A Novel Multitarget Mu- and Delta-Opioid Receptors Agonist HAGD Has Peripherally Restrictive Potent Analgesia with Less Side Effects in Mice and Minimal Impact on Human Sperm Motility

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Abstract

Objective

Pain is a common clinical symptom. Although a variety of opioid analgesics have been developed, the side effects including negative impact on human sperm motility still hinder their application. Aim of this study is to develop a novel opioid analgesic, a multitarget peptide HAGD (H-Tyr-D-Ala-Gly-Phe-NH_2) which has less side effects and minimal impact on sperm motility.

Methods

The peripheral antinociceptive effects of HAGD were appraised in a series of preclinical mice pain models, including the tail-flick test, carrageenan-induced inflammatory pain, acetic acid-induced writhing test and formalin test. In conditioned place preference experiment, open field test, gastrointestinal transit test and rotarod test, the side effects of HAGD in mice were assessed. The impacts of HAGD on sperm motility in vitro were investigated.

Results

HAGD produced equipotent antinociception compared with morphine. HAGD was stronger in terms of analgesia intensity in chemical stimulation pain of formalin test phase I. The antinociception was mediated by mu- and delta-opioid receptors. HAGD didn't induce conditioned place preference and hyperlocomotion, but morphine did. Both HAGD and morphine had no impacts on motor coordination. HAGD had a limited side effect in gastrointestinal transit, while morphine inhibited gastrointestinal transit to a greater extent. However, HAGD had minimal impact on human sperm motility, whereas morphine declined sperm motility at concentrations of 1×10^{-7} mol/l and 1×10^{-8} mol/l at 3.5 h of incubation.

Conclusion

HAGD may be a better candidate for future development of novel multitarget opioid analgesics with less side effects and minimal impact on human sperm motility.

Introduction

Infertility is defined as the failure of gestation after more than 12 months of regular unprotected sexual intercourse in International Classification of Diseases [1]. In 2010, the number of infertile couples was between 45.0 million and 52.6 million globally [2]. According to World Health Organization (WHO), infertility affects up to 15% of reproductive age couples universally, in which male infertility accounted for 50% [3]. Male infertility arose from assorted aspects, including reproductive system disorders (i.e., infection, anatomical defects and genetic disorders) [4, 5], lifestyle (i.e., smoking, excessive alcohol intake and obesity), environmental pollutants and toxins [6, 7], psychological stress [8] and drugs (like opioid analgesics) [9]. In reproductive system, ejected semen is the most common matter of infertility [1], while poor sperm motility is the major one.

Most of injuries and diseases are accompanied by pain. Pain is also the most common clinical symptom for which patients pay medical attention [10]. Analgesics usually used include non-opioid analgesics (e.g., non-steroidal anti-inflammatory drugs, antidepressants and anticonvulsants) and opioid analgesics (e.g., morphine, tramadol and remifentanil). Opioid drugs have proven to be potent against moderate to severe acute and chronic pain [10]. They are typical mu (μ) opioid receptor agonists, led by morphine, as well as fentanyl and oxycodone [11, 12]. However, the utilization of opioid analgesics is partly limited due to their side effects, such as addiction, constipation, nausea, respiratory depression and poor sperm motility [13, 14, 15, 16]. According to previous researches, the activation of μ opioid receptor may decrease sperm motility, while the activation of delta (δ) opioid receptor may maintain sperm motility [15, 16, 17].

In 1997, Zadina et al. [18] discovered and isolated endomorphin-1(EM-1, Tyr1-Pro2-Trp3-Phe4-NH_2) and endomorphin-2 (EM-2, Tyr1-Pro2-Phe3-Phe4-NH_2) from bovine frontal cortex, which are highly selective for μ opioid receptor and produce more potent and prolonged analgesia in contrast with morphine in mice. Soon after, Czapla et al. [19] elucidated that respiratory and cardiovascular effects of EMs are less severe than morphine in rats; besides, findings demonstrated the inability of EM-1 to cross the blood-brain barrier (BBB) into the central nervous system (CNS) as an analgesic [20]. Therefore, EM-1 could provide the basis for evolving novel and safer analgesics, such as multitarget ligands [21]. Recently, our group developed a multi-targeting peptide HAGD (H-Tyr-D-Ala-Gly-Phe-NH_2) by structural optimization. HAGD can activate μ-/δ-opioid receptors and peripherally act potent analgesic with limited side effects and minimal impact on sperm motility.

Here, antinociceptive effects of peripheral HAGD were investigated in a series of preclinical mice pain models, including the tail-flick test, carrageenan-induced inflammatory pain, formalin test and acetic acid writhing. Meanwhile, side effects of peripheral HAGD were evaluated in conditioned place preference (CPP), open field test, gastrointestinal transit (GIT) and rotarod test. The impact of HAGD on human sperm motility in vitro was evaluated.

Materials And Methods

Drugs and preparations

HAGD (H-Tyr-D-Ala-Gly-Phe-NH_2) was obtained by solution-phase methods with segment-coupling peptide synthesis strategy as our previous reports [22, 23]. Morphine hydrochloride was produced by Shenyang First Pharmaceutical Factory (Shenyang, China). Naloxone (Nal), naloxone methiodide (NALM), β-funaltrexamine hydrochloride (β-FNA), nor-binaltorphimine dihydrochloride (nor-BNI) and naltrindole (NTI) were purchased from Sigma-Aldrich (St. Louis, MO, USA).G-MOPS^{TM} PLUS was obtained from Vitrolife Sweden AB (Goteborg, Sweden). Drugs for mice were dissolved in physiological saline, and for human sperm were dissolved in G-MOPS^{TM} PLUS. All drugs were stored at −20 ℃. G-MOPS ^{TM} PLUS was stored at 4 ℃.

In animal experiments, HAGD or morphine was administered subcutaneously (s.c.) in conscious mice. The selective antagonists for μ-, δ- and κ-receptors, i.e., β-FNA (1 mg/kg), NTI (1 mg/kg) and nor-BNI (1 mg/kg), respectively, were s.c. injected 4 h, 10 min or 30 min, respectively, prior to s.c. injection of HAGD (10 mg/kg). Nal (1 mg/kg, s.c.) or NALM (1 mg/kg, s.c.; 5 nmol, intracerebroventricular (i.c.v.)) was injected 10 min, respectively, prior to s.c. injection of HAGD (10 mg/kg).

Animals

Adult Male Kunming mice (18–22 g) were provided by the Experimental Animal Centre of Lanzhou University. Mice were housed in a standard animal room that was maintained at 22 ± 1 °C with a 12/12-h dark/light cycle. Food and water were available freely. All animal experimental protocols were approved by the Ethics Committee of Lanzhou University (permit number: SYXK Gan 2009-0005) and were carried out in accordance with the European Community guidelines for the use of experimental animals (2010/63/EU). Animals were randomly assigned into the experimental groups. Each group included 5 mice at least with best efforts to minimize the numbers of animals used. All mice were used only once in this study. Before experiments, mice were allowed to acclimatize to the environment in the behavior room for 30 min. The study was blinded to treatment assignment and date analysis.

The tail–flick test

The radiant heat tail–flick test has been widely used as an acute pain model to judge effects of analgesics [24]. Mice were gently stabilized by hand, and dorsal surface of the tail 3 cm from the distal end was placed on the radiant heat source. To avert tissue damage, the cut-off time was set at 10 s. The radiant heat intensity was adapted to engender a basal latency within 3-5 s in naive mice. The tail–flick latency was defined as the time when the mouse flicked its tail away from the radiant heat source.

Carrageenan-induced inflammatory pain

The carrageenan was used as an inflammatory pain model to estimate antinociception of drugs [25]. Mice were placed into a clear plastic chamber on the glass surface of the radiant heat equipment (PL-200, Chengdu Technology & Market Co., Ltd., Chengdu, China). The cut-off time was set at 25 s to avoid tissue damage. On the first day, the paw withdrawal threshold was tested, and then the right hind paw was intraplantarly injected with 20 μl of 2% λ-carrageenan. Twenty-four hours later, thermal hyperalgesia was measured. Subsequently, the paw withdrawal threshold was measured 3 times at approximately 2-min intervals at every point after administration.

Acetic acid-induced writhing test

Acetic acid-induced writhing test was performed to investigate visceral pain [26]. Mice were acclimatized in a transparent acrylic chamber (20 × 20 × 30 cm) for 15-20 min. First of all, the mice were s.c. injected with drugs. Five minutes later, the mice got intraperitoneal injection (i.p.) of 0.6% (v/v) acetic acid solution (10 ml/kg. Within the 5-15 min after injecting acetic acid, the number of writhes was written down. A writhe was defined as a contraction of abdominal muscles together with a stretching of hind limbs.

Formalin test

Formalin test was conducted following the previous research [27]. Mice were acclimated in a transparent plexiglass chamber (20 × 20 × 30 cm) for 15-20 min. Firstly, mice received s.c. injection of drugs. Then, the right hind paw was intraplantarly administrated with 20 μl of 5% formalin at 5 min post-injection. Immediately, the mice were placed back in the chamber. Simultaneously, the time spent licking, biting and shaking injected paw within 0-5 min and 15-30 min was recorded.

Conditioned place preference experiment

CPP experiment was a classical model for evaluating dependence [25]. The CPP device consisted of three compartments. Two large compartments (20 × 20 × 20 cm) were connected by a narrower compartment (5 × 20 × 20 cm). The large compartments were visually and tactually distinct (white walls with a rough floor versus black walls with a smooth floor). Before the experiment, mice could move freely for 15 minutes to accommodate to the apparatus. On the first day, the time when mice stayed in each compartment was documented within 15 min. Over the next three days, mice were s.c. administrated with drugs and limited to one of two large compartments. About 6 hours later, mice were s.c. injected with saline and confined to the opposite compartment. On the fifth day, mice moved freely again for 15 min and the time spent in each compartment was logged.

Open-field test

The open-field test was implemented to assess mouse locomotor activity [28]. Apparatuses were an uncovered black plexiglass arena (50 × 50 × 40 cm) and a video tracking system (PMT-100, Chengdu Technology & Market Co., Ltd., Chengdu, China). At the beginning, mouse was placed in the center of this arena and permitted to explore freely for 30 min. After that, mouse was s.c. injected with saline, HAGD or morphine, and locomotor activity was monitored for another 150 min. Experiment arena was wiped with 75% ethanol to eliminate scent.

Gastrointestinal transit test

GIT test was carried out according to previous reports [24, 29]. Briefly, mice were fasted for 16 h with free access to water. Drugs were s.c. injected 15 min before oral administration of a charcoal meal (an aqueous suspension of 5% charcoal and 10% gum arable, 10 ml/kg). Thirty min after oral administration, animals were sacrificed. The farthest traveled distance of charcoal meal (L_1) and total length of small intestine (L_2) were measured. The result was represented by the percent of gastrointestinal transit: GIT% = (L_1/L_2) ×100.

Rotarod test

Motor coordination and equilibrium were determined using a rotarod apparatus (ZB-200, Chengdu Technology & Market Co., Ltd., Chengdu, China) [28]. Mice were trained on the rotating rod at 16 revolutions per min (rpm) in three consecutive trials for 2 days. Animals remaining on the rod for 180 s at

least were used. The cut-off time was 300 s. On the third day, mice received s.c. administration. The latency to fall off the rod was recorded at 15, 30, 45, 60 and 90 min after administration.

Sperm preparation

Freshly ejaculated semen was obtained by masturbation after abstinence (3-7 days). All donors (18-40 years old) had normal sperm parameters according to the reference of the WHO manual 2010 [30]. Related experimental protocols were approved by the Ethics Committee of the First Hospital of Lanzhou University. Semen ejaculated into sterile containers was liquefied at 37 ℃ for 30 min. Samples were processed by a swim-up technique [15, 16]. One 15-ml centrifuge tube contained fresh semen (2 ml) and G-MOPS™ PLUS medium (2 ml). After approximately 45 min incubation at 37 ℃, most of the upper medium layer was transferred to another new centrifuge tube and centrifuged at 1500 rpm for 4 min. Subsequently, the sperm sediment was resuspended to 20 × 10^6 cells/ml with G-MOPS™ PLUS. Spermatozoa suspension with at least 60% of forward motility was used for subsequent.

Incubation medium

Spermatozoa suspension was divided into 20-μl aliquots. The control aliquot was treated with G-MOPS™ PLUS, and the others were incubated with different doses of HAGD (180 μl) or morphine (180 μl) for 4 hours at 37 °C. All media were made on the day of use and maintained at 37 ℃. All incubations were done at 37 °C.

Motility analysis

Motility analysis was carried out by automatic sperm analysis system (Beijing Suiplus Software Co., Ltd., Beijing, China) at time 0, 0.5, 1, 3.5 and 4 h after drug addition to the medium. To measure sperm concentration and motility, a wet preparation was made. A minimum of 200 sperm from at least 5 different fields was analyzed. The motility of each spermatozoon is graded as WHO (2010): Progressive motility (PR): spermatozoa moving actively, either linearly or in a large circle, regardless of speed; non-progressive motility (NP): all other patterns of motility with an absence of progression; immotility (IM): no movement.

Statistical analysis

All data were presented as the mean ± SEM (GraphPad Prism 7.0). Data were analyzed with one-way ANOVA followed by Dunnett's or Bonferroni's post hoc test. The dose- and time-response was analyzed by Two-way ANOVA. $P < 0.05$ was considered statistically significant.

Results

Antinociceptive effects of subcutaneous HAGD in the tail-flick test

HAGD produced dose- and time-dependent increases in the tail-flick latencies, which peaked at 15 min **(Fig. 1A)**. HAGD (10 mg/kg) produced equipotent antinociception compared with morphine (10 mg/kg) in the area under the curve (AUC) **(Fig. 1B)**. Our previous researches indicated that HAGD acts as an opioid receptor agonist to activate μ- and δ-opioid receptors [23]. To further explore whether opioid receptors are involved in HAGD-induced antinociception, mice were pretreated with antagonist Nal or peripheral antagonist NALM [31]. Pretreatment with Nal or NALM (s.c.) completely blocked antinociception of HAGD **(Fig. 1C, D)**. On the contrary, HAGD-induced antinociception was not affected by NALM (i.c.v.) **(Fig. 1E)**. The figure shows that the peripheral antinociception of HAGD was significantly blocked by μ- and δ-opioid receptor antagonists β-FNA and NTI, but not by κ-opioid receptor antagonist nor-BNI **(Fig. 1F-H)**.

Antinociceptive effects of subcutaneous HAGD in carrageenan-induced inflammatory pain Carrageenan isolated from sea plants, is a polysaccharide, and λ-carrageenan has been prevalently used to induce an inflammatory pain. HAGD displayed dose- and time-dependent anti-allodynic activities in carrageenan-induced inflammatory pain model. The anti-allodynic activities of HAGD lasted for 90 min, with a peak effect of 30 min post-injection **(Fig. 2A)**. Morphine (10 mg/kg) and HAGD (10 mg/kg) exhibited equipotent antinociception in the AUC **(Fig. 2B)**.

Antinociceptive effects of subcutaneous HAGD in acetic acid writhing test

As a widely used pain model, the acetic acid writhing test is to assess visceral pain induced by irritant chemical stimulation. Intraperitoneal injection of 10 ml/kg 0.6% acetic acid produced significant abdominal constrictions, and the average writhing number was 34 ± 2.49 in saline group in 5-15 min after i.p. injection of acetic acid. HAGD produced a dose-dependent inhibition of the writhing response, and the average number of 1 mg/kg HAGD was 5.83 ± 1.94; subcutaneous injection of 1 mg/kg of morphine also inhibited the writhing response, and the average number was 4.60 ± 2.43. There was no significant difference between HAGD (1 mg/kg) and morphine (1 mg/kg) in inhibiting writhing response **(Fig. 3)**.

Antinociceptive effects of subcutaneous HAGD in the formalin test

In the formalin test, intraplantar injection of 5% formalin performs a biphasic pain response (phase I: 0–5 min and phase II: 15–30 min) during 30 min observed period. It is thought that phase I is induced by the direct activation of nociceptors, whereas the subsequent phase II represents the combined effects of nociceptor input and spinal cord sensitization. In Figure 4, HAGD dose-dependently inhibited licking/flinching/biting behaviors induced by formalin in both phase I and phase II. In line with the inhibitory results of HAGD (10 mg/kg), subcutaneous injection of 10 mg/kg of morphine also attenuated nociceptive responses in both phase I and phase II; moreover, HAGD (10 mg/kg) had better analgesic effects than morphine (10 mg/kg) in phase I, but there was no significant difference in the phase II **(Fig. 4)**.

Side effects evaluation of subcutaneous HAGD

In CPP, saline did not induce a place preference change; there was a significant place preference change in 10 mg/kg of morphine with CPP scores of 140.50 ± 43.96, compared to saline. On the contrary, 10 mg/kg of HAGD did not induce a significant place preference difference. Furthermore, there was a significant difference in the CPP scores between HAGD and morphine **(Fig. 5A)**. Activation of the dopaminergic reward circuits partially contributes to the addiction properties of opioid analgesics, which also produces an acute hyperlocomotive response in the open field test [32, [33]. In the open field test, 10 mg/kg of HAGD did not show apparent effect on locomotion compared with saline. In contrast, 10 mg/kg of morphine enhanced the locomotion activity of mice and significantly increased the total distance travelled compared with saline or HAGD **(Fig. 5B)**. In GIT, 10 mg/kg of HAGD induced a significant delay in mice when compared to saline. 10 mg/kg of morphine also significantly weakened GIT, and exhibited a greater inhibition effect than HAGD **(Fig. 5C)**. The potential influence of 10 mg/kg of HAGD on the motor function was evaluated in the rotarod test. Compared with saline group, there was no significant difference in the HAGD or morphine group in the endurance time on the rotating rod in the mice **(Fig. 5D)**.

Effects of HAGD on human sperm motility in vitro

As showed in Figure 6, compared to the G-MOPS^{TM} PLUS control group, the addition of μ-/δ-receptor agonist HAGD to the incubation medium did not produce significant changes in PR of sperm during the 4-hour incubation process, no matter what concentration HAGD was (0.0001, 0.001, 0.01, 0.1, 1 or 10 μM) **(Fig. 6A-F)**. The addition of μ-receptor agonist morphine did not also change the PR of sperm at 0.5, 1 3.5 or 4 h, when the concentrations of morphine were 0.0001 or 0.001 μM **(Fig. 6A, B)**. However, when spermatozoa were incubated with 0.01 or 0.1 μM of morphine for 3.5h, the PR of sperm was significantly reduced compared with vehicle (45.75 ± 2.89 vs. 57.29 ± 2.22; 46.16 ± 1.15 vs. 57.29 ± 2.22; P 0.05.) **(Fig. 6C, D)**. Higher doses of morphine (1 or 10 μM) did not significantly alter PR of sperm when compared to the control **(Fig. 6E, F)**.

Discussion

In the clinic, morphine performs an important role in the treatment of moderate to severe degrees of acute and chronic pain. The number of infertile couples also continues to increase in society. There is an urgent need for novel compounds that can provide opioid-like analgesia without side effects bothering doctors and patients. In this study, our results elucidated that peripheral HAGD might be a promising compound for developing multi-targeting opioid analgesics with less side effects and minimal impact on human sperm motility.

In the mouse radiant heat tail-flick test, compared to morphine, s.c. administration of HAGD could produce equipotent analgesia effects in acute heat irritation pain model. The antinociception induced by s.c HAGD was blocked by the opioid receptor antagonist Nal and NALM when they were peripherally but not centrally administrated. The antinociception of s.c. morphine was not blocked by peripheral administration of NALM [24]. These findings support that HAGD has poor inability to cross the BBB into the CNS. Furthermore, the peripheral analgesic effects of HAGD were blocked by μ- and δ-opioid receptor

selective antagonist β-FNA and NTI, respectively, which presented that peripheral antinociception of HAGD were mainly mediated by opioid receptors, namely μ- opioid receptor and δ-opioid receptor.

In other preclinical mice pain models tested, HAGD appeared to still maintain similar potent analgesic effects when compared with morphine. In carrageenan-induced inflammatory pain model, the maximum analgesic dose of s.c. HAGD and morphine was both 10 mg/kg in the thermal hyperalgesia mice. During the observed period, the AUC values of MPE % of HAGD (10 mg/kg) and morphine (10 mg/kg) had no significant differences, which was consistent with other inflammatory pain in formalin test phase II. Although there was a significant difference either HAGD (10 mg/kg) or morphine (10 mg/kg) compared to vehicle, HAGD was still stronger than morphine in terms of analgesia intensity (41.11 ± 6.40 vs 67.36 ± 3.93, P 0.05) in chemical stimulation pain of formalin test phase I. This illustrated HAGD was better than morphine in the pain of chemical irritation. In visceral pain, the analgesic strength of s.c. HAGD and morphine increased by 10 times compared to other pain models above, and the maximum analgesic dose became 1 mg/kg in acetic acid-induced writhing test.

In general, the analgesic effect of HAGD was equivalent to that of morphine, and the former was even better in the chemically stimulated mice model. Previous studies have shown that EMs is better than morphine in a cold water allodynia test in rats with a sciatic nerve injury [34]. Pasquinucci et al. and Vicario et al. also reported that simultaneous activation of μ- and δ-opioid receptors exhibited a potent antinociceptive property in inflammatory and neuropathic pain modulation [35, 36, 37].

Although opioid-like analgesics play an important role in relieving human pain, the unfavorable side effects also limit their application. From a long-term perspective, the safety of drugs must be guaranteed. Addiction is an aspect that must be considered in the development of opioid drugs. In our study, addiction was assessed in CPP experiment, and only the analgesic dose of morphine presented significant conditioned place preference. There was no significant difference between HAGD and saline. Addiction properties of opioid analgesics were partly associated with the activation of dopaminergic reward circuits, which also induced an acute hyperlocomotive response in mice injected with morphine but not HAGD in the open field test. HAGD only had a limited side effect in the GIT, whereas morphine inhibited GIT to a greater extent (44.83 ± 4.14 vs 24.11 ± 3.79, P 0.05). HAGD and morphine didn't perform remarkable impacts in mice on the rotating rod. Taken together, HAGD didn't have significant effects on CPP, acute hyperlocomotive response and motor coordination at potent analgesic doses in mice. Dietis and colleagues have also reviewed that targeting multiple opioid receptors simultaneously may improve side effects [21].

For reproductive andrologists, the most concerned issue is the effect of opioid analgesics on male sperm motility. As early as 30 years ago, Ragni et al. [38] has proposed that reduced sperm motility (asthenozoospermia) is a common abnormality in opiate drug addicts. In 2006, the presence of functional μ-, δ-, and κ-opioid receptors in human sperm membranes, was reported for the first time by Agirregoitia and colleagues [16]. Until today, the effects of exogenous and endogenous opioids on human sperm motility by three classical opioid receptors have been studied in lots of literature [15, 17, 22,

23]. In our present study, all the different concentrations of HAGD (10, 1, 0.1 0.01, 0.001 and 0.0001 μMol/L) did not have a significant effect on human sperm motility within the duration of the experiment. Consistent with previous researches [16], 0.1 μMol/L (namely 1×10^{-7} Mol/L) morphine greatly reduced human sperm motility at 3.5 hours of the experiment. At the same time, we found that there was also a significant inhibition at 3.5h when the morphine concentration was 0.01 μMol/L (namely 1×10^{-8} Mol/L). However, this phenomenon did not appear in other concentrations of morphine tested. This may be related to the ratio of μ and δ opioid receptors activated by different concentrations of morphine. In summary, HAGD, a multitarget mu-/delta- opioid receptors agonist, had minimal impact on human sperm motility.

Conclusions

HAGD as a multitarget μ- and δ-opioid receptors agonist, HAGD may be a better candidate for future development of novel multitarget opioid analgesics with less adverse effects and minimal impact on human sperm motility.

Abbreviations

WHO: World Health Organization

EM-1: endomorphin-1

EM-2: endomorphin-2

BBB: blood-brain barrier

CNS: central nervous system

CPP: conditioned place preference

GIT: gastrointestinal transit

NaI: aloxone

NALM: naloxone methiodide

β-FNA: β-funaltrexamine hydrochloride

nor-BNI: nor-binaltorphimine dihydrochloride

NTI: naltrindole

s.c.: subcutaneous

i.c.v.: intracerebroventricular

i.p.: intraperitoneal

rpm: revolutions per min

PR: Progressive motility

NP: Non-progressive motility

IM: Immotility

AUC: area under the curve

MPE%: percentage maximal possible effect

Declarations

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Authors' contributions

YQW, SQH and XHZ designed the study. FFL carried out experiments. YQW, FFL, FY, WZ and BX analyzed and interpretated the data. YQW and FFL wrote the paper. All authors read and approved the final manuscript.

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Availability of data and materials

All data from this study are available from the corresponding author on reasonable request.

Ethics approval and consent to participate

All animal experimental protocols were approved by the Ethics Committee of Lanzhou University (permit number: SYXK Gan 2009-0005) and were carried out in accordance with the European Community

guidelines for the use of experimental animals (2010/63/EU). Human sperm experimental protocols were approved by the Ethics Committee of the First Hospital of Lanzhou University.

Consent for publication

All authors have approved the manuscript for submission.

Competing interests

The authors declare that they have no conflict of interest.

Footnotes

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Figures

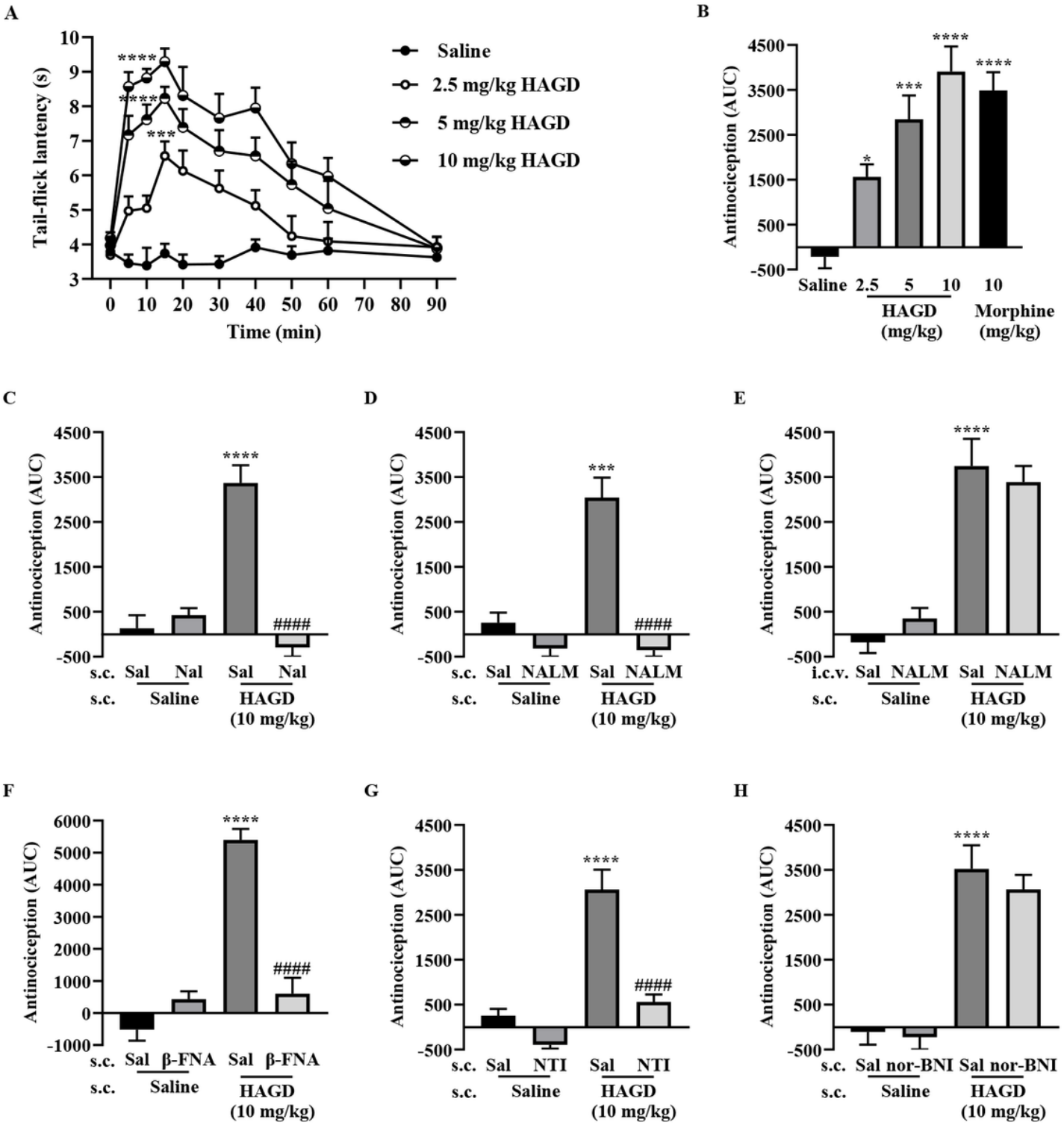

Figure 1

Antinociceptive effects of HAGD in tail-flick test after subcutaneous administration. (A) Antinociceptive dose- and time-response curve for HAGD; n = 6, 5, 5, 6 mice; *** P < 0.001 and **** P < 0.0001 indicate significant differences compared with Saline group, according to two-way ANOVA, followed by Bonferroni's post hoc test. (B) The AUC values of percentage maximal possible effect (MPE%) of HAGD and morphine during the observed period; n = 6, 5, 5, 6, 6 mice; * P < 0.05, *** P < 0.001 and **** P <

0.0001 indicate significant differences compared with Saline group, according to ono-way ANOVA, followed by Dunnett's post hoc test. (C) Effect of pretreatment with Nal on the HAGD antinociception; n = 5, 5, 5, 6 mice; (D) Effect of pretreatment with NALM (s.c.) on the HAGD antinociception; n = 5, 5, 5, 6 mice; (E) Effect of pretreatment with NALM (i.c.v.) on the HAGD antinociception; n = 5, 5, 5, 6 mice; (F) β-FNA blocked the antinociception of HAGD; n = 5, 6, 5, 6 mice; (G) NTI blocked the antinociception of HAGD; n = 5, 5, 5, 6 mice; (H) nor-BNI had no effect on the antinociception of HAGD; n = 5, 5, 5, 5 mice ; *** P < 0.001 and **** P < 0.0001 indicate significant differences compared with the Saline + Saline group, #### P < 0.0001 indicates significant differences compared with the Saline + HAGD group, according to ono-way ANOVA, followed by Bonferroni’s post hoc test.

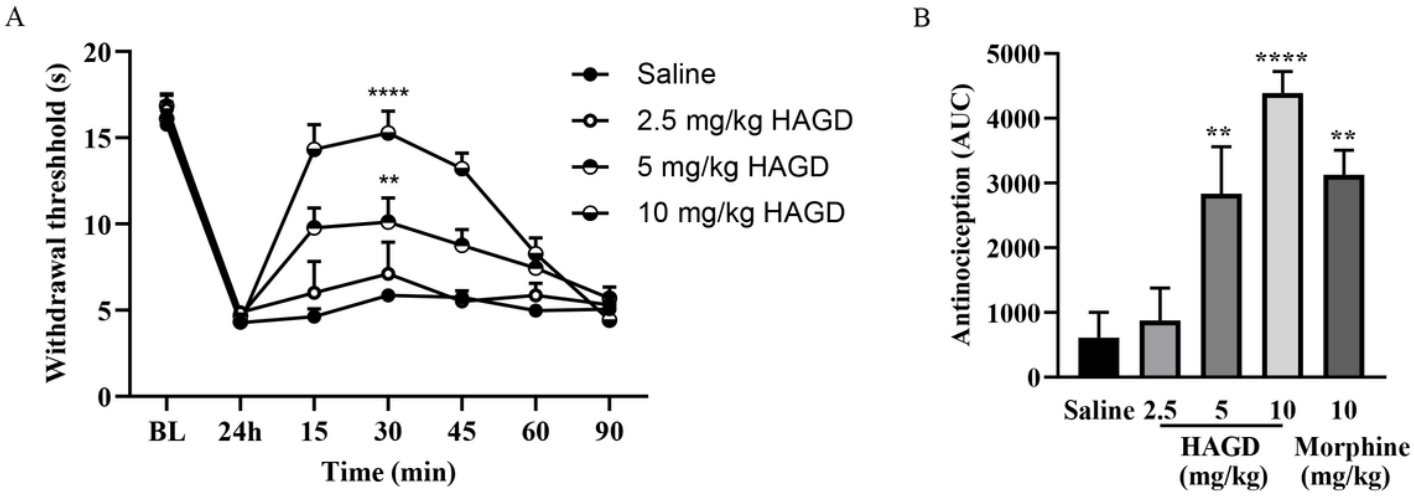

Figure 2

Antinociceptive effects of HAGD in Carrageenan-induced inflammatory pain mice after subcutaneous administration. (A) Antinociceptive dose- and time-response curve for HAGD; n = 9, 8, 9, 8 mice; ** P < 0.01 and **** P < 0.0001 indicate significant differences compared with Saline group, according to two-way ANOVA, followed by Bonferroni’s post hoc test. (B) The AUC values of MPE % of HAGD and morphine during the observed period; n = 9, 8, 9, 8, 9 mice; ** P < 0.01 and **** P < 0.0001 indicate significant differences compared with Saline group, according to one-way ANOVA, followed by Dunnett's post hoc test.

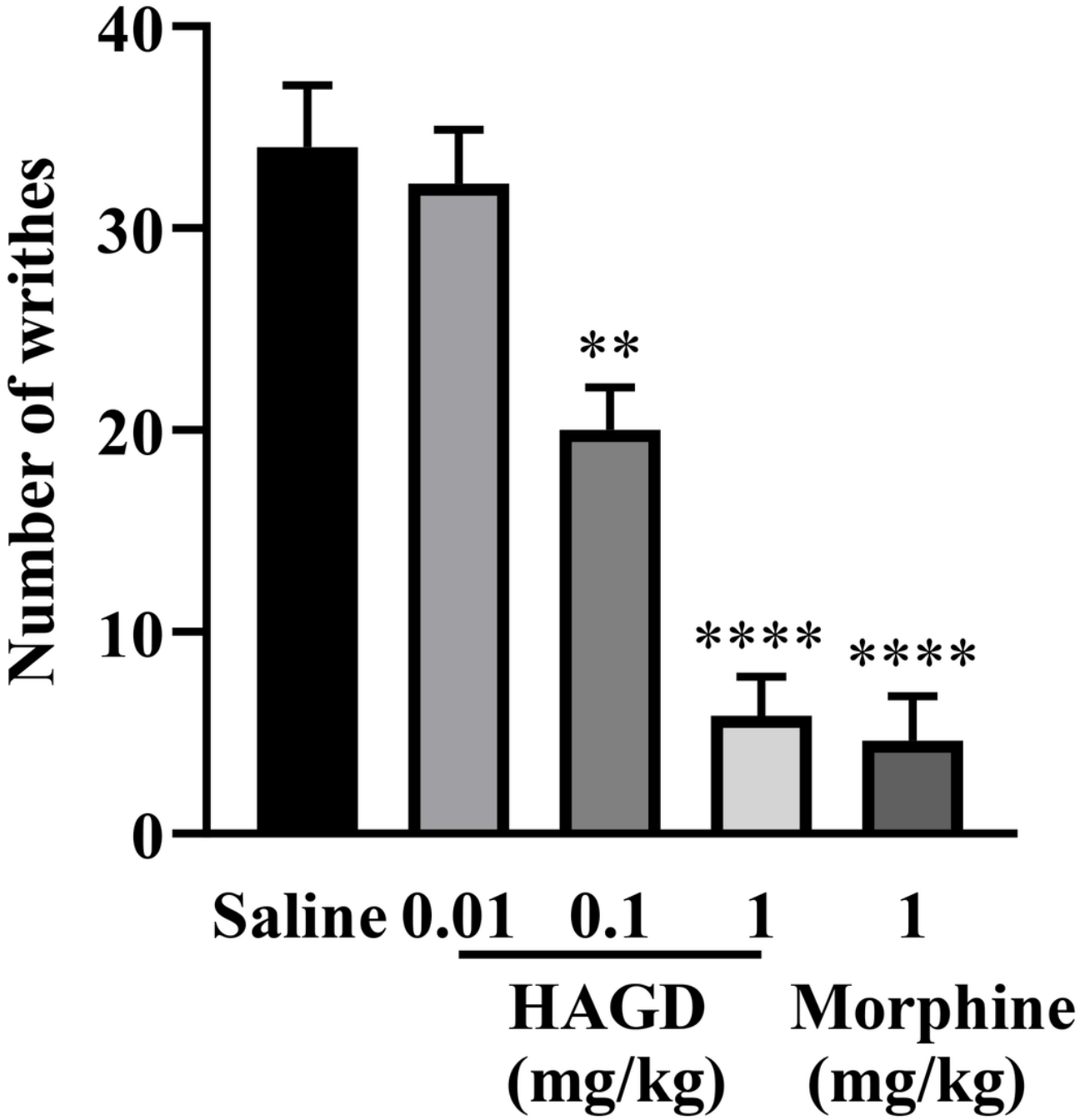

Figure 3

Antinociceptive effects of HAGD in the acetic acid-induced writhing test after subcutaneous administration. The AUC values of MPE % of HAGD and morphine during the observed period; n = 5, 6, 6, 5, 5 mice; ** P < 0.01 and **** P < 0.0001 indicate significant differences compared with Saline group, according to ono-way ANOVA, followed by Dunnett's post hoc test.

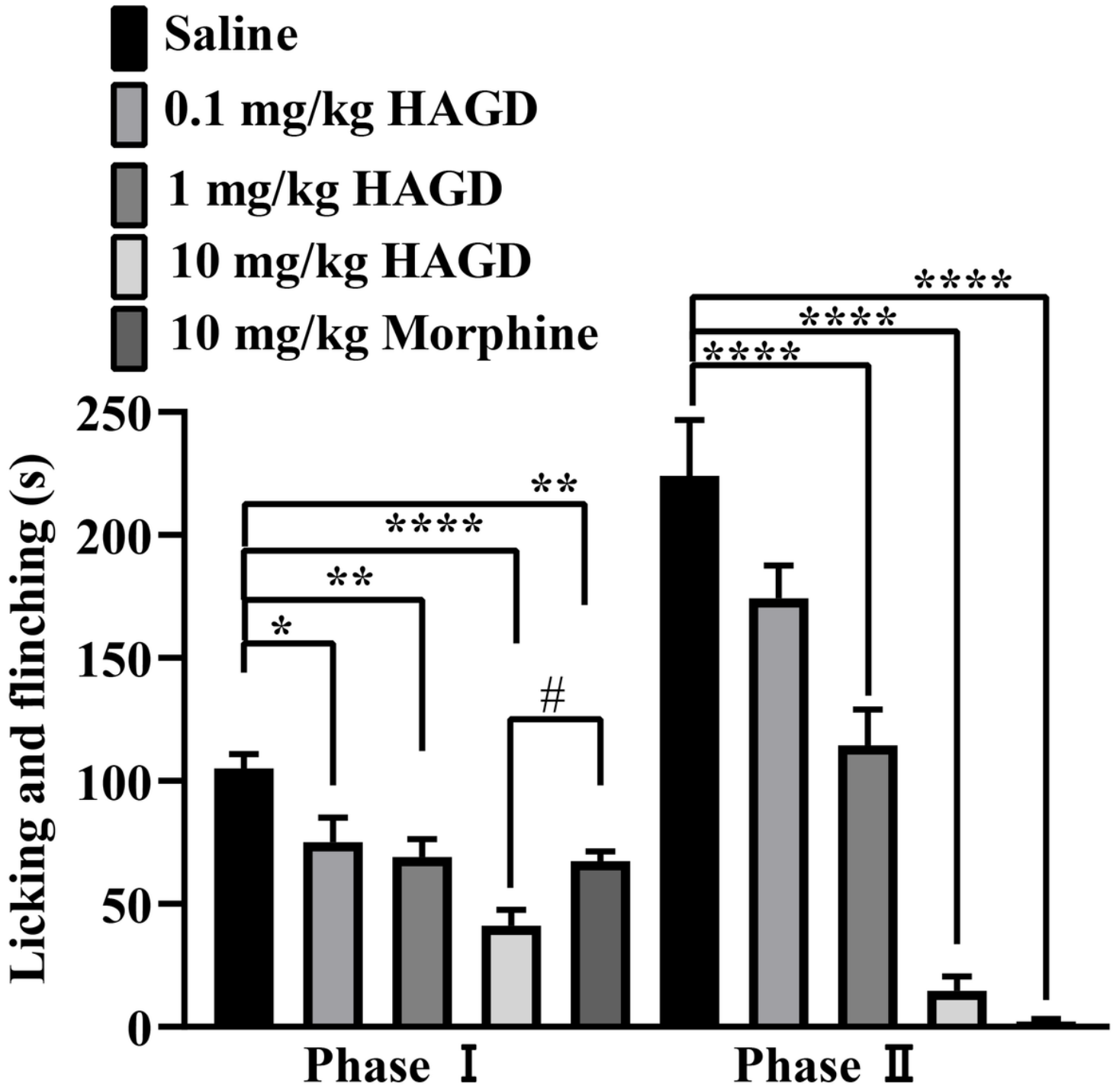

Figure 4

Antinociceptive effects of HAGD in the formalin test after subcutaneous administration. The AUC values of MPE % of HAGD and morphine during the observed period; n = 6, 5, 7, 7, 6 mice; * $P < 0.05$, ** $P < 0.01$ and **** $P < 0.0001$ indicate significant differences compared with Saline group, # $P < 0.05$ indicated significant differences compared with morphine group, according to ono-way ANOVA, followed by Dunnett's post hoc test.

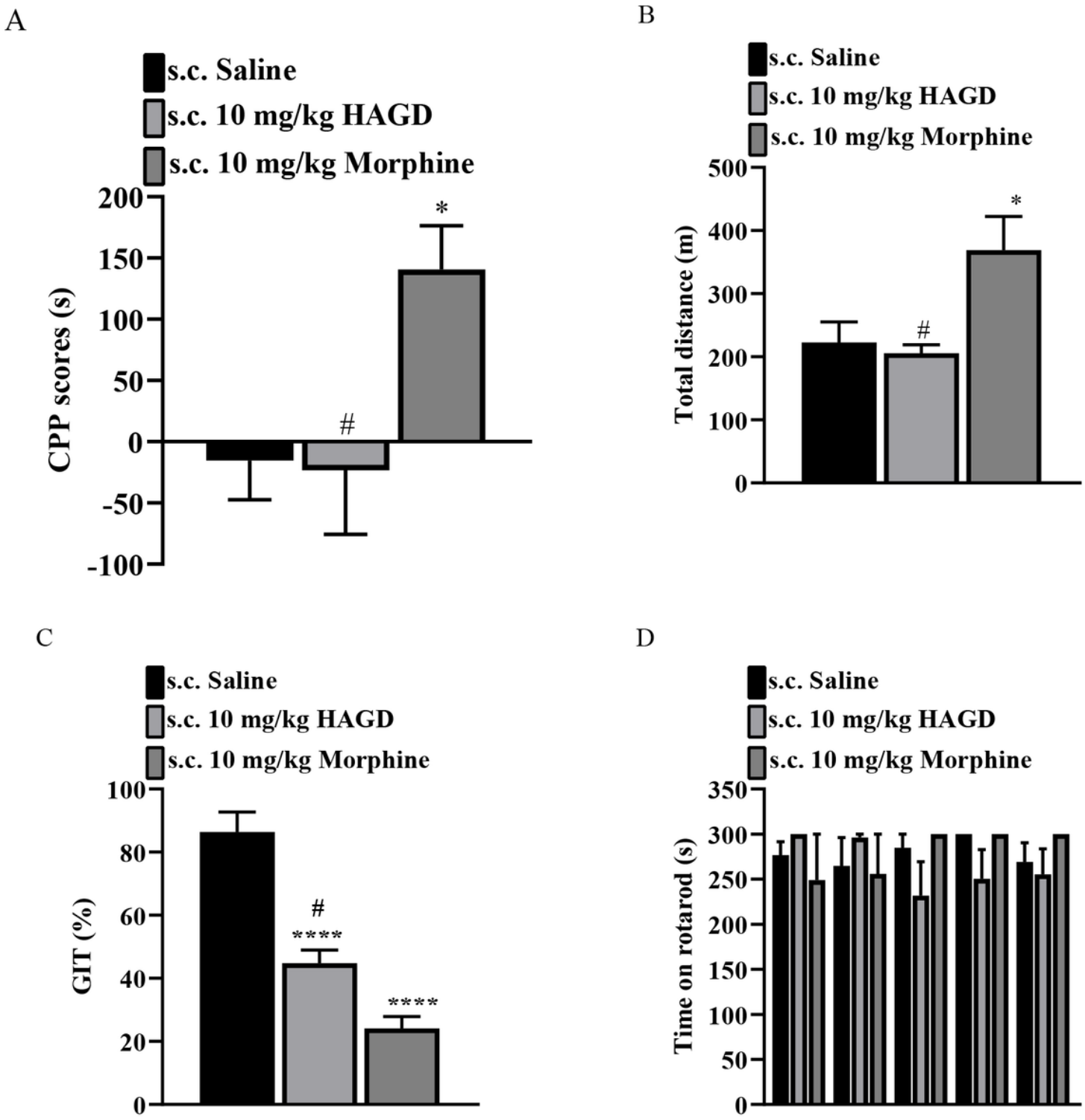

Figure 5

Evaluation of side effects of subcutaneous administration of HAGD and morphine. (A) Effects of HAGD in CPP; n = 5, 5, 8 mice. (B) Effects of HAGD in open field test; n = 6, 6, 6 mice. (C) Effects of HAGD in GIT; n = 6, 6, 6 mice. (D) Effects of HAGD in rotarod test; n = 6, 6, 5 mice. * $P < 0.05$ and **** $P < 0.0001$ indicate significant differences compared with Saline group, # $P < 0.05$ indicates significant differences compared with morphine group, according to ono-way ANOVA, followed by Bonferroni's post hoc test.

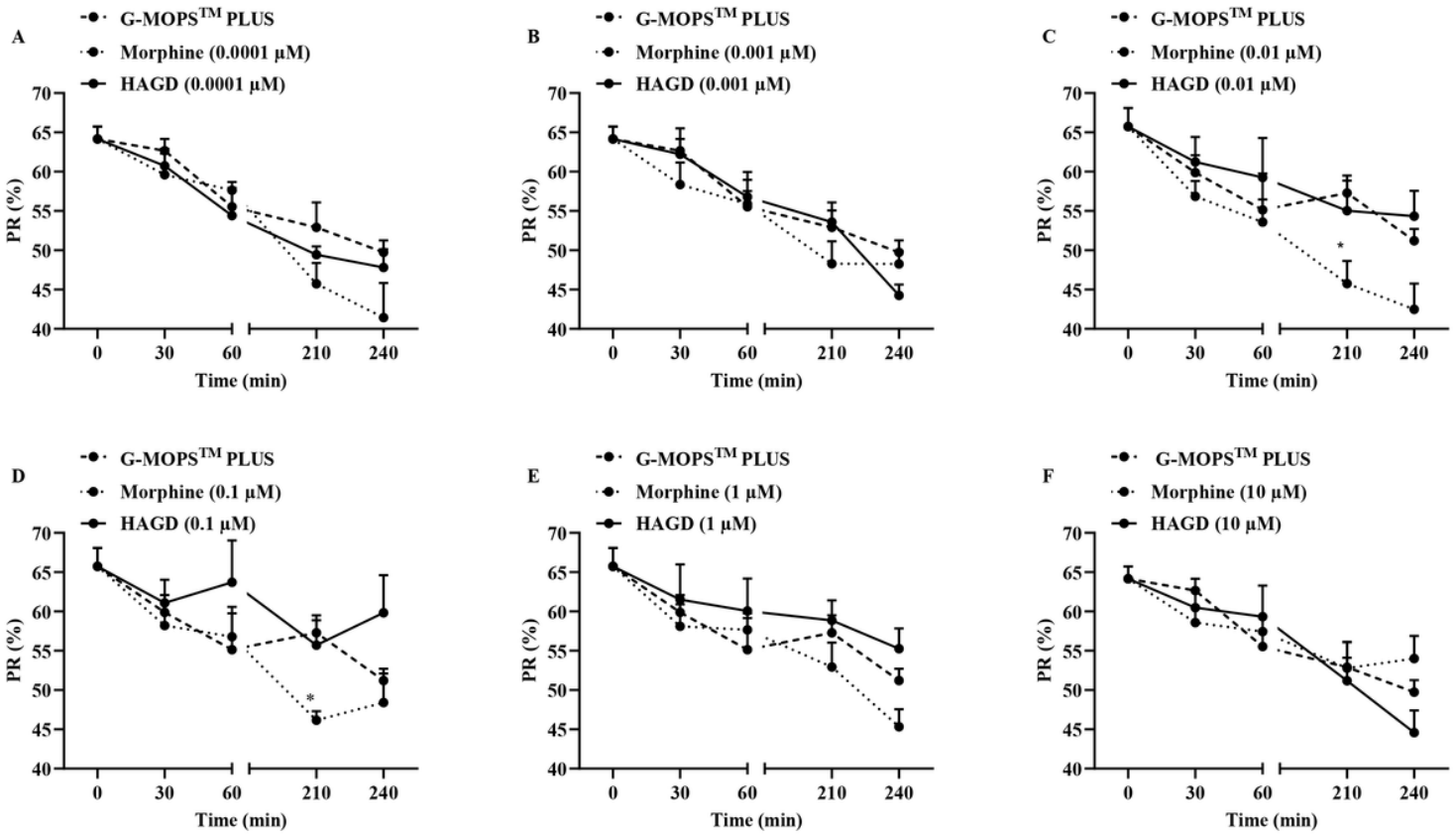

Figure 6

Evaluation of the effects on sperm motility of HAGD. (A) To evaluate the effect of 0.0001 µM of HAGD on sperm motility; n = 5. (B) To evaluate the effect of 0.001 µM of HAGD on sperm motility; n = 5. (C) To evaluate the effect of 0.01 µM of HAGD on sperm motility; n = 5. (D) To evaluate the effect of 0.1 µM of HAGD on sperm motility; n = 5. (E) To evaluate the effect of 1 µM of HAGD on sperm motility; n = 5. (F) To evaluate the effect of 10 µM of HAGD on sperm motility; n = 5. * $P < 0.05$ indicates significant differences compared with the G-MOPSTM PLUS group, according to two-way ANOVA, followed by Bonferroni's post hoc test. Drug dosage was in µMol/L per sperm sample.