Sterile water; a novel and promising human experimental craniofacial muscle pain model

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Abstract

Background: The aim of this study was to investigate if intramuscular injection of sterile water can be used as a human experimental pain model that resembles clinical craniofacial muscle pain and to analyse if the effects differ between sexes.

Methods: This randomised, double-blind, placebo-controlled cross-over study included 30 healthy age-matched women and men (23.6 ± 2.4 years). At three sessions, with at least one week of washout in between, 0.2 mL of either sterile water (test-substance), hypertonic saline (58.5 mg/mL; active control) or isotonic saline (0.9 mg/mL; passive control) was randomly injected into the right masseter muscle. Pain intensity (VAS) was continuously assessed during 5 min whereafter pain duration (s) and pain area (au) were calculated; pressure pain thresholds (PPT; kPa) were recorded every 5 minutes during 30 minutes.

Results: Sterile water evoked pain of similar intensity (74.5 ± 49.9) as hypertonic saline (74.0 ± 50.5); whereas, isotonic saline evoked low-intensity pain (11.4 ± 23.4). The pain induced by sterile water and hypertonic saline had higher intensity (P < 0.001), longer duration (P < 0.001) and larger pain area (P < 0.001) than isotonic saline. There were no significant differences in any pain variable between sterile water and hypertonic saline. The PPT did not change significantly after any substance, except for in women 5 minutes after sterile water injection (P < 0.002). Pain duration was longer in the men for all substances (P < 0.006), while the pain area was larger in women after injection of hypertonic saline (P < 0.003).

Conclusion: These results indicate that pain evoked by sterile water resembles clinical muscle pain and may offer a novel and simpler alternative to hypertonic saline injections.

Keywords: experimental pain model, hypertonic saline, pain, sterile water

Abbreviations: DC/TMD, Diagnostic Criteria for temporomandibular disorders; GAD-7, Generalised anxiety disorder; PCS, Pain catastrophizing scale; PHQ-15, Patient health questionnaire 15; PHQ-9, Patient health questionnaire 9; PPT, Pressure pain threshold; PSS-10, Perceived stress scale; SD, Standard deviation; TMD, Temporomandibular disorders; VAS, Visual analogue scale.

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1 | INTRODUCTION

Painful temporomandibular disorder (TMD) is the second most common musculoskeletal disorder after chronic back pain, affecting up to 5%-12% of the adult population and twice as many women as men. TMD includes conditions such as chronic muscular and joint pain, impaired jaw function, as well as articulating sounds from the temporomandibular joint. TMD myalgia is often described as a dull, pressing muscle pain of moderate intensity, which can be intensified to a more intense and sharp pain when provoked. Studies have shown that chronic pain of moderate to severe intensity requires specific care and treatment. Further, patients with TMD myalgia often report pain upon chewing, mouth opening difficulties, as well as headache, but also suffer from depression and anxiety. Taken together, TMD myalgia affects the quality of life negatively.

To investigate pathophysiological aspects and therapeutic approaches of painful TMDs in a standardised situation to minimise confounding factors, one have to mimic the clinical setting using experimental pain models. In other words, the experimental pain model should: (i) evoke pain in a controlled situation; (ii) be fully reversible; (iii) be homogenous across participants; and (iv) short lasting (but long enough to evaluate specific pathophysiological aspects and therapeutic approaches as well as facilitating assessment of various pain effects on the sensory-motor system).

In experimental pain models, nociceptors in the structure that will be investigated, such as muscles and joint, are activated. After the stimulus is administered, pain pathways and pain characteristics can be assessed, but also therapeutic agents as well as the effect pain has on function can be studied. There are several types of stimuli used and they can be divided into two groups; endogenous and exogenous. The endogenous pain models use a natural stimulus such as ischaemia or exercise to induce muscle pain. Exogenous pain models, on the other hand, use external stimuli such as thermal, mechanical and chemical. Injections with glutamate, hypertonic saline, acidic saline, serotonin, bradykinin and nerve growth factor (NGF) have been used to chemically induce muscle pain and/or allodynia/hyperalgesia. However, all these models have limitations. Hence, none of them can fully mimic the actual pain experience of chronic myalgia. For example, hypertonic saline induces short-lasting (minutes) sharp and deep pain that resembles clinical myalgia, but with a higher intensity and no allodynia/hyperalgesia. Finding an exogenous experimental pain model that better mimics the characteristics of clinical myalgia could broaden the knowledge of the pathophysiological aspects as well as increase the trustworthiness of the findings when investigating new therapeutic approaches.

Cutaneous injection of sterile water into the lower back region is commonly used during labour with the purpose to direct pain from internal organs to the skin. The cutaneous injection evokes a sharp pain sensation which in turn is believed to activate the conditioned pain modulation (CPM) pathways to inhibit organ pain during labour. Due to its hypotonic sodium content, water generates an osmotic gradient which causes intense pain in biological tissues. When compared with other chemical substances as well as isotonic saline injected intramuscularly, studies in rabbits and rodents have shown that sterile water causes less muscle necrosis and haemorrhage. Therefore, sterile water injection into muscles could perhaps offer an alternative to other human experimental pain models, but to the best of our knowledge, this has not been investigated. In contrast to other substances, it is cheap and does not require production on demand by a pharmacy (glutamate, acidic saline) or dilution before injection (hypertonic saline).

Therefore, the aims of this study were to investigate if intramuscular injection of sterile water (hypotonic solution) can be used as a human experimental pain model that resembles clinical craniofacial muscle pain and to analyse if the effects differ between men and women.

2 | MATERIALS AND METHODS

The study followed the present guidelines according to the Declaration of Helsinki and was approved by the Regional Ethical Review Board in Stockholm, Sweden (2019/3:1). The experiments were conducted at the clinical research laboratory of the Department of Dental Medicine, Karolinska Institutet, Huddinge, Sweden, between February 2019 to May 2019. Verbal and written information of the study was provided to all participants, and their written consent was obtained before the start of study.

2.1 | Participants

The study comprised of fifteen healthy women with a mean (SD) age of 23.5 (1.9) years and 15 healthy age-matched men with a mean (SD) age of 23.7 (2.9) years. The volunteers were recruited by flyers placed at different floors at the Department of Dental Medicine, Karolinska Institutet, as well as Södertörn University, both in Huddinge, Sweden.

The inclusion criteria were: 1) age between 18 to 40 years; and 2) good general health. Participants were excluded if they had: a) any current pain from the oro-facial region; b) a pain diagnosis of TMD according to the Diagnostic Criteria for TMD (DC/TMD); c) current headache of any type; d) diagnosed systemic muscular and/or joint diseases, such as fibromyalgia or rheumatoid arthritis; e) whiplash-associated disorders; f) neuropathic pain or neurological disorders; g) severe psychiatric conditions (diagnosed by a physician); h) pregnancy or lactation; i) use of any kind of medication except for contraceptives 48 hours preceding the study day; and j) any previous negative reactions to either of the substances to be injected.

2.2 | Study protocol

The study protocol had a randomised, double-blind, placebo-controlled and cross-over design, where each participant was his/her own control. The study comprised of three separate sessions, with at
least one week of washout between each session (Figure 1). The sessions, in which sterile water (test-substance), hypertonic saline (active control) and isotonic saline (passive control) respectively were injected into the right masseter muscle were in a random order. To randomise the order of injections, a randomisation list was generated using a web-based randomisation tool (www.randomization.com, seed 8010) by one of the researchers not participating in data collection (NCh).

The participants were seated in the same conventional dental chair, in a quiet environment, during all three sessions. At the first visit, the participants were interviewed for trial suitability, and they completed the DC/TMD Axis II protocol. This contains questions regarding the presence of oro-facial pain and headache during the last 30 days and screening instruments for stress (Perceived Stress Scale; PSS-10), depression (Patient Health Questionnaire; PHQ-9), pain catastrophizing (Pain Catastrophizing Scale-13; PCS-13), anxiety (Generalised Anxiety Disorder Scale-7; GAD-7), and nonspecific physical symptom (Patient Health Questionnaire; PHQ-15). A clinical examination according to the DC/TMD Axis I protocol followed to confirm that the participants did not fulfill any TMD pain diagnosis, that is, arthralgia, myalgia or headache attributed to TMD. The women were asked about use of contraceptives or if normally menstruating the date of the first day of the last menses. Baseline recordings of pain characteristics and pressure pain threshold (PPT) were conducted. After the baseline recordings, injection of one of the substances was done, where after pain assessments and recordings of PPT were repeated. Side effects after injections were recorded. At each subsequent visit, participants were asked about presence of oro-facial pain and headache, and the masseter was palpatated before baseline recordings and injections.

2.3 Injections

The substances used were as follows: (a) sterile water (0 mOsm/l; B. Braun Melsungen AG, Melsungen, Germany)\(^2\); (b) hypertonic saline (NaCl 58.5 mg/mL) that was prepared from a concentrated solution of sodium chloride (NaCl 234 mg/mL, 2024 mOsm/l; B. Braun Melsungen, Germany) by mixing it in a 1:3 proportion with sterile water (B. Braun Melsungen AG, Melsungen, Germany) shortly before injection; and (c) isotonic saline (NaCl 9 mg/mL, 308 mOsm/l; B. Braun Melsungen AG, Melsungen, Germany). Preparation and blinding of injection substances was made by one of the researchers (JS, ES, NCh) not participating in data collection. All liquids were clear, colourless and odourless. The syringes, therefore, appeared identical making it impossible for the researchers collecting and registering the data (RE, CK), and for the participants to differentiate one substance from another.

The most prominent point of the right masseter muscle was used for injections (in the midline, approximately 2 cm superior to the mandibular border). This point was determined by manual palpation during contraction of the muscle. The point of injection was thereafter marked on a translucent paper where the participant’s nose, outline of the eye and jawline were marked. This was done to ensure that the injections were given at the same site in the masseter muscle. The skin over the injection site was cleaned with an injection swab (B. Braun, Isopropyl Alcohol 70%) and left to dry for at least 30 seconds before injection. The sterile needle used was a 27 Gauge (Grey), with a diameter of 0.4 mm and a length of 19 mm (BD Microlance, ref 302 200; Becton Dickinson Microlance™ 3, CE-marked). The needle was inserted into the relaxed muscle to a depth of approximately 15 mm, and 0.2 mL of each substance was injected during 10 sec.

![Flowchart displaying the study protocol. The flowchart shows the time-points in minutes for every registration: inclusion examination, baseline (BL1-3) for each session and the experimental assessments of pain intensity (VAS; visual analogue scale), pressure pain threshold (PPT), pain area as well as the time-points for injections of test substances, in 15 healthy, pain-free women and in 15 healthy, pain-free and age-matched men [Colour figure can be viewed at wileyonlinelibrary.com]](image-url)
2.4 | Assessment of pain characteristics

A 0-100 mm visual analogue scale (VAS) was used for assessment of pain intensity. The scale was drawn as lines on sheets of paper that the participant had in front of him/her. The end points were marked with ‘no pain’ on the left side and ‘worst pain experienced’ on the right side. Pain intensity was assessed directly after injection (time 0) and then every 15th second (monitored by the examiner using a timer) until pain had subsided or a maximum of 5 minutes. If pain persisted 5 minutes after injection the participant was instructed to inform the experimenter when it had subsided, and this time-point was recorded. From these data, the maximal pain intensity (peak pain intensity), pain duration (s) and the area under the curve (VAS auc), that is, the cumulated pain intensities multiplied by the pain duration (expressed in arbitrary units, au) were extracted and used in the analysis.

Pain drawings were used to assess the distribution (spread) of pain caused by the injection, that is, the pain area. Five min after injection or when pain had subsided, the participants were asked to recall the maximal pain distribution from their memory and encircle it on an extra-oral lateral view of the head and a lateral view of the head highlighting the jaws and teeth (both on the side of injection). All pain drawings were scanned separately using the printer Ricoh MP C6004ex with a resolution of 300 dpi, and the pain area was calculated (au) using an area calculation function in a photo editing program (Adobe Photoshop CC2019, Adobe Systems Incorporated, USA).

2.5 | Assessment of pressure pain threshold

PPT was recorded (kPa) using an electronic pressure algometer (Somedic Sales AB, Hörby, Sweden) with a 1 cm² probe tip covered with a 1 mm thick rubber disc. The pressure algometer was placed perpendicular to the skin overlaying the masseter muscle at the most prominent point of the masseter muscle. The tip of the right index finger was used as reference point. The rate of increase of pressure was set to 50 kPa/s. When the participants perceived that the sensation of pressure turned into pain, they pressed a signal button to stop the increase of pressure. The participants were instructed beforehand and to ensure correct procedure, the PPT was first practised for the recording on the dorsal side on the right thumb. The pressure algometer was balanced after each measurement. The PPT was assessed at baseline, immediately after injection, and then at 5, 10, 15, 20, 25 as well as 30 minutes after injection. Baseline PPT was calculated as the mean value of three assessments, while the PPT at the other time-points was calculated as the mean value of two assessments (to reduce the risk of temporal summation).

2.6 | Data analysis and statistics

The SigmaPlot for Windows version 14.0 software (Systat Software Inc, San Jose, CA, USA) was used for data analysis. The Shapiro-Wilk’s test was used to test the normality of the data. Mean and standard deviation (SD) or median and interquartile range (IQR) were used for descriptive statistics depending on distribution of data. Data regarding pain intensity and PPT were normally distributed; whereas, data regarding peak pain intensity, pain duration, VAS auc and pain area from pain drawings were not normally distributed. An attempt to log transformation still did not result in normally distributed data. Hence, parametric statistical methods were used for PPT data, while non-parametric statistics were used for other data.

Two-way RM ANOVA (2-way RM ANOVA) with substance as the independent factor and time as the repeated factor was used to analyse differences over time. This was done for the whole group but also for each sex separately. When the RM ANOVA indicated a significant time difference, the Tukey test for multiple comparison versus a control group (baseline) was used as a post-hoc test, and to test differences between substances and interactions at the different time-points. The PPT values were normalised to baseline, that is, the relative changes (%) were used in the statistical analyses.

The Friedman repeated measures (RM) analysis of variance (ANOVA) on ranks was used to test for differences between substances. When a significant difference was indicated, the Dunn’s method for multiple comparisons versus a control group (isotonic saline) was used as a post-hoc test. Sex differences for these variables were analysed with Mann-Whitney U-test.

The significance level was set to $P < 0.05$ for all tests.

3 | RESULTS

All 30 participants completed all three sessions; thus, there were no dropouts. None of the participants reported any oro-facial pain or headache or had any palpatory pain in the masseter muscle at any visit. None reported any adverse event for any of the included test substances. Further, there were no significant sex differences in any of the background variables or psychosocial scores according to the Axis II questionnaire (Table 1). Eight of the women used contraceptives. The seven normally menstruating women were in different phases of their menstrual cycle (visit 1: day 4-27; visit 2: day 3-24); visit 3: day 1-30).

3.1 | Pain intensity

Figure 2 shows the pain intensity evoked by the different injections for all subjects combined (Figure 2A) and for women and men separately for the different injections (Figure 2B–D). Although sterile water evoked pain of similar intensity as hypertonic saline, the pain induced showed another temporal pattern. Isotonic saline evoked only low-intensity pain (Figure 2A). The 2-way RM ANOVA showed a time effect ($df = 21; F = 84.053; P < 0.001$), an effect of substance ($df = 2; F = 69.226; P < 0.001$) and an interaction between time and substance ($df = 42; F = 38.481; P < 0.001$). The post-hoc test showed that sterile water evoked pain that was higher at all time-points after injection, that is, 0-300 s, than at baseline ($P < 0.001$, Tukey test). Further, the post-hoc test showed that hypertonic saline...
evoked pain of higher intensity compared with baseline 0-285 seconds after injection \((P = 0.022, \text{Tukey test})\). For isotonic saline, the post-hoc test showed higher pain intensity than baseline during the first 15 seconds after injection \((P = 0.024, \text{Tukey test})\). Finally, when the substances were compared, hypertonic saline-induced pain with a higher intensity than sterile water at 0 s and 30-165 s after injection \((P < 0.015, \text{Tukey test})\) and isotonic saline 0-285 s after injection \((P < 0.036, \text{Tukey test})\). Also, sterile water induced pain with a significantly higher intensity than isotonic saline 0-300 seconds after injection \((P < 0.001, \text{Tukey test})\), all shown in Figure 2A.

### 3.2 Other pain characteristics

The peak pain intensity for sterile water was 74.5 ± 49.9, for hypertonic saline 74.0 ± 50.5 and for isotonic saline 11.4 ± 23.4. The median (IQR) pain duration was 660 (889) s for sterile water, 285 (361) s for hypertonic saline and for isotonic saline 98 (128) s. The VAS auc was 1231 (1815) au for sterile water, 814 (1144) au for hypertonic saline and 5 (45) au for isotonic saline. The pain area, finally, was 195 (177) au, 176 (253) au and 13 (71) au for sterile water, hypertonic saline and isotonic saline, respectively.
The Friedman RM ANOVAs showed that all variables differed significantly between substances ($P < 0.001$). The post-hoc test revealed significantly higher peak pain intensity longer pain duration and larger VAS auc as well as pain area after injection of both sterile water and hypertonic saline compared with isotonic saline ($P < 0.001$, Dunn's method). There were no significant differences between sterile water and hypertonic saline ($P > 0.135$) for any variable (Figure 3A-D).

There were no sex differences in peak pain intensity for sterile water ($P = 0.798$), hypertonic saline ($P = 0.721$) or isotonic saline ($P = 0.901$). Women displayed a significantly shorter pain duration than men after injection of all substances ($P < 0.006$). Women further reported a significantly larger pain area than men after injection of hypertonic saline ($P = 0.003$) but not after sterile water ($P = 0.281$) or isotonic saline ($P = 0.368$). There were no differences in VAS auc between sexes for any of the substances ($P > 0.269$) (Figure 3B-D).
3.3 | Pressure pain threshold

None of the substances affected the PPT. The 2-way RM ANOVA showed no significant time effect (df = 6; F = 0.525; P = .789), no effect of substances (df = 2; F = 0.0849; P = .919) and no interaction between time and substances (df = 12; F = 1.520; P = .115), as shown in Figure 4A.

Since men and women displayed a somewhat different profile in PPT changes after injections, data were also analysed for each sex separately. In the women, the 2-way RM ANOVA showed a significant time effect (df = 6; F = 3.607; P = 0.003), but no differences between substances (df = 2; F = 0.816; P = 0.922) or any interaction between time and substances (df = 12; F = 1.771; P = 0.057). The post-hoc test showed that PPT had decreased by 20% compared with baseline 5 minutes after injection of sterile water (P = 0.002, Tukey test), but there was no significant change for the other substances. In the men, there was no significant time effect (df = 6; F = 0.804; P = 0.569), no difference between substances (df = 2; F = 0.144; P = 0.866) or interaction between time and substances (df = 12; F = 0.441; P = 0.944) after injections (Figure 4B-D).

There were no significant changes in the PPT over the reference point in response to any injection, as shown in Figure 5.

4 | DISCUSSION

To our knowledge, this study is the first in which intramuscular injections of sterile water were investigated scientifically in humans. The main findings were that injections of sterile water into the masseter muscle induced similar pain intensity (peak pain...
intensity), pain duration, VAS auc and pain area as hypertonic saline, that is, significantly higher pain intensity, longer duration and larger pain spread compared with isotonic saline. These findings suggest that sterile water can be used as an alternative acute experimental pain model to hypertonic saline (Figure 3). However, in similarity to hypertonic saline, sterile water did not affect the PPT, why it does not fully resemble clinical masseter myalgia (Figures 4 and 5).

Intramuscular injections of sterile water caused a short-lasting pain of a moderate to high intensity (VAS 74.5 ± 49.9). This is in line with cutaneous injections of sterile water and acupuncture given during labour which causes a sharp pain intensity (sterile water: VAS 72.9 ± 15.4; acupuncture: VAS 72.9 ± 18.2) before its pain relieving effect.20 Also, it is in similarity with other commonly used experimental pain models, such as hypertonic saline in this (74.0 ± 50.5) and previous studies or glutamate injections into the masseter muscle that cause pain less than 10 minutes with an average peak pain intensity around NRS 5-6.10,16,29 However, sterile water had a somewhat different pain profile than hypertonic saline. Peak pain intensity was reached directly after injection and then the pain immediately started to decline but with a slower rate than hypertonic saline. Hypertonic saline, on the other hand, reached its peak pain intensity after about one min after injection. The pain then stayed on a higher level than for sterile water for approximately 1.5 minutes.

FIGURE 4 Differences between substances in PPT over the masseter muscle. The mean (SEM) percentage changes in pressure pain threshold (PPT; kPa) to baseline (BL) after intramuscular injections of hypertonic saline, isotonic saline and sterile water in 30 healthy, pain-free participants (A) and divided by sex into 15 women and 15 age-matched men (B-D). Assessments displayed were made every 5th min beginning 5 min after injection up to 30 min after injection. PPT did not differ between sexes after injections (B-D), although PPT decreased by 20% 5 minutes after injection of sterile water in women (P = 0.002, Tukey test) [Colour figure can be viewed at wileyonlinelibrary.com]
There were both similarities and differences between sterile water and hypertonic saline in pain characteristics. For example, both sterile water and hypertonic saline evoked pain of significantly higher intensity and pain area when compared with isotonic saline. Regarding hypertonic saline, this is in accordance with previous studies. On the other hand, sterile water induced pain with a 120% longer duration than hypertonic saline (NS), which is an advantage over hypertonic saline (Figure 3). Another advantage is that no preparation of the solution is needed since sterile water is bought ready-made in sterile plastic ampules. Hypertonic saline, however, needs to be diluted to the correct concentration in the clinic/laboratory. This entails a risk for varying concentrations and the sterility of the solution. Thus, sterile water is simpler and may be safer to use than hypertonic saline.

In contrast to the effect on pain variables, our findings showed no significant changes in PPT values after injections of any substance (Figures 4 and 5). Also regarding PPT after hypertonic saline injection, previous studies show contradictory results. A few studies report reduced muscle PPT, that is, mechanical sensitisation, while other studies report no mechanical sensitisation. These findings indicate that experimentally induced muscle pain with hypertonic saline do not necessarily lead to muscle sensitisation. Based on our findings, sterile water appears to be a suitable alternative to hypertonic saline for inducing experimentally induced muscle pain.

**Figure 5** Differences between substances in PPT over the reference point (the right index fingertip). The mean (SEM) percentage changes in pressure pain threshold (PPT; kPa) to baseline (BL) after intramuscular injections of hypertonic saline, isotonic saline and sterile water in 30 healthy, pain-free participants (A) and divided by sex into 15 women and 15 age-matched men (B-D). Assessments displayed were made every 5th min beginning 5 min after injection up to 30 min after injection. There were no significant differences in PPT over time or any between substances for the entire group, or when the sexes were analysed separately (P < 0.05) [Colour figure can be viewed at wileyonlinelibrary.com]
on the findings from this single study, it seems that sterile water shows the same lack of mechanical sensitisation. This is a disadvantage for an experimental model for clinical myalgia. Nevertheless, these results together show that sterile water may be used as an alternative experimental pain model to hypertonic saline.

The pain mechanism for hypertonic saline is not clear but is theorised that pain is caused by a shift in sodium concentrations leading to depolarisation of excitable membranes, which in turn cause free muscle afferents to be activated by a higher osmolality. In a previous study, sodium concentrations were recorded to be 40.5% higher after injections of hypertonic saline. Hypertonic saline has an osmolality of 2002 mOsm/l, which is higher than that of plasma (308 mOsm/l). According to other studies, solutions with different osmolarity than blood irritate biological tissues, and thus may cause pain. Similar, a shift in the osmolarity may explain the pain evoked by sterile water. However, sterile water is a hypotonic solution with an osmolality lower than plasma. When a hypotonic solution is injected in biological tissues, pain will occur due to a lower osmolarity as well as a lower osmotic pressure. After injection of a hypertonic saline solution, water from the extracellular fluid flows into the cell causing it to swell. This influx of water is due to a change in ion concentrations as well as a change in osmotic pressure. To restore the osmotic gradient balance, sodium and potassium channels are opened, and an outflow of sodium and potassium will occur. The ions will then affect the muscle afferents to induce pain. When the osmotic imbalance is restored, the cell will return to its normal size.

There were some sex differences regarding pain characteristics with a longer pain duration in men for all substances and larger pain area in women after experimentally induced pain by hypertonic saline. However, there were no differences between sexes in peak pain intensity or VAS auc. Earlier studies report inconsistent results regarding sex differences in pain characteristics after algesic injections. For example, in one study, women had a longer pain duration and larger pain area than men after experimentally induced muscle pain with acidic saline. Yet, other studies report no differences in pain duration, but larger pain area in women after hypertonic saline injection. Furthermore, although results from this study report no differences in peak pain intensity, other experimental studies show that women rate pain higher than men after glutamate-induced pain in the masseter muscle. The reason and possible mechanisms for sex differences regarding pain response can be discussed. Since pain is multifactorial, several factors are thought to be involved and contribute to sex differences such as biological, psychological and sociocultural factors. For example, it has been shown in adolescence that more girls than boys develop TMD pain, suggesting that sex hormones play an important role in the development of oro-facial pain. Furthermore, previous study showed that women have three times higher odds of developing chronic TMD pain compared with men, thus viewed as a risk factor. Another possible explanation for the differences in this study could be the differences in the particular group of individuals included in this study, that is, the group was somewhat younger compared with previous studies, hence with less or no previous pain experiences in life. Since most experimental studies also include quite few participants sample selection probably could explain the somewhat different results across studies.

In general, PPT did not differ significantly between sexes after injections (Figure 4), although PPT decreased significantly by 20% 5 minutes after injection of sterile water in women (Figure 4C). Hence, the results are in concordance with a few studies that reported no sex difference in PPT after hypertonic saline injection, and glutamate injection. However, the PPT values were generally higher in men, which supports results from some previous studies of sex differences in masseter PPT. Taken together, our results regarding sex differences support data from previous systematic reviews with meta-analysis that show that women in general rate similar pain stimuli as more painful and are more sensitive to pain than men.

In accordance with previous studies, there was no adverse effects by the injected solutions. Hypertonic saline and isotonic saline have previously been used in other studies without any side effects. Since sterile water often is used to dilute hypertonic saline, no side effects were anticipated. The pain induced by the injected solutions had vanished after a few minutes and had no long-lasting effects. In animal studies on rabbits and rodents, sterile water showed the less tissue damage than isotonic saline.

Some strengths and limitations of this study need to be addressed. One strength was that the experiment used a randomised, double-blind and placebo-controlled design which is considered to be the ‘gold standard’ of human experimental studies. Also, using both a passive and an active control substance verifies the outcome of sterile water. Furthermore, the one-week washout in-between sessions allowed the muscle tissue to restore in order not to cause any false pain experiences by remaining tissue damage. Another strength is that the injection depth in the masseter muscle was controlled, so placement and depth of the needle could not affect the results. A limitation of this study was that the menstrual cycle for women was not considered. However, all women participating in the experiment were in different phases of the menstrual cycle, and thus, not likely to affect the results.

5 | CONCLUSION

The study has shown that intramuscular injection of sterile water into the masseter muscle of young healthy men and women evokes pain with similar characteristic as hypertonic saline. Yet, there were no changes in PPT. These results indicate that pain evoked by sterile water resembles clinical muscle pain and may offer a novel and simpler alternative to hypertonic saline injections. However, more studies are needed to further validate the results. It would also give a more in-depth knowledge of the pain mechanism of sterile water.
CONFLICT OF INTEREST
The authors declare that they have no competing interests. The authors alone are responsible for the content and writing of the paper. All authors have read and approved the final version of the manuscript.

AUTHORS' CONTRIBUTIONS
Sofia Louca Jounger and Johanna Svedenlöf were responsible for the data collection and wrote the manuscript and involved in statistical analysis. Reija Elenius, Christoffer Källkrans and Emil Scheid were involved in the data collection and statistical analysis and edited the manuscript in general. Malin Ernberg was responsible for the design of the study and statistical analysis and edited and revised the manuscript in general. Nikolaos Christidis was responsible for the design of the study, data collection and statistical analysis and wrote and revised the manuscript in general.

DATA AVAILABILITY STATEMENT
The data sets used and/or analysed during the current study are available from the corresponding author on reasonable request.

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