.6
0.3
0
50
100
200
Applied pressure (mmHg)

Figure 3


A
MaBF (ml/min)
2
0
Applied pressure (mmHg)
100
0
20 s
B
Peak amplitude
10
8
6
4
2
0
0.5
1
2
5
C
HRT
TTP
Half-return- time (s)
12
10
8
6
4
2
0
1.8
1.5
1.2
0.9
0.6
0.3
0
Time to peak (s)
0.5
1
2
5
Stimulus duration (s)