A new hypertonic saline assay for analgesic screening in mice: effects of animal strain, sex, and diurnal phase

Nouveau test avec solution saline hypertonique pour l'évaluation analgésique chez la souris : effets de la souche animale, du sexe, et de la phase diurne

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Abstract

Purpose *There exists a pressing need for the identification of novel analgesics. We recently reported on a new preclinical assay for rapid analgesic screening based on intraplantar (i.pl.) injection of 10% hypertonic saline (HS)*

in female outbred (CD-1) mice. Herein, we characterized the HS assay's performance in inbred (C57BL/6) mice, sensitivity to sex differences, and effects of diurnal rhythm phase.

Methods *In randomized, controlled, blinded in vivo animal experiments, we studied nociceptive responses induced by i.pl. HS in C57BL/6 (vs CD-1) mice of both sexes (n = 240) and determined diurnal rhythm phase effects in female animals. We established the HS assay's sensitivity to morphine by constructing dose-response curves and calculating half-maximal inhibitory doses (ID₅₀s).*

Results *The injection of i.pl. HS produced nociceptive (licking and biting) responses in all C57BL/6 mice tested. In both C57BL/6 and CD-1 mice, the mean (95% confidence interval [CI]) response magnitudes were greater in females vs males (C57BL/6: 87 sec [64 to 110] vs 45 sec [29 to 61]; difference in means, 42 sec; 95% CI, 17 to 68; P < 0.001; n = 10/group; CD-1: 110 sec [95 to 126] vs 53 sec [32 to 74]; difference in means, 57 sec; 95% CI, 34 to 79; P < 0.001; n = 10/group). The mean (95% CI) nociceptive responses were greater at 24:00 hr than at 12:00 hr in C57BL/6 mice (64 sec [40 to 88] vs 37 sec [24 to 51]; difference in means, 27 sec; 95% CI, 7 to 47; P = 0.007; n = 10/group), but not in CD-1 mice (P = 0.97). Intravenous morphine dose-dependently attenuated nociceptive responses of both C57BL/6 and CD-1 mice (ID₅₀, 0.6 and 2.5 mg·kg⁻¹, respectively; P = 0.41).*

Conclusion *These findings in inbred and outbred mice solidify the utility of the HS assay as an effective, rapid, robust, and versatile preclinical tool for analgesic screening.*

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Résumé

Objectif Il existe un besoin impérieux d'identification de nouveaux analgésiques. Nous avons récemment publié les conclusions d'un nouveau test préclinique portant sur le dépistage analgésique rapide basé sur l'injection intraplantaire (i.pl.) d'une solution saline hypertonique à 10 % (HS) chez des souris femelles croisées (CD-1). Dans notre présente étude, nous avons caractérisé la performance du test de HS chez des souris consanguines (C57BL/6), la sensibilité aux différences de sexe, et les effets des phases de rythme diurne.

Méthode Dans le cadre d'expériences animales in vivo en aveugle randomisées contrôlées, nous avons étudié les réponses nociceptives induites par une i.pl. de HS chez des souris C57BL/6 (vs CD-1) des deux sexes ($n = 240$) et déterminé les effets des phases du rythme diurne chez les animaux femelles. Nous avons établi la sensibilité du test HS à la morphine en construisant des courbes de dose-réponse et en calculant des doses inhibitrices semi-maximales (DI_{50}).

Résultats L'injection i.pl. de HS a produit des réponses nociceptives (léchage et morsure) chez toutes les souris C57BL/6 testées. Chez les souris C57BL/6 et CD-1, les magnitudes de réponse moyenne [intervalle de confiance (IC) 95 %] étaient plus élevées chez les femelles que chez les mâles (C57BL/6 : 87 [64 à 110] vs 45 [29 à 61] sec; différence de moyennes, 42 sec; IC 95 %, 17 à 68; $P < 0,001$; $n = 10$ /groupe; CD-1: 110 [95 à 126] vs 53 [32 à 74] sec; différence de moyennes, 57 sec; IC 95 %, 34 à 79; $P < 0,001$; $n = 10$ /groupe). Les réponses nociceptives moyennes [IC 95 %] étaient plus importantes à minuit (24 h) qu'à midi (12 h) chez les souris C57BL/6 (64 [40 à 88] sec vs 37 [24 à 51] sec; différence de moyennes, 27 sec; IC 95 %, 7 à 47; $P = 0,007$; $n = 10$ /groupe), mais pas chez les souris CD-1 ($P = 0,97$). La morphine intraveineuse a atténué de façon dose-dépendante les réponses nociceptives chez les souris C57BL/6 et CD-1 (DI_{50} , 0,6 et 2,5 mg·kg⁻¹, respectivement; $P = 0,41$).

Conclusion Ces résultats chez les souris croisées et consanguines appuient l'utilité du test de HS comme un outil préclinique efficace, rapide, robuste et polyvalent pour le dépistage analgésique.

Keywords Pain measurement/drug effects · analgesics · drug screening · analgesics · disease models/animals · mice · drug evaluation · preclinical

There continues to be a pressing need for the identification of novel non-opioid analgesics, evidenced by the ongoing opioid crisis and efficacy constraints combined with the adverse event profile of currently available agents.^{1–7} The

realization of this goal hinges in part upon the availability of reliable *in vivo* analgesic screening assays, which are among the most important features of preclinical pain research.⁸ The success of a preclinical nociceptive assay requires consistency in the magnitude of control or pretreatment responses, the ability to detect clinically used analgesics, and reproducibility within species.⁹ We recently reported on the development of a new nociceptive assay in mice based on the intraplantar (i.pl.) injection of 10% hypertonic saline (HS),^{10,11} previously shown to produce nociceptive stimulation in a study of transient receptor potential vanilloid 4 (TRPV4) channels.¹² The HS assay evokes nociceptive responses that are reproducible in the same animal regardless of repetitive testing and rapidly detects antinociceptive activities of a range of established centrally and peripherally acting analgesics belonging to the principal classes.¹¹ This allows each animal to serve as its own control, thereby reducing response variability and the number of animals needed. At the same time, in contrast to formalin-based assays, the HS assay produces no discernible tissue damage and hence improves animal welfare.¹⁰ Collectively, these attributes speak to the principles of the 3Rs (Replacement, Reduction, and Refinement) in the development of more humane animal research.¹³

Nevertheless, the HS assay was developed exclusively using mice from an outbred stock, CD-1. To show assay versatility, robustness, and broad utility, it is imperative to investigate its performance using mice from a common inbred strain. Inbred mice are generally preferred over outbred mice in biomedical research. One reason is that outbred mice possess greater genetic variability due to cross-breeding. In contrast, inbred mice are isogenic to one another and therefore possess less phenotypic variability. Thus, fewer animals are required to maintain similar statistical power (*Reduction*), and response variability may be reduced (*Refinement*).¹⁴ Inbred mice are also preferred because of their central role in creating transgenic knockout mice. Typically, embryonic stem cells from the inbred 129 strain are microinjected into embryo cells of the inbred C57BL/6 strain.¹⁵ Since most transgenic knockout mice originate from an inbred genetic background¹⁶ and have been widely employed in preclinical pain research,¹⁷ testing the HS assay with C57BL/6 mice is a priority.

Differences in nociceptive responses and analgesic sensitivity within rodent species have been well documented in many pain assays.¹⁸ In one study involving 11 inbred mice strains, large variations in baseline nociceptive sensitivity were found with C57BL/6 in a variety of nociceptive, thermal, and chemical pain assays.¹⁹ With regard to analgesic sensitivity, the C57BL/6 strain is known to be resistant to many analgesics compared with other inbred mouse strains.^{16,20} These differences likely arise from genetic influences²⁰ and the nature of noxious

stimuli. To complicate matters, some assays also display sex-specific differences within strains.²¹ In thermal pain assays, C57BL/6 males have lower baseline nociceptive sensitivities compared with females²² whereas no sex differences have been observed with outbred CD-1 mice.²³ Differences also exist in chemical nociceptive assays. For example, both sexes of C57BL/6 mice exhibit larger nociceptive responses than CD-1 mice in phase I and II of the standard formalin foot test.²¹ Because the HS assay was recently developed, response variability due to strain and sex effects have not yet been thoroughly investigated.

The goal of the present study was to determine the nociceptive responses of male and female C57BL/6 vs CD-1 mice to i.pl. injection of the nociceptive stimulus (HS) using 0.9% normal saline (NS) as a control.¹⁰ We further sought to investigate the effects of sex and diurnal rhythm phase on nociceptive responses and assess the ability of the HS assay to detect the antinociceptive activity of the “gold standard” opioid analgesic, morphine, in C57BL/6 mice. Our principal hypothesis was that the HS assay would evoke morphine-sensitive nocifensive licking and biting behaviour in inbred C57BL/6 mice.

Methods

We conducted a randomized, controlled, blinded *in vivo* laboratory animal study. The experimental protocol and procedures were approved by The University of British Columbia Animal Care Committee (Vancouver, BC, Canada) and conducted in accordance with the guidelines of the Canadian Council on Animal Care. Animals were used once and euthanized immediately at the end of testing using isoflurane followed by CO₂. We followed the Animal Research Reporting In Vivo Experiments 2.0 (ARRIVE 2.0) guidelines²⁴ for the reporting of this study.

Animals

Adult male and female CD-1 (25–30 g) and C57BL/6 (20–25 g) mice (total $n = 240$; Charles River Laboratories, Senneville, QC, Canada) were group-housed (five per cage) at 24°C and 45–55% relative humidity under a 12-hr light–dark cycle where lights are turned on at 07:00 hr. Animals were given free access to laboratory food and water. Experiments were performed between 09:00 and 17:00 hr, with the exception of those performed at 24:00 hr (see below).

Drugs and chemicals

Sodium chloride (NaCl; BDH Chemicals, Toronto, ON, Canada) was dissolved in distilled water to produce saline

at different concentrations. NaCl 0.9% (normal saline [NS]) was employed as the negative control throughout the study. Morphine sulfate (Toronto Research Chemicals, Toronto, ON, Canada) was dissolved in NS.

Hypertonic saline analgesic assay

Animals were randomly assigned to control and treatment groups using an internet-based true random number service.²⁵ Mice were habituated for two hours in a quiet and temperature-controlled (23°C) room. Individual mice were then placed in an opaque Plexiglas tube (height, 19 cm; diameter, 9 cm; Associated Plastics and Supply, Vancouver, B.C., Canada). Animals received injections into the middle of the right hind paw with 10 μ L of either HS or NS (0.3-mL disposable insulin syringes with 29G needle; BD, Mississauga, ON, Canada) by an experimenter blinded to group assignment. Mice were immediately placed back into their respective Plexiglas tube and nociceptive responses were recorded for 30 min using a video camera with night vision capability (Lorex by FLIR, ON, Canada) situated below the clear glass bottom of the observation chamber. The primary outcome was the time animals spent exhibiting nociceptive responses, defined as licking and biting of the injected hind paw, and assessed *post hoc* in five-minute bins by a separate blinded investigator from the video recordings. Other behavioural responses such as flinching were not considered for the primary endpoint.

Assessment of diurnal rhythm phase effects

Female C57BL/6 and CD-1 mice were randomly allocated to two groups to be tested separately at 12:00 and 24:00 hr, respectively. These time points represent the peaks of rest and activity and are known to influence animal responses to nociceptive stimuli.^{26,27} After the habituation period, mice received an injection of HS or NS into the surface of the right hind paw and were video recorded for five minutes. For animals tested at 24:00 hr, the entire experiment was performed in a dark room; red light was used briefly when administering solutions.

Assessment of sensitivity to the antinociceptive activity of morphine

Female C57BL/6 and CD-1 mice were randomly allocated to receive an intravenous injection of morphine into the tail vein (30 μ L; 0, 0.6, 2, 6, and 20 mg·kg⁻¹; each animal received only one dose). After five minutes, 10 μ L of HS were injected into the surface of the right hind paw (i.pl.) as described above and nociceptive responses over the following five minutes were recorded. We used the

duration of time animals spent licking and biting the injected paw to establish dose-response relationships to assess the HS assay's sensitivity to the antinociceptive activity of morphine in the inbred vs outbred animal strains.

Statistical analysis

Group responses are plotted with individual data points where feasible and reported as means (95% confidence intervals [CIs]); effect sizes are reported as differences in means with their 95% CIs. The amount of time mice spent licking and biting the affected paw during the first five minutes period after i.pl. injection was used for the primary analysis. Two-way analysis of variance (ANOVA) (factors: saline concentration $\times$ sex) followed by the Tukey multiple comparisons test was utilized to determine the effect of sex and saline concentration. To determine the effects of strain and sex on nociceptive responses in C57BL/6 vs CD-1 mice, we used two-way ANOVA (factors: stock/strain $\times$ sex) followed by the Tukey multiple comparisons test. To determine the effects of diurnal rhythm phase and HS in female C57BL/6 and CD-1 mice, we employed two-way ANOVA (factors: time of measurement $\times$ saline concentration) followed by the Sidak multiple comparisons test.

For assessment of the antinociceptive activity of morphine, we constructed dose-response curves using non-linear regression. Specifically, we first normalized dose-dependent nociceptive responses to the mean of control (NS) responses and then fitted the data to a four-parameter (variable slope) logistic equation ($\log[\text{inhibitor}]$ vs response):

$$Y = \text{Bottom} + [\text{Top} - \text{Bottom}] / [1 + 10^{([\log \text{ID}_{50} - X] * \text{Hill slope})}],$$

where Y is the response, Top and Bottom are the minimum and maximum nociceptive responses (plateaus), ID_{50} is the dose that produces 50% of the maximal inhibition (i.e., a response half-way between Top and Bottom), X is the dose, and Hill slope is the slope factor (i.e., the steepness of the curve). We calculated estimated ID_{50} s with their 95% CIs from the respective curves and used the extra sum of squares F test for statistical comparison.

Individual target sample sizes for each group were $n = 10$, which was consistent with and adapted from our previous studies.^{10,11} Based on previously observed mean durations of nocifensive licking and biting behaviour in female CD-1 mice due to HS and NS of 96 sec (95% CI, 76 to 116) and 10 sec (95% CI, -1 to 21) sec, respectively,¹⁰ and assuming a common standard deviation of 22, a sample size of ten animals per group would yield 99% power to detect a 45-sec difference between HS and NS and 80%

power to detect a 30-sec difference ($\alpha = 5\%$). For the morphine dose-response experiments, we studied a sample size of convenience of $n = 8$ per dose (total, $n = 80$).

Analyses were carried out using Prism software (GraphPad Software Inc., San Diego, CA, USA). $P < 0.05$ was set as the criterion for statistical significance.

Results

Nociceptive responses elicited by intraplantar hypertonic saline and effects of sex

The i.pl. injection of HS induced robust (i.e., in all animals tested) nociceptive (licking and biting) responses in both male and female C57BL/6 mice (Fig. 1). Two-way ANOVA revealed significant sex ($F_{[1,36]} = 8.7$, $P = 0.005$), saline concentration ($F_{[1,36]} = 79.6$, $P < 0.001$), and interaction effects ($F_{[1,36]} = 11.3$, $P = 0.002$). Female mice exhibited significant increases in the mean (95% CI) duration of nociceptive licking and biting responses to HS compared with NS during the first five-minute interval (87 sec [64 to 110] vs 5 sec [1 to 9], respectively; difference in means, 82 sec; 95% CI, 57 to 107; Tukey multiple comparison test, $P < 0.001$; $n = 10/\text{group}$) (Fig. 1A). Male mice also exhibited significant increases in mean licking and biting responses to 10% HS compared with NS during the first five minutes (45 sec [29 to 61] vs 8 sec [-2 to 18], respectively; difference in means, 37 sec; 95% CI, 12 to 63; Tukey multiple comparison test, $P = 0.002$; $n = 10/\text{group}$) (Fig. 1A). The mean (95% CI) magnitude of the nociceptive responses in female mice injected with HS was significantly higher than in male mice during the first five minutes (87 sec [64 to 110] vs 45 sec [29 to 61], respectively; difference in means, 42 sec; 95% CI, 17 to 68; Tukey multiple comparison test, $P < 0.001$; $n = 10/\text{group}$) (Fig. 1A). As shown in Fig. 1B, the licking and biting response in C57BL/6 mice was at a maximum during the first five-minute period after injection and gradually subsided over 15 min.

Hypertonic saline induced licking and biting nociceptive responses in both sexes of CD-1 mice, confirming our previous observations.¹⁰ As revealed by two-way ANOVA, there was a significant sex effect ($F_{[1,36]} = 20.9$, $P < 0.001$), saline concentration effect ($F_{[1,36]} = 184.1$, $P < 0.001$), and interaction between both parameters ($F_{[1,36]} = 26.5$, $P < 0.001$). HS induced robust (i.e., in all animals tested) mean (95% CI) nociceptive responses in female mice compared with NS control (110 sec [95 to 126] vs 1 sec [-1 to 2], respectively; difference in means, 109 sec; 95% CI, 87 to 132; Tukey multiple comparison test, $P < 0.001$; $n = 10/\text{group}$) (Fig. 1C). Males exhibited similar

Fig 1 Nociceptive responses of C57BL/6 (inbred) vs CD-1 (outbred) mice induced by intraplantar (i.pl.) 10% hypertonic saline (HS). **A**) Comparison of the duration of nocifensive licking and biting behaviour induced by i.pl. HS in male (blue) and female (red) C57BL/6 mice. HS evoked nocifensive licking and biting behaviour in both sexes compared with 0.9% normal saline (NS) control (two-way analysis of variance followed by the Tukey multiple comparison test: males, $P = 0.002$ [**]; females, $P < 0.001$ [***]). Female mice exhibited greater responses to HS than male mice ($P < 0.001$ [***]). There was no difference between the sexes in their responses to NS control ($P = 0.99$). Each data point represents an individual animal response during the first five-minute interval. Horizontal bars denote mean responses and vertical error bars represent 95% confidence intervals (CIs). **B**) Time course of observed licking and biting

responses after i.pl. injection of HS vs NS in male vs female C57BL/6 mice. Each point denotes the mean of five-minute interval responses; vertical error bars represent 95% CIs (same animals as in panel A). **C**) Comparison of the duration of nocifensive behaviour induced by i.pl. HS in male (blue) and female (red) CD-1 mice. Consistent with previous observations,¹⁰ HS evoked nocifensive behaviour in both sexes compared with NS control (both, $P < 0.001$ [***]), with females exhibiting greater responses than males ($P < 0.001$ [***]). Similar to C57BL/6 (panel A), there was no difference between the sexes in their responses to NS ($P = 0.98$). **D**) Time course of licking and biting responses after i.pl. injection of HS vs NS in male vs female CD-1 mice. Each point denotes the mean of five-minute interval responses; vertical error bars represent 95% CI (same animals as in panel C). All groups, $n = 10$ (total, $n = 80$).

responses with HS vs NS (53 sec [32 to 74] vs 4 sec [−1 to 10]; difference in means, 49 sec; 95% CI, 27 to 71; Tukey multiple comparison test, $P < 0.001$; $n = 10$ /group) (Fig. 1C). The mean (95% CI) duration of the nociceptive responses of female CD-1 mice to HS was twice that of males (110 sec [95 to 126] vs 53 sec [32 to 74], respectively; difference in means, 57 sec; 95% CI, 34 to 79; Tukey multiple comparison test, $P < 0.001$; $n = 10$ /group) (Fig. 1C). The nociceptive responses were at a maximum during the first five minutes post-injection and returned to a control level over 10–15 min (Fig. 1D). Similar to the sex differences observed with C57BL/6 mice and consistent with our previous findings,¹⁰ the mean licking and biting response magnitude in CD-1 mice was larger in females than in males.

When comparing the licking and biting response magnitudes between males and females of C57BL/6 and CD-1 mice, there was a significant sex effect ($F_{[1,36]} = 34.3$, $P < 0.001$), no strain effect ($F_{[1,36]} = 3.5$, $P = 0.07$), and no interaction between strain and sex ($F_{[1,36]} = 0.7$, $P = 0.39$). There were no differences in mean (95% CI) licking and biting responses of female C57BL/6 vs CD-1 mice following i.pl. HS injection (87 sec [64 to 111] vs 110 sec [95 to 126], respectively; difference in means, 23 sec; 95% CI, 9 to 55; Tukey multiple comparison test, $P = 0.24$; $n = 10$ /group). We similarly observed no differences in mean (95% CI) licking and biting responses of male C57BL/6 vs CD-1 mice following HS injection (45 sec [29 to 61] vs 54 sec [33 to 74], respectively; difference in means, 9 sec; 95% CI, 24 to 41; Tukey multiple comparison test, $P = 0.89$; $n = 10$ /group).

Effects of diurnal rhythm phase on nociceptive responses to intraplantar hypertonic saline

The licking and biting responses of female C57BL/6 mice to HS were sensitive to the time of measurement relative to the diurnal rhythm phase (Fig. 2A). We found a significant time effect ($F_{[1,36]} = 5.2$, $P = 0.03$), saline concentration effect ($F_{[1,36]} = 66.1$, $P < 0.001$), and interaction ($F_{[1,36]} = 4.5$, $P = 0.04$) between the two in the nociceptive responses. The mean (95% CI) licking and biting responses in animals that were tested with HS at 24:00 hr were significantly higher than those tested at 12:00 hr (64 sec [40 to 88] vs 37 sec [24 to 51], respectively; difference in means, 27 sec; 95% CI, 7 to 47; Sidak multiple comparisons test, $P = 0.007$; $n = 10/\text{group}$) (Fig. 2A). For CD-1 mice, the results of two-way ANOVA indicated a significant saline concentration effect ($F_{[1,36]} = 51.8$, $P < 0.001$) with no time of day effect ($F_{[1,36]} = 0.1$, $P = 0.80$), and no interaction between treatment and time of day ($F_{[1,36]} = 0.004$, $P = 0.95$). In other words, in contrast to C57BL/6 mice, there was no effect of diurnal rhythm phase on the nociceptive responses in female CD-1 mice (Sidak multiple comparisons test, $P = 0.97$; $n = 10/\text{group}$; Fig. 2B).

Comparative sensitivity to the antinociceptive activity of morphine

Intravenous morphine dose-dependently attenuated the licking and biting responses triggered by i.pl. injection of HS in both C57BL/6 and CD-1 female mice. Figure 3A shows the dose-response relationship of morphine in C57BL/6 mice with an estimated ID_{50} of $0.6 \text{ mg}\cdot\text{kg}^{-1}$ (95% CI, 0.3 to 1.5). Following administration of HS in

female CD-1 mice, morphine similarly produced a dose-dependent reduction in licking and biting response duration (Fig. 3B), with an estimated ID_{50} of $2.5 \text{ mg}\cdot\text{kg}^{-1}$ (95% CI, 1.5 to 4.2) that was not different from that in C57BL/6 mice (extra sum of squares F test, $F_{[1,72]} = 0.7$, $P = 0.41$).

Discussion

Our study lays the groundwork for future analgesic screening studies as it shows that the HS assay is applicable to inbred (C57BL/6) mice. Indeed, our aim was to test the HS assay using an inbred mouse strain that is extensively used in transgenic pain research. We found that the nociceptive responses of C57BL/6 mice, induced by i.pl. injection of HS, were similar and exhibited minimal phenotypic variability compared with an outbred (CD-1) stock. In both CD-1 and C57BL/6 mice, nociceptive responses occurred as early as five minutes after injection of HS and slowly recovered over approximately 15 min. We also found that the HS assay was sensitive to sex differences and morphine. Similar to CD-1 mice, female C57BL/6 mice were more sensitive to HS than male mice; as well, morphine dose-dependently produced antinociception with comparable potency. To our surprise, the nociceptive responses of female C57BL/6 mice were greater during the dark phase of the diurnal rhythm which was not observed in CD-1 mice. These findings solidify the practicality of the HS assay to induce reliable nocifensive behaviours and detect antinociceptive effects in both outbred and inbred mice.

In both C57BL/6 and CD-1 mice, the nociceptive responses induced by HS presented as licking and biting behaviours whose response magnitude depended on sex.

Fig 2 Diurnal rhythm phase effects on nociceptive responses induced by intraplantar 10% hypertonic saline. **A**) Nociceptive responses in C57BL/6 (inbred) mice tested at night (24:00) were significantly greater than those of animals tested during the day (12:00; two-way analysis of variance followed by the Sidak multiple comparisons test, $P = 0.007$ [**]). There was no difference between the groups in their responses to 0.9% normal saline control ($P = 0.99$). Each point represents individual animal responses during the first five-minute

interval. Horizontal summary bars denote means and vertical error bars represent 95% CIs. **B**) Nociceptive responses in CD-1 (outbred) mice tested at night (24:00) were not significantly different from those tested during the day (12:00; $P = 0.95$). There was no difference between the groups in their responses to 0.9% saline control ($P = 0.97$). All groups, $n = 10$ (total, $n = 80$).

Fig 3 Effects of morphine on nociceptive responses induced by 10% hypertonic saline. Shown is a dose-dependent attenuation of nociceptive licking and biting responses by intravenous morphine in C57BL/6 (inbred; *panel A*) mice and CD-1 (outbred; *panel B*) mice. Each animal received only one dose. Individual responses were normalized to the mean response of the 0.9% normal saline control group during the first five-minute interval. Horizontal bars and

vertical error bars represent means and 95% confidence intervals, respectively. There was no significant difference between the two (inbred vs outbred) strains in the estimated ID_{50} of morphine ($P = 0.41$; details, see body text). Each dose/group, $n = 8$ (total, $n = 80$).

Female C57BL/6 mice were more sensitive to the HS stimuli than male mice, consistent with both present and previous observations in CD-1 mice.¹⁰ The ability to detect sex differences in two of the most commonly used laboratory mice highlights the versatility of the HS assay. Several laboratories have used various preclinical pain assays to show the propensity for female rodents, compared with males, to exhibit greater nociceptive responses.^{28–31} The mechanistic basis of the sex differences in preclinical pain assays is not well understood. Nevertheless, sex-dependent modulation of nociceptive signals^{32,33} may involve the influence of sex hormones on the activities of neurotransmitter and immune systems.^{34,35} Future studies on the effects of sex hormones, and, in particular, γ -aminobutyric acid signalling,³⁶ may shed light on the mechanism of the sex differences observed with the HS assay. The clinical observation that human female subjects exhibit higher sensory scores compared with males after subcutaneous or intramuscular injection of 5% HS further corroborates our findings in mice.³⁷

Diurnal rhythm phase had differential effects on licking and biting responses induced in the HS assay. We found that inbred C57BL/6 mice exhibited greater licking and biting responses during the dark phase than the light phase. In the formalin foot assay, however, licking and biting responses of C57BL/6 mice during the first ten minutes were similar in both dark and light phases.³⁸ Indeed, this may reflect different physiologic mechanisms associated with formalin and hypertonic saline-induced inflammatory pain. It should be noted that while the HS assay did not detect diurnal rhythm phase effects on nociceptive responses of CD-1 mice, other outbred stocks (Deer and CF-1 mice) exhibit higher nociceptive response thresholds during the dark phase.^{39,40} The variable effects of diurnal

rhythm phase on nociceptive responses induced by HS assay warrant closer investigation.

Systemic morphine decreased licking and biting responses in both female C57BL/6 and CD-1 mice. This is noteworthy as evidence in the literature indicates that C57BL/6 mice are resistant to many analgesics, including morphine.¹⁶ Further, it has been reported that females require greater morphine doses than males for the same analgesic effect magnitude.²² Thus, based on previous studies, one might have expected lower potency estimations for female C57BL/6 mice. In the present studies, we found no significant difference in the estimated potencies between female C57BL/6 and CD-1 mice. This may be due to differences between the type of nociceptive stimulus used and the primary outcome that was being assessed. Nevertheless, the present findings demonstrate the HS assay's sensitivity to morphine in female C57BL/6 mice, similar to CD-1 mice and with comparable potency.

This study has several strengths. It provides the basis for the extension of the HS assay and its advantages—including those on the domains of *Reduction* and *Refinement*—for use in C57BL/6 mice, the most popular inbred murine strain for biomedical research involving gene modification and utilized in models of human disorders. The potential benefits for animal welfare, screening efficiency, cost, and drug discovery are immense. The randomized and blinded study design is key to the production of high-quality data and validity. The Table summarizes the attributes of the HS assay and provides a comparative overview of the advantages and disadvantages of existing assays.

The study also has a number of limitations. First, our goal was not to investigate the effects of a wide range of analgesic classes. Future studies that show the HS assay's ability to detect antinociceptive effects in inbred mice of

Table Overview of common preclinical rodent analgesic screening assays in comparison with the hypertonic saline assay

Assay	Advantages	Disadvantages
<i>Thermal</i>		
Tail flick ⁴⁹	- Simple, rapid	- Affected by stimulus location ^{51–53}
- Radiant heat applied to tail	- Low risk of tissue injury	- Prone to habituation ⁵⁴
	- Reliable after repeated testing ⁵⁰	- Narrow range of sensitivity to analgesics (predominantly opioids) ^{9,55}
		- Animal restraint required ⁵⁶
Hargreaves ⁵⁷	- Low risk of tissue injury	- Affected by stimulus location ⁵⁰
- Radiant heat applied to hind paw	- No restraint required	- Lengthy habituation time ⁵⁸
	- Animal serves as own control ^{58,59}	- Expensive equipment
Hot plate ⁶⁰	- No restraint required	- Sensitivity to analgesics dependent on temperature ⁶¹
- Animal placed on heated plate	- Multiple behaviours for assessment ⁵⁹	- Reproducibility issues ⁶²
<i>Mechanical</i>		
von Frey	- Simple, rapid	- Affected by stimulus location ⁵⁹
- Filaments applied to hind paw	- Low risk of tissue injury	- Lengthy habituation time
	- Animals serve as own control	- Subjective assessment
		- Expensive equipment
<i>Chemical</i>		
Writhing ⁶³	- Simple, rapid	- Response dependent on algogen used ⁶⁴
- i.p. injection into abdominal region	- Low risk of tissue injury	- Analgesic specificity difficult to detect ⁹
Formalin ⁶⁵	- Simple	- Time-consuming ⁶⁶
- s.c. injection into hind paw	- Sensitive to wide range of analgesics ⁵⁸	- Produces tissue injury;
	- No restraint required	- inappropriate for repeated testing ^{67,68}
		- Affected by ambient temperature ⁶⁹
Hypertonic Saline ^{10,11}	- Simple	- Validity among other murine strains to be defined
- i.pl. injection into hind paw	- Rapid (< 10 min)	- Mechanism of hypertonic saline-induced nociception unclear
	- Low risk of tissue injury ¹⁰	
	- Low number of animals required	
	- Sensitive to wide range of analgesics ¹⁰	
	- No restraint required	
	- Consistent reproducible results after repeated testing ¹¹	
	- Similar responses in outbred (CD-1) and inbred (C57BL/6) mice	
	- Sensitive to sex differences	

i.p. = intraperitoneal. s.c. = subcutaneous. i.pl. = intraplantar.

such agents as non-steroidal anti-inflammatory drugs or local anesthetics would encourage further adoption of the assay in preclinical analgesic research. Second, only female animals were used in diurnal rhythm phase and morphine studies. An argument can be made that daily tracking of the four-day estrous cycle was necessary to determine hormonal influence on nocifensive behaviours and responses to morphine. Nevertheless, one study found that female mice tested at random were no more variable than males in terms of nociceptive traits.⁴¹ Another area of future research may be to investigate diurnal rhythm phase

and non-opioid analgesic effects in males to supplement our present findings. Third, we assessed only one nocifensive behavioural modality induced by the HS assay. Other behaviours that may be indicators of spontaneously evoked pain include analgesic self-administration, conditioned place preference, or flinching.⁴² To further support widespread translatability of the HS assay, it may be worthwhile for future studies to assess these behaviours as well. Fourth, we recognize that the phenotypic nociceptive responses to many other murine strains may not be reflected by our results and thus, any

future research utilizing the HS assay would benefit from method validation for each specific strain. Finally, the precise mechanistic basis of the nociceptive responses due to HS remains unsolved. While it has been long been reported that HS produces acute and transient pain in studies of human muscle and joint pain,^{43–48} room for future mechanistic studies exists to explore the relationship between HS-induced nociception in rodents and acute pain states.

In conclusion, the 10% HS analgesic assay produced licking and biting nocifensive behaviours in C57BL/6 mice, detected sex-dependent differences, and was sensitive to the antinociceptive activity of morphine. For preclinical pain assays to be useful, nociceptive responses must be specific, reliable, and sensitive to known analgesics. The present findings extend the utility of the HS assay as an easy, effective, and robust preclinical tool for analgesic screening to inbred mice, thus increasing its generalizability in preclinical research.

Author contributions Yahya I. Asiri was involved in the conception and design of the study, conduct of the experiments, data collection, data analysis, and manuscript preparation; he co-wrote the first draft of this paper. Desmond H. Fung was involved in data analysis and manuscript preparation; he co-wrote the first draft of this paper. Timothy Fung helped with the conduct of the experiments, data collection, and manuscript preparation. Alasdair M. Barr, Ernest Puil, Bernard A. MacLeod, and Stephan K. W. Schwarz were involved in the conception and design of the study, data analysis, manuscript preparation, and critical editing for final submission. All authors provided intellectual contributions to this work and approved the final manuscript for submission.

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