Influence of glutamate-evoked pain and sustained elevated muscle activity on blood oxygenation in the human masseter muscle

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Running title: Blood oxygenation in masseter muscle

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Abstract

This study aimed to investigate the effect of glutamate-evoked masseter muscle pain on intramuscular oxygenation during rest and sustained elevated muscle activity (SEMA). Seventeen healthy individuals participated in two sessions in which they were injected with glutamate and saline in random order. Each session was divided into three, 10-min periods. During the first (period 1) and the last (period 3) 10-min periods, participants performed five intercalated 1-min bouts of masseter SEMA with 1-min periods of ‘rest’. At onset of the second 10-min period, glutamate (0.5 ml, 1 M; Ajinomoto, Tokyo, Japan) or isotonic saline (0.5 ml; 0.9%) was injected into the masseter muscle and the participants kept the muscle relaxed in a resting position for 10 min (period 2). The hemodynamic characteristics of the masseter muscle were recorded simultaneously during the experiment by a laser blood-oxygenation monitor. The results demonstrated that glutamate injections caused significant levels of self-reported pain in the masseter muscle; however, this nociceptive input did not have robust effects on intramuscular oxygenation during rest or SEMA tasks. Interestingly, these findings suggest an uncoupling between acute nociceptive activity and hemodynamic parameters in both resting and low-level active jaw muscles. Further studies are needed to explore the pathophysiological significance of blood-flow changes for persistent jaw-muscle pain conditions.

Key words: blood flow, experimentally evoked muscle pain, hemodynamic parameters, maximal voluntary occlusal bite force, pain measurement.
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Introduction

Orofacial pain disorders are highly prevalent and debilitating conditions associated with significant psychosocial distress and high levels of health care utilization (1-3). Among the various orofacial pain conditions, pain-related temporomandibular disorders (TMD) are some of the most common causes of orofacial pain (1), and they have traditionally been assumed to be linked to hyperactivity or abnormal contraction of the masticatory muscles (4-8). The background for this assumption is the frequent clinical observation that many patients with TMD apparently exhibit a tendency to clench or grind their teeth (9-11), although tooth grinding is not necessarily associated with a higher risk of transient pain; thus the relationship between bruxism and TMD pain is not likely to be a linear one (12, 13). Nevertheless, a large number of studies have described TMD-like pain evoked by prolonged sustained elevated muscle activity (SEMA) such as the experimental bruxism models (14-20). Interestingly, when such experimental clenching tasks are repeated over three or five days, the pain and fatigue ratings decrease after the first day indicating that SEMA can, indeed, cause muscle symptoms but that these are self-limiting in healthy individuals (21).

Moreover, human experimental-muscle pain models are also used to investigate underlying mechanisms of the myalgic TMD pain. It has been shown that injections of glutamate (0.1 to 1 M) into the masseter muscle evokes pain through activation of peripheral N-methyl-D-aspartate (NMDA) receptors in healthy individuals and that this pain resembles some of the aspects of the muscle pain in myalgic TMD patients (22-28).

In terms of underlying muscle pain mechanisms, localized intramuscular hemodynamic disturbances leading to a lower level of oxygen distribution and hypoxia have been proposed to play an important role in the chronic painful muscle (29-31). Using a near-infrared method, DELCANHO et al. (32) demonstrated lower concentrations of total hemoglobin (Total-Hb) in the masseter muscle immediately after submaximal isometric contractions in patients with chronically painful masticatory muscles compared to the levels seen in healthy participants.
ACERO et al. (33) also utilized the near-infrared method, and assessed hemodynamic changes in chronic localized muscle pain patients and showed that the intramuscular hemoglobin levels were significantly reduced during cold pressor stimulation. This phenomenon may represent only a secondary characteristic of the pain-inducing mechanism or might be a fundamental aspect of the primary process causing pain (34). Our recent study suggested that different levels of repeated (30 times in each level) SEMA of the masseter muscles in healthy participants were associated with distinct hemodynamic characteristics in addition to muscle symptoms such as pain and fatigue (35). However, it is still unknown how an acute painful condition may influence the hemodynamic parameters and the previously observed differences in hemodynamic parameters caused by SEMA.

The aim of this study was, therefore, to investigate the effect of glutamate-evoked masseter muscle pain on intramuscular oxygenation during rest and SEMA. It was hypothesized that glutamate-evoked pain following SEMA may lead to a perturbation of the hemodynamic parameters in the masseter muscles.
Materials and Methods

Participants

A total of 17 healthy participants, 11 men and 6 women, mean age 27.6 ± 6.6 yr (mean ± SD) were enrolled. The participants were recruited by advertising the experiment by posting flyers in Aarhus University, Denmark, and in the webpage www.forsoegsperson.dk. The participants were included if they were between the ages of 18 to 40 yr old. Questionnaire-based exclusion criteria were: ongoing pain, chronic pain during the last 6 months, systemic diseases (e.g., metabolic diseases, neurogenic diseases and cardiovascular disorders), previous radiotherapy or chemotherapy, intake of any medicine affecting the nervous system in the 24 h before the experiments, physical or mental disorders. The participants had no signs or symptoms of TMD according to the DC/TMD screener (36). It was confirmed that none of the participants took medication that could influence psychological and / or cardiovascular responses. The experimental protocol was approved by the local ethics committee Region Midt (Denmark) (case No.1-10-72-201-15) in accordance with the Declaration of Helsinki. The participants were informed about their right to withdraw from the study anytime and they signed an informed consent form.

Study design

In this cross-over experiment, the participants were requested to attend two sessions (glutamate or isotonic saline) (Fig. 1). Each session was divided into three periods (period 1, 2, and 3) of 10 min each. During specific time points, the participants were asked to perform two different tasks (SEMA or rest). During the first 10 min (period 1), the participants performed intercalated SEMA and rest tasks. After period 1, an intramuscular injection of glutamate (0.5 mL, 1 M) or isotonic saline (0.5 mL; 0.9%) was given into the right masseter muscle. After the injection, the participants kept the masticatory muscle relaxed in a resting position without having the teeth in contact for 10 min (period 2). Subsequently (period 3), they repeated the intercalated SEMA and
rest tasks, which were the same as for period 1. Assessment of pain, fatigue, and unpleasantness was performed during the experiment. Intramuscular blood oxygenation of the masseter muscle was recorded continuously throughout the duration of the experiment. In the second session, participants went through the exact same tasks and procedures with the other type of solution being injected. The two sessions were scheduled at least five days apart and the order of injection (glutamate or saline) was randomly selected.

**SEMA**

The participants performed a maximal voluntary occlusal bite force (MVOBF) test at the beginning of the experimental session following the methodology described by Dawson et al. (39). The MVOBF was measured in Newton (N) by asking the participants to bite on a bite force transducer (41.0 × 12.0 × 5.0 mm, length × width × height; Aalborg University, Aalborg, Denmark) between the first or second molars on the right side. The analog output of the amplifier was connected to an analog-to-digital converter (BNC-2090; National Instruments, Austin, TX, USA), and the bite force signal was analog-to-digital converted, sampled at 2 kHz and stored on a personal computer for later analysis. To stabilize and hold the end of the transducer between the upper and lower teeth, a layer of acrylic resin with 2-mm thickness and silicone impression material (President Putty; Coltene, Altstätten, Switzerland) were added on the bite force transducer and fitted to the occlusion. The MVOBF was determined as the mean calculated from three consecutive MVOBF efforts for 2-3 s with an interval of about 3 s. The assessment was done in triplicate and the mean was calculated. The mean value of the three MVOBF assessments served to define the 40% of MVOBF as the target force level. This force level was decided based on our previous study (35), which showed that the relative changes of Total-Hb were greater during SEMA at 40% MVOBF than at 10% MVOBF.

The participants underwent five 1-min bouts of 40% MVOBF as SEMA (SEMA 1-5) with intervals of 1-min rest (rest 1-5) between the bouts (Fig. 1). They were then instructed to
observe the bite force display as a feedback to ensure that the biting force was kept constant during the SEMA. The examiner continuously supervised and encouraged the participants to maintain the target force level.

**Intramuscular injection of glutamate and isotonic saline**

The participants received an injection on the right side of the masseter muscle in each session in the beginning of period 2 (Fig. 1). The injected solution was either sterile solutions of monosodium glutamate (0.5 ml, 1M) or isotonic saline (0.5 ml; 0.9%). Sterile monosodium glutamate solution for injection was prepared by Skanderborg Hospital Pharmacy (28) (pH = 6.9 (assessed by PHM 93 Reference pH Meter, Radiometer, Brønshøj, Denmark). All injections were given manually over a 10-s period with a 27-gauge hypodermic needle (Sterican; B. Braun Medical, Frederiksberg, Denmark) and disposable syringe into the masseter muscle. The needle was inserted about 20 mm into the muscle at a 45-degree angle to the skin surface, so that the tip of the needle was around 14-15 mm in depth from the skin surface. The site of application of the injection was approximately 15 mm behind the bulkiest point of the masseter muscle on which the detector 1 of the BOM device was placed.

Both participant and experimenter were blinded with respect to the order of the solution injected, which was randomly determined. An assistant who had no clinical function in this experiment performed the randomization process using the website "http://www.randomization.com".

**Assessment of pain, fatigue, and unpleasantness**

The participants were asked to constantly score the pain intensity on electronic visual analog scale (eVAS) (Foresee-IMC Scale (VAS); Interactive Minds Centre, Aarhus, Denmark) of 0 to 10, with the lower end of the scale was marked “no pain” and the upper end was marked “most pain imaginable” during period 2 (Fig. 1). The software was run on a Windows PC and the
participant controlled the scale with a mouse. The participants were able to see the score on the
cscreen of the PC thereby providing live feedback. The data was sampled every 1-s and stored
automatically in an Excel table.

During periods 1 and 3, the perceived levels of pain, fatigue and unpleasantness were
evaluated intermittently by the participants using 3 separate 0 - 10 numerical rating scales
(NRS) with the “0” end representing “no pain, no fatigue or no unpleasantness” and the “10” end
representing “the highest pain, highest fatigue or highest unpleasantness imaginable” at pre-
determined time points. The registrations of the NRS of pain during SEMA were assessed
during rest periods immediately after the SEMA period had finished, as a “recall assessment of
pain during SEMA” (Fig. 1).

Intramuscular blood oxygenation
The hemodynamic characteristics were assessed from the right masseter muscle with the use
of a laser blood-oxygenation monitor (BOM, BOM-L1TRW; OMEGAWAVE, Tokyo, Japan) using
the methodology previously described (35). The BOM recording device had one probe and two
detectors. The lights from the probe were scattered and absorbed in the tissue, and part of
those scattered lights were detected by the two detectors. The distance from the probe to the
nearest detector was 1 cm and 2 cm to the second detector. The absorption in the tissue was
mainly caused by hemoglobin, and the absorption spectra of oxygenated hemoglobin and
deoxygenated hemoglobin were different. The oxygenated blood volume and deoxygenated
blood volume were calculated from the changes of the detected laser light intensity (37, 38).
The device was placed in the middle of the right masseter muscle parallel to the main directions
of muscle fibers as determined from palpation of the muscles. The detector 1, which was closer
to the light source was placed close to the bulkiest point of the muscle found in the lower 1/3 of
the muscle as determined by manual palpation while asking the subject to clench for a few
seconds (35).
The following parameters were derived from the BOM: i) oxygenated hemoglobin (Oxy-Hb), ii) deoxygenated hemoglobin (Deoxy-Hb), iii) total hemoglobin (Total-Hb), and iv) tissue blood oxygen saturation (StO₂). Total-Hb and StO₂ were calculated based on the values obtained from Oxy-Hb, and Deoxy-Hb (38). These analog outputs were connected to a data acquisition system (IX-404E; iWorx Systems, Dover, New Hampshire, U.S.A.), which was managed by a recording software (LabScribe2; iWorx Systems), sampling at a rate of 10 samples/s per channel in each of its four channels, and stored on a personal computer for later analyses.

**Statistics**

All data are expressed as mean ± SEM (standard error of the mean).

**Intramuscular blood oxygenation**

The baseline values, which was defined as a 1-min period before starting the SEMA task on the first period of the first session (Fig. 1), of each parameter (Oxy-Hb, Deoxy-Hb, Total-Hb and StO₂) were tested by t-tests to determine if there were any gender differences.

For the analysis of hemodynamic parameters, the data were averaged for every 1 min to observe changes over time. Each BOM parameter (Oxy-Hb, Deoxy-Hb, Total-Hb and StO₂) was analyzed using relative changes from their baseline value. The 5-s transition periods from rest to SEMA and from SEMA to rest were excluded for all the periods in order to avoid the influences of the opening and closing the mouth during the SEMA tasks (period 1 and 3). The BOM data obtained was analyzed using a two-way ANOVA with session and time as factors to determine if there were statistical significant differences between the two different injections at any of the 4 BOM outcomes. The two-way ANOVA was performed separately for each period (period 1, 2, and 3) and for each task (SEMA, rest) with the exception of period 2, which was recorded with the jaws at rest.
The average values of the five values for each task in each period (period 1 and 3) were calculated. Differences between tasks were tested within period 1 and 3. Differences between periods were compared using paired t-tests within sessions (glutamate or saline).

Bite force
The results of the MVOBF were compared using a two-way analysis of variance (ANOVA) with gender (men and women) and session (glutamate and saline) as factors.

The results of the bite force recorder during SEMA were averaged for each 1-min SEMA, and tested by three-way ANOVA with the following factors: session (glutamate and saline), period (period 1-3) and time (SEMA 1-5).

Experimentally evoked pain
The results of eVAS during period 2 were also averaged for every 1 min (overall pain) and compared using a two-way ANOVA using sessions (glutamate and saline) and time (0-10 min) as factors. The eVAS outcome parameters: peak of the pain (eVAS peak), duration of the pain (eVAS duration), and area under the curve (eVAS AUC) were tested by paired t-tests.

A two-way ANOVA was used to analyze the NRS scores of pain, fatigue, and unpleasantness in each of the periods (period 1-3) separately with session (glutamate and saline) and time (SEMA 1-5/ rest 1-5) as factors.

Post hoc comparisons were performed using Tukey Honestly Significant Difference (HSD) tests with correction for multiple comparisons. The STATISTICA software (StatSoft, Tulsa, OK, USA) was used for all analyses. The statistical significance level was 0.05. There was no missing data.

Results
SEMA

The mean MVOBF was 377.3 ± 79.2 N (mean ± SEM), (men: 391.7 ± 26.6 N, women: 351.0 ± 23.3 N). Two-way ANOVA showed that there were no statistically significant effects of gender or session (F < 1.030; P > 0.327) for MVOBF and no interactions between the factors. The mean 40% MVOBF corresponded to 150.9 ± 76.8 N (men: 151.1 ± 15.2 N, women: 140.4 ± 93.4 N).

Fig. 2 shows that the results of the actual percentage of the bite force during the SEMA tasks period. All participants completed the SEMA tasks with the bite force average corresponding to 37.9 ± 0.1% of the MVOBF. Three way ANOVAs did not show any significant effects of any factor nor interactions (F < 0.962; P > 0.435).

Intramuscular injection of glutamate and isotonic saline

Fig. 3 demonstrates the overall pain assessed on the eVAS for period 2. As for the overall pain averaged for 1 min, the two-way ANOVA showed an interaction between session and time, and an overall effect of session and time (F > 16.411; P < 0.001), with significantly higher pain scores for glutamate than for isotonic saline at every time point (P < 0.001, Tukey HSD test).

Regarding eVAS peak, duration and AUC, the t-tests showed significantly greater values in the glutamate session compared with the isotonic saline session (P < 0.001, Fig. 4).

Fig. 5 shows the mean NRS scores in period 1 and 3 of the recall pain during SEMA (A), and pain (B), fatigue (C), and unpleasantness (D) during rest. The two-way ANOVA analyses for period 1 showed only an overall time effect for recall pain during SEMA and for pain, fatigue and unpleasantness during rest (F > 5.649; P < 0.028). In period 3 there were overall time effects (F > 4.495; P < 0.003) for all parameters and there were overall session effects (F > 4.735; P < 0.045) for all parameters with exception of unpleasantness (F = 4.138; P = 0.059). There were no statistical significant interactions (F < 1.476; P > 0.220) with the exception of period 1 in pain during rest (F = 5.857; P < 0.001).
Intramuscular blood oxygenation

Gender differences

There were no significant differences between genders for any of the BOM parameters at baseline (t-tests; P > 0.486).

Effects of SEMA on intramuscular oxygenation

In period 1, the two-way ANOVA analyses of the relative changes of BOM parameters showed no session effects for SEMA or for rest tasks (F < 3.081; P > 0.097). Overall, there were significant time effects for SEMA (F > 2.937; P < 0.028) with the exception of deOxyHb (F = 2.343; P = 0.064). There were also significant time effects for the rest tasks (F > 3.406; P < 0.014) with the exception of Total Hb (F = 1.869; P = 0.126). There were no significant interactions (F < 1.582; P > 0.189).

T-tests showed that the relative changes of the SEMA task values were significantly higher than the relative changes measured during the rest tasks for deOxy-Hb and total-Hb (P < 0.001) and significantly lower for Oxy-Hb and StO2 (P < 0.001) (Fig. 6).

Effects of glutamate evoked pain on intramuscular oxygenation

In period 2, the two-way ANOVA analyses of the relative changes of BOM parameters showed interactions between session and time for Oxy-Hb and StO2 (F > 1.960; P < 0.049). There were no main effects of session (F < 4.366; P > 0.052) for all BOM parameters but there was an overall effect of time for all BOM parameters (F > 2.148; P < 0.030) with the exception of deOxy-Hb (F = 0.503; P = 0.871). Post-hoc tests showed that the relative changes of Oxy-Hb was significantly increased 3 min after the glutamate injection compared to min 1 (P < 0.047). Post-hoc tests for StO2 showed that the relative changes were significantly higher at min 3 and 4 after injection with isotonic saline (P < 0.032). Meanwhile this increase in StO2 was sustained from
min 2 only in the glutamate session (P < 0.005). This resulted in significant differences between sessions from min 6 and onwards (P < 0.011) (Fig. 6).

**Effect of SEMA on intramuscular oxygenation after glutamate evoked pain**

In period 3, the two-way ANOVA analyses of the relative changes of BOM parameters showed no session effects for SEMA or for rest tasks (F < 3.411; P > 0.082). Overall, there were no time effects of SEMA (F < 2.342; P > 0.064) with the exception of OxyHb (F = 3.427; P = 0.013). For the rest tasks there were only time effects for Oxy-Hb and StO2 (F > 3.566; P < 0.011). There were no significant interactions (F < 1.044; P > 0.391) (Fig. 6).

**Effects of SEMA on intramuscular oxygenation before and after glutamate evoked pain**

Overall, the average values for SEMA and rest after the glutamate injections (period 3) were significantly increased compared to before the injections (period 1) (P < 0.013) with the exception of deOxy-Hb which was significantly decreased (P = 0.006) during SEMA (Fig. 6). The values for SEMA were also significantly increased in period 3 when compared with before period 1 (P < 0.001) in the isotonic saline session with the exception of deOxy-Hb (P = 0.102)
Discussion

The present results indicated that there were no robust effects of session (i.e. differences between glutamate or isotonic saline) either following standardized SEMA or the relaxed jaw muscle tasks in the hemodynamic parameters.

Sustained elevated muscle activity

Several and diverse factors such as anatomical characteristics (e.g. the cranio-facial morphology and thickness of the cranio-facial muscles), physiological characteristics (e.g. age and gender) and mechanical characteristics (e.g. the bite-force recording system) are known to influence measurements of the bite force (40, 41). In addition to these factors, measurements of MVOBF are also dependent on the motivation and cooperation of the participants (42). Perhaps therefore, different studies have found a wide range of values in terms of MVOBF. Unilateral measurement of MVOBF in the molar region averages between 300 to 600 N in healthy adults with natural teeth (43, 44).

The mean MVOBF in this study was 279.9 ± 38.5 N (men: 320.0 ± 55.2 N, women: 241.0 ± 49.2 N) that was higher than the reported one in our previous study (35). The target percentage of MVOBF to be achieved by the participants during SEMA tasks was 40%. In the present study, the mean of the actual percentage of the bite force was 37.9 ± 0.1% during the SEMA tasks (Fig. 2) and this value was slightly higher than in our previously reported study (32.7 ± 0.4%) (35), even though the same targets of 40% MVOBF were requested. These minor discrepancies showed that despite similar experimental setups (same MVOBF, same researchers, same set ups, similar participant characteristics, etc.), the actual forces might vary between individuals and even within the same individuals.

Many studies reported that the MVOBF is higher in men than in women because the jaw dimensions are larger in men than in women (45-48). Moreover, the muscle fibers might be
different between genders, as a greater bite force in men appears to be linked to a greater diameter and cross-sectional-area of the type II fibers in the masseter muscle (49). However, in the present study and in our previous study (35) there was no significant gender difference in the MVOBF. This lack of difference may be explained by the relatively small samples sized used in both studies and that the studies were not designed to test specifically for gender-differences.

\textit{Electronic visual analog scale (eVAS) for pain after the injections}

As expected, the present study showed that an intramuscular injection of glutamate (1.0 M, 0.5 mL) into the masseter muscle evoked pain (Fig. 3 and 4). The present results suggested that the duration of the glutamate evoked pain seemed to be longer when it was preceded by a SEMA task. In previous studies, different volumes and concentration of injections of glutamate (0.1 to 1 M and 0.2 to 0.5 mL) into the masseter muscle have been found to evoke about 5 to 10 min of muscle pain in healthy human (23, 24, 28, 50-53). In the present study, almost all the participants reported pain until the end of the measurement of eVAS (10 min) (Fig. 3). Even though we cannot compare our results directly with previous studies due to different concentrations and volumes it has been reported that similar intensity levels of spontaneous pain can be evoked by glutamate injection of 0.5 M and by 1.0 M glutamate into the human masseter muscle (23). It has also been reported previously that different volumes (0.6 mL vs. 0.4 mL) of the same concentration (0.5 M) glutamate injections into the splenius muscle did not cause any different pain intensity (50). Moreover, it has been reported that 0.5 mL, that is the same volume as in present study did not last longer than seven min using three different kinds of temperature injection of 0.5 M glutamate (52). GAMBAROTA et al. suggested that it is the clearance of glutamate that determines the duration of glutamate-evoked pain response, and that the clearance of glutamate in the masseter muscle is rapid and appears to be completed within 10 min after the injection in rats (54). They proposed that increased muscle blood flow
would accelerate the rate at which intramuscularly injected glutamate could be absorbed into the systematic circulation and thus removed from the muscle tissue. Moreover, another previous animal study found that injection of hypertonic solutions such as 1.0 M glutamate into the masseter muscle significantly increased muscle blood flow (55).

In our present study, we showed that after injections there was an increase in some of the hemodynamic parameters that may imply an increased blood flow and therefore an increase of clearance would be expected with a consequent decrease in the pain-evoked parameters. However, our study did not show this decrease in self-reported pain scores. We speculate that the lack of pain reduction even in the presence of a possible increase in blood flow may be due to a disturbance in capillary function to exchange O₂ by CO₂ that was proposed by ØSTERGAARD et al. (31).

DAWSON et al. suggested that substances such as K⁺ and H⁺ (56, 57), prostaglandins (58, 59), cytokines (60, 61), neuropeptides such as bradykinin (62), and calcitonin gene-related peptide (63) might be released in association with an experimental SEMA task and could contribute to the activation of muscle nociceptors (64).

It could be speculated that the SEMA task before the glutamate injection might have influenced on the length of the duration of glutamate-evoked pain in our present study although further study is needed to determine whether the SEMA before the glutamate injection would interfere with the normal glutamate clearance.

NRS scores for pain, fatigue and unpleasantness during the SEMA tasks
The NRS scores for the recall pain during SEMA, and the NRS scores of pain and fatigue during rest were significantly higher in the glutamate session than in the isotonic saline session in period 3 (Fig. 5). A previous crossover experimental study design, in which the participants
performed a 10-min sustained 25% MVOBF before glutamate or isotonic saline injections showed that there were no significant overall differences in NRS scores of pain and fatigue (65). Nevertheless, in this cited study, higher pain scores were reported in the glutamate session than in the isotonic saline session in the first 2 min following the injections (65). This previous study also showed higher NRS scores for pain and fatigue in the first 10-min of prolonged 25% MVOBF task before the injection when compared to our study. However, in our present study we applied 10 min intermittent MVOBF of 1-min 40% MVOBF repeated 5 times with 1-min interval. Taking all this information together, lower force levels (25% MVOBF) than our study (40% MVOBF) induced higher NRS scores for pain and fatigue. These findings do however not align with our previous study report that indicated that the 40% MVOBF task caused higher NRS scores for pain and fatigue than similar SEMA tasks with lower MVOBF (10% MVOBF) (35). These differences may be attributed to the specific subtype of MVOBF used in these studies i.e. sustained versus intermittent MVOBF.

**Intramuscular blood oxygenation**

The present study showed that SEMA and rest tasks are associated with different hemodynamic responses in the human masseter muscle. The relative changes evoked by the SEMA tasks were higher compared to the rest task for deOxy-Hb and total-Hb and lower for Oxy-Hb and StO2 (Fig. 6). These results are in concordance with our previously published work (35).

In terms of the effect of glutamate-evoked pain on blood flow, CAIRNS et al. showed that injection of hypertonic solutions such as 1.0 M glutamate into the rat’s masseter muscle significantly increased muscle blood flow compared with isotonic saline for 10 min after the injection (55). Our present study showed that the relative changes for all BOM parameters increased from baseline, not only in the glutamate session but also in the isotonic saline session, with the exception of deOxy-Hb which decreased only in the glutamate session) (Fig. 6B). The influence of bleeding / micro trauma in the muscle caused by the injection of either solution
might have affected these results. However, the relative changes of StO$_2$ also increased in both the glutamate and isotonic saline session after the injection, but with significantly higher scores in the glutamate session at 6, 8, 9 and 10 min (Fig. 6D). More research will be needed to find out the duration of blood oxygenation increases triggered by intramuscular administration of glutamate. These observed differences may nevertheless indicate that painful glutamate injections into the human masseter muscle can lead to minor and delayed differences in hemodynamic characteristics.

In terms of the effects of glutamate-evoked pain on the blood flow during the SEMA and rest tasks then there were no overall significant differences between sessions in the relative changes of the BOM parameters.

It is pertinent to discuss that there are some limitations of the near-infrared method, which should be discussed in this study. Firstly, the precise pathway of the reflected light through the illuminated tissue and the absolute blood volume can be assessed but not exactly known (30). Secondly, the signals from the skin vasculatures and blood vessels cannot be avoided; the device used in this present study may have measured not only the hemodynamic changes in the muscle but also some from the skin (30). Moreover, FALLOW et al. suggested that the difference of the skin types tended to influence diffuse reflectance even though this trend was not statically significant (66). The sample size was relatively small but the study was designed as a cross-over trial minimizing the influence of inter-individual variability. Furthermore, the study was not specifically designed or powered to examine gender-related differences in hemodynamic characteristics. Further studies may be needed to test such aspects.

In conclusion, these findings suggest an uncoupling between acute nociceptive activity and hemodynamic parameters in both resting and low-level active jaw muscles. Further studies
will be needed to explore the pathophysiological significance of blood flow changes for persistent jaw muscle pain conditions.

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**Figure legends**

**Fig. 1. Schematic illustration of the experimental protocol**
Participants took part in 2 sessions in total. Each session was divided into 3 periods; they performed sustained elevated muscle activity (SEMA) tasks in period 1 and 3, and they received injections of glutamate or isotonic saline at the start of period 2. Numerical rating scales (NRS) scores for pain, fatigue, and unpleasantness were rated during rest periods (1-minute duration after each SEMA). Electronic visual analogue scales (eVAS) were assessed in period 2. A laser blood-oxygenation monitor (BOM) recorded the hemodynamic characteristics during the whole experimental session. “BL” = Baseline.

**Fig. 2. Actual percentage of the bite force level**
Average of the actual percentage of bite force level compared with the individual maximal voluntary occlusal bite force (MVOBF) during sustained elevated muscle activity (SEMA) tasks recorded by bite force transducer.

**Fig. 3. Perceived pain after injection**
Electronic visual analogue scales (eVAS) during 10 min after intramuscular injection with the “0” extreme representing “no pain” and “10” extreme with “the highest pain imaginable”. Each value represents the means ± SEM (n=17). (*): Significant main effects of session and time (P < 0.001, two-way ANOVA).

**Fig. 4. Peak, duration and area under the curve (AUC) of pain after injection**
Bar graphs illustrate the mean ± SEM for the various electronic visual analogue scales (eVAS) outcomes; peak of the pain (eVAS peak) (A), duration of the pain (eVAS duration) (B), and area under the curve (eVAS AUC) (C) in response after the intramuscular injection
of glutamate and isotonic saline. (†): Significant differences between sessions (P < 0.001, t-tests).

**Fig. 5. Numerical rating scales (NRS) scores for pain, fatigue and unpleasantness**

Numerical rating scales (NRS) scores for recall pain during SEMA (A), pain (B), fatigue (C), and unpleasantness (D) during rest. Each value represents the mean ± SEM (n=17). There was an overall time effect in all parameters in both periods. (*): Significant overall session effect in period 3.

**Fig. 6. Relative changes of intramuscular blood oxygenation**

Relative changes of oxygenated hemoglobin (Oxy-Hb) (A), deoxygenated hemoglobin (Deoxy-Hb) (B), Total hemoglobin (Total-Hb) (C) and tissue blood oxygen saturation (StO$_2$) (D). Each plot represents the means ± SEM (n=17) of 1 min. SEMA: sustained elevated muscle activity. (‡): Significant differences between session (P < 0.046, Tukey HSD tests).
<table>
<thead>
<tr>
<th></th>
<th>Period 1</th>
<th>Period 2</th>
<th>Period 3</th>
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<tr>
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</table>

**DC/TMD pain screener**

- ○

**Recall pain during SEMA**

- ●●●●● ●●●●●

**Pain during rest**

- □□□□□ □□□□□

**Fatigue**

- ■■■■■ ■■■■■

**Unpleasantness**

- △△△△△ △△△△△

**eVAS**

- -

**BOM**

- -

*Fig. 1* Injection (glutamate or isotonic saline)
Fig. 2

Percentage of bite force (%)

- Glutamate
- Isotonic saline

Period 1
SEMA1 | SEMA2 | SEMA3 | SEMA4 | SEMA5

Time

Period 3
SEMA1 | SEMA2 | SEMA3 | SEMA4 | SEMA5
Fig. 3

A graph showing the changes in eVAS (0-10) over time (s) for Isotonic saline and Glutamate treatments.
Fig. 4

A. eVAS peak

B. eVAS duration

C. eVAS AUC

<table>
<thead>
<tr>
<th></th>
<th>Glutamate</th>
<th>Isotonic saline</th>
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<tbody>
<tr>
<td>eVAS peak (eVAS score units)</td>
<td>9 ± 1</td>
<td>3 ± 1</td>
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<tr>
<td>eVAS duration (s)</td>
<td>600 ± 100</td>
<td>200 ± 100</td>
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<tr>
<td>eVAS AUC (eVAS score units)</td>
<td>3500 ± 500</td>
<td>1000 ± 500</td>
</tr>
</tbody>
</table>

† Indicates significant difference compared to isotonic saline.
Fig. 5

**A. Recall pain during SEMA**

**B. Pain during rest**

**C. Fatigue**

**D. Unpleasantness**