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Pregabalin, Duloxetine, and Diazepam Selectively Modulate Acid-Induced Hyperalgesia and Anxio-Depressive Comorbidity in Rats

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Abstract

Objective:

Patients with chronic widespread pain (CWP) syndrome often have comorbid with depression/anxiety symptoms and unsatisfied. Effects of pregabalin, duloxetine and diazepam in the well-established acid-induced CWP model remain unclear. This study aimed to assess these medicines on mechanical hyperalgesia and psychiatric comorbidity in this CWP model.

Methods:

The CWP model was elicited by repeated injections of acidic solution into unilateral gastrocnemius muscle of a rat. Each medicine was administered orally via gavage twice per day for >2 weeks. Paw withdrawal threshold (PWT) was evaluated by von Frey filaments. Anxiety-like behavior was assessed by an elevated plus maze. Despair mood and anhedonia of depression-like behavior were evaluated using the forced swimming and sucrose preference tests, respectively.

Results:

Rats receiving acid injections displayed bilateral hyperalgesia and anxio-depressive comorbidity. Pregabalin significantly increased PWTs and ameliorated anxiety-like and anhedonic behaviours. Duloxetine significantly increased PWTs and ameliorated depression-like but not anxiety-like behaviour. Diazepam did not alter PWTs but remedied anxiety-like and anhedonic behaviours. Pregabalin, duloxetine, and diazepam revealed different effects on hyperalgesia and anxio-depressive comorbidity in the acid-induced CWP model.

Conclusion:

Our results strengthen the acid-induced pain model as humans with CWP on additional evidences of face and predictive validities. This study provides information of these medicines on selective treatment of pain and psychiatric comorbidity.

Keywords:

Muscle pain, Fibromyalgia, Pregabalin, Duloxetine, Diazepam, Anxiety, Depression

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Introduction

Chronic widespread pain (CWP) syndromes, such as fibromyalgia (FM) and myofacial pain syndrome, are characterized by widespread and long-lasting musculoskeletal pain. FM affects 2% of the general population and a considerable proportion of patients present with symptoms of mood disorders, particularly anxiety and depression [1,2]. Thus, CWP syndromes are associated with significant disability, self-injury and medical costs [3]. Several medicines, such as duloxetine and pregabalin, are used in patients with CWP syndromes [4]. Both pregabalin and duloxetine fibromyalgia clinical trials reveal that the pain relief is not related to alleviation of anxiety or depression symptom [5]. Recently, a clinical study indicates that combination of pregabalin with duloxetine for fibromyalgia increases multiple clinical outcomes [6]. However, there is little guideline on these medicines to manage pain and psychiatric comorbidity in fibromyalgia patients because of divergent comorbid symptoms (e.g. psychiatric disorder, sleep disturbance, etc.) [7,8]. Animal models with psychiatric comorbidity play an important role in elucidating the psychophysiology of CWP syndromes in terms of various medicines [9].

An animal model with CWP syndromes has been developed using repeated acid injections to the gastrocnemius muscle in two- to five-day intervals to produce a long-lasting, bilateral, mechanical and muscular hyperalgesia but not thermal hyperalgesia, without motor deficits or tissue damage [10,11]. Recently, acid-induced CWP animal model has shown comorbid anxiety- and depression-like behaviors [12]. This CWP animal model has been used for studying the mechanisms of peripheral and central sensitization [13-15]. Previous studies have shown acute effect of pregabalin on reducing hyperalgesia in the acid-induced CWP model [11]. Acute administration of diazepam has shown little effect on acid-induced hyperalgesia [16]. In contrast to acute treatment of these medicines on hyperalgesia, there is however not systematically investigated about the effects of chronic administration of pregabalin, duloxetine and diazepam on both hyperalgesia and psychiatric comorbidity in the CWP animals.

In the current study, effects of pregabalin, duloxetine and diazepam were investigated on both acid-induced hyperalgesia and anxio-depressive behaviors in the CWP animal model through a chronic oral administration via gavage. We hypothesized that these drugs would selectively modulate hyperalgesia and comorbidity of anxiety- and depression-like behaviors in the acid-induced CWP animal model. The results of this study provided predictive validity between acid-induced muscle pain in CWP animal model and patients with CWP syndromes and also extended our understanding of these medicines on CWP syndromes and psychiatric comorbidity.

Methods

Male Sprague-Dawley rats (8-9 weeks old) were kept in a sound-attenuated room under a 12/12-h light/dark cycle (lights on at 06:00-18:00) with food and water provided *ad libitum*. The rats were randomly assigned into a group receiving the vehicle (pH 7.2) or acidic saline (pH 4.0) at a ratio of 1:1.1 because of unsuccessful pain induction in previous studies [10,16]. The Institutional Animal Care and Use Committee reviewed and approved the experimental procedure. All experiments complied with the guidelines for the ethical use of animals of the US National Institutes of Health. The entire experimental design was conducted according to institutional guidelines and followed the 3R's principles (i.e., replacement, refinement, and reduction) for animal welfare. According to our previous study [12], ten rats with successful pain induction in each group were used.

■ Induction of muscle pain

The method used to induce muscle-mediated chronic pain was as previously described in detail [10]. All rats were briefly anesthetized with vaporized isoflurane (3-5%). After the skin covering was shaved, left gastrocnemius muscle was injected with 100 μl neutral saline (pH 7.2) in the control group or 100 μl acidic saline in the experimental group, on days 1 and 6. Normal saline was adjusted with an 2-[**N**-morpholino] ethanesulfonic acid to pH 4.0±0.1 as acidic saline.

■ Von Frey filament testing

Rats were placed in a Lucite cubicle on an elevated metal grid allowing for the stimulation on plantar surface of the paw. The grid hole diameter was 3 mm and the center distance between two consecutive holes was 5 mm. Rats were placed on the platform for adaptation to the apparatus for 5-10 min prior to the experimental measurement. Discrete von Frey filaments of varying bending forces (2, 4, 6, 8, 10, 15, and

26 g) were applied to plantar surface of the paw to assess the withdrawal response. A "response" to the stimuli was defined as an abrupt lifting of the foot upon application of the von Frey filament. A trial contained 5 von Frey stimuli, with an inter-stimulus interval of 5-6 minutes to reduce possible habituation. Von Frey testing forces were performed in an ascending sequence. Paw withdrawal threshold (PWT) was defined as the lowest force that elicited ≥3 withdrawals in 5 consecutive stimuli.

Von Frey nylon hairs were calibrated both prior to and throughout the time course of the study to ensure that consistent bending forces were routinely applied. The withdrawal threshold of the ipsilateral left hind paw was measured first, and then the contralateral right hind paw. Von Frey tests were employed at the times of D1, D2, D6, D7, D13, D14, D20, D21, D27, D28, D34, and D35.

■ Body weight assessment

Body weight was assessed using a commercial scale at particular time points similar to those described for von Frey filament testing **(Figure 1)**.

■ Behavioral tests

Three behavioral tests were used to evaluate anxiety- and depression-like behaviours of rats [12,17]: elevated plus maze test, forced swimming test, and sucrose preference test. The elevated plus maze test was used to measure anxious status for animals facing open environmental stimulus. The forced swimming test was used to assess the duration of immobility, which was analogous to a despair-like status. The sucrose preference test was a measure of the 'hedonic' state of an animal or the ability to experience pleasure. A decreased sensitivity to reward (i.e., anhedonia) is a fundamental feature of clinical depression. All behavior measures were taped, digitized, and analyzed using video tracking record system (TM-01, Singa, Taiwan).

■ Elevated plus maze test

The elevated plus maze consisted of black polypropylene plastic which was elevated 68 cm above the floor. Each maze arm extended 45 cm from the junction area, which measured 13×13 cm. The open and closed arms were 13 cm wide, and the closed maze arms had walls extending 25 cm from the junction area. During testing, each rat was placed in the central square facing an open arm and was allowed 5 minutes to freely explore the maze. The alleys of the maze were thoroughly cleaned with an ethanol solution (60% volume) after the removal of each rat. Time spent on different sections of the maze (including the central area) and the numbers of open and closed arm entries were measured. The percentage of entries in open arms was calculated according to the following formula: percentage of open arm entry = (number of entries into the open arms/number of open+closed arm entries) × 100; percentage of time spent in open, centre, and closed sections of the maze = (location × 100/300). The percentage of venturing into open arms is validated for anxiety measure [18].

■ Sucrose preference test

In the sucrose preference test, rats were placed in an unfamiliar test cage supplied with two identical bottles, one contained drinking water and the other sucrose solution (20%). All rats were allowed freely to approach the bottles for a period of 1 h after 23 h of food and water deprivation. The results were expressed as a percentage of sucrose preference according to the following formula: preference (%) for sucrose over water (calculated as [sucrose intake/total fluid intake] × 100%). The preference for sucrose solution is a reliable hedonic index in rats [12,19].

■ Forced swimming test

The forced swimming test apparatus was a plastic cylinder (47 cm height, 38 cm inside diameter) containing 38 cm of water at 25±1 °C. The forced swimming test consisted of two phases. In the initial 15-min habituation session of the first day, which was excluded from the data analysis, the rats were individually forced to swim in a plastic cylinder. After a period of vigorous swimming, all of the rats reduced their movements to only those necessary to maintain their head above the water level, with no other displacement. The 5-min test sessions began 24 h later. The duration of immobilization was measured. After the test, rats were removed and dried with a towel before being returned to their home cages. Increased immobility in the forced swimming test was indicative of depression-like behavior [20].

■ Drug administration

A chronic oral administration was used via gavage. Dosage used in rats based on the following equation: human dosage × human Km/animal Km, where Km= weight/body surface area. In general, dosage of the rat was 6 folds as
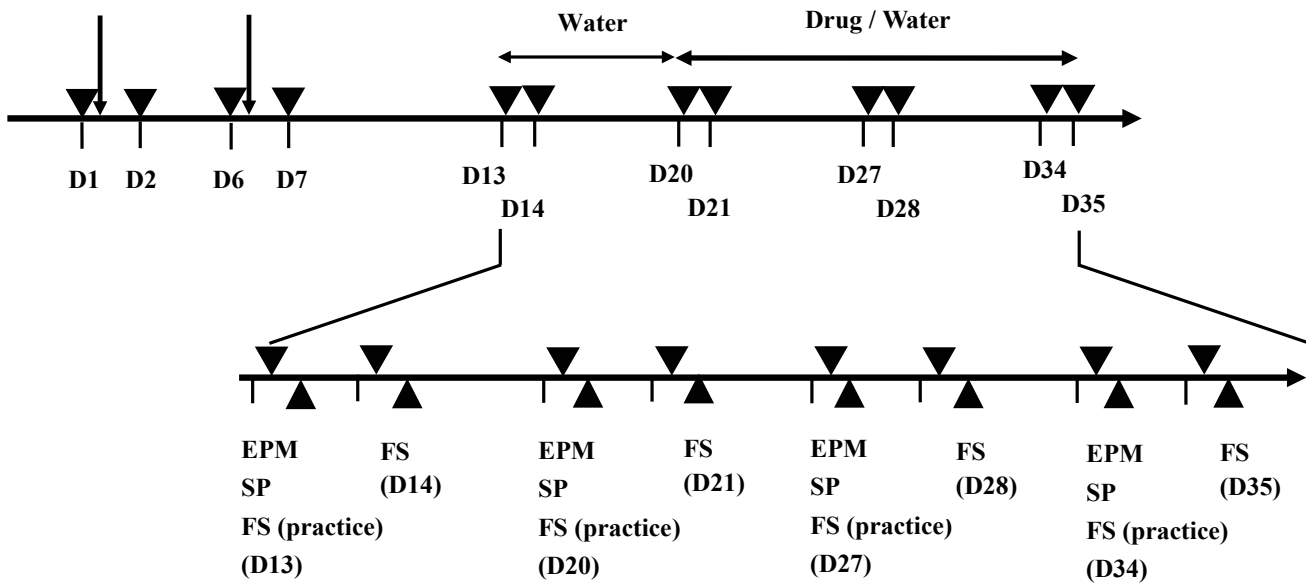
Figure 1: Experimental procedure of induction of chronic pain and its behavioral assessment.

Body weight and paw withdrawal threshold were measured at particular times (▼), while anxiety- and depression-like behavioral tests were measured in sequence (▲). Vehicle or acidic saline was injected into the left gastrocnemius muscle at D1 and D6 (arrows). All rats received water for 1 week via gavage from one week after 2nd injection (D13). Thereafter, all rats received water or drugs (pregabalin, duloxetine or diazepam) for 3 weeks. Drug or water was administered by gavage twice per day during D13-D35. Time points were: the 1st injection (D1), 24 h after the 1st injection (D2), the 2nd injection (D6), 24 h after the 2nd injection (D7), and weekly after the 2nd injection for 4 weeks (D13, D14, D20, D21, D27, D28, D34, and D35), respectively. EPM, elevated plus maze; FS, forced swimming; SP, sucrose preference.

used in human [21]. Pregabalin (LYRICA 75 mg capsule; Pfizer), duloxetine (Cymbalta 30 mg capsule; Eli Lilly), and diazepam (Diapin 2 mg tablet; Souriree) were administered orally by gavage in a dose of 15 mg/kg [11], 3 mg/kg [22], and 0.5 mg/kg [16], respectively. A single dose was administered throughout the experiment. All medicines mixed with 2 ml water were administered twice daily via gavage for >2 weeks in the acid group. The vehicle group received the same amount of water twice daily via gavage. The interval between the two drug/water administrations in any given day was 4 h.

■ Experimental procedure

All rats were placed in the recording room 1 week prior to the experiment for adaptation. Rats were placed in a Lucite cubicle on an elevated metal grid allowing stimulation of plantar surface of the hind paw for at least 20 min to allow them to become acclimatized to the test environment. This was done for 3 days before von Frey filament testing. Two intramuscular injections of pH 7.2 or pH 4.0 saline were employed at D1 and D6. Body weight and paw withdrawal threshold were measured at particular time points **(Figure 1)**. All behavioral evaluations were performed at 14:00-17:00 to minimize circadian influences.

The study was designed explore long-term effect of drugs on hyperalgesia and anxio-depressive comorbidities. To this aim, anxio-depressive behavioral tests were repeatedly employed as previous studies [22-24]. The elevated plus maze, sucrose preference, and forced swimming tests were carried out 1 week after 2nd intramuscular injection in sequence. All rats received water twice daily per orally for 1 week. Subsequently, rats received water or drug via gavage from D20 for >2 weeks. Three behavioral tests were carried out once per week. A detailed flowchart of the entire experimental procedure is shown in **Figure 1**. Measures were performed before the 1st injection (D1), 24 h after the 1st injection (D2), before the 2nd injection (D6), 24 h after the 2nd injection (D7), and weekly after the 2nd injection for 4 weeks (D13, D14, D20, D21, D27, D28, D34 and D35), respectively.

Statistical analysis

Changes in body weight and indexes of the elevated plus maze test, the sucrose preference test, and forced swimming test in all groups were analyzed using two-way repeated measures analysis of variance (ANOVA) with one factor repetition, if appropriate, followed by post hoc

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Fisher's LSD test. Temporal changes of the paw withdrawal thresholds in all groups were assessed by Friedman repeated measures ANOVA on rank, if appropriate, followed by Dunnett's test. The paw withdrawal threshold among groups at a particular time point was analyzed by one-way ANOVA on rank (H-test) followed by post hoc Dunnett's test. To further assess the stability of a within-subject experimental design, all indexes of the vehicle group throughout the recording period were analyzed by one-way repeated measure ANOVA. Data were expressed as the mean ± standard error of the mean (SEM). All statistics were carried out using Sigmaplot statistics. Statistical significance was set at $p < 0.05$.

Results

In the present study, 56 rats (vehicle $n=10$, acid $n=46$) were used. Six rats receiving intramuscular injections of acidic saline failed to exhibit mechanical hyperalgesia. A hyperalgesia induction rate was 87% in the acid group. Thus, 40 rats with hyperalgesia were randomly assigned into the acid groups treated with water ($n=10$), pregabalin ($n=10$), duloxetine ($n=10$), or diazepam ($n=10$), respectively.

■ Paw withdrawal threshold

The withdrawal thresholds of bilateral hind paws of rats in all groups are shown in **Figure 2**. Paw withdrawal threshold of the vehicle group was consistent in bilateral hind limbs, and the threshold was primarily at 8 g throughout the measured period. There was no significant difference throughout the recording period in the vehicle group (ipsilateral, $\chi^2(11)=6.261$, $p=0.855$; contralateral, $\chi^2(11)=9.432$, $p=0.582$). In contrast, the paw withdrawal threshold of the acid group was at the same level as that of the vehicle group before the 2nd injection of acid saline. The paw withdrawal thresholds of bilateral hind limbs were reduced to 2-4 g after the 2nd injection of acid saline. This hyperalgesic response lasted for 4 weeks. Intramuscular administration of acidic saline produced long-lasting and significant decreases in the paw withdrawal threshold (ipsilateral, $\chi^2(11)=86.637$, $p<0.001$; contralateral, $\chi^2(11)=87.174$, $p<0.001$). Paw withdrawal thresholds from D7 to D35 were significantly lower than its baseline (D1) in the acid group treated with water. In addition, significant differences between the vehicle and acid groups were also found at the same time points.

Paw withdrawal threshold of the acid group treated with pregabalin revealed significantly temporal change (ipsilateral, $\chi^2(11)=76.508$, $p<0.001$; contralateral, $\chi^2(11)=83.001$, $p<0.001$). Compared with the vehicle group, the acid group treated with pregabalin had significantly lower paw withdrawal thresholds at D7, D13, and D14 prior to drug administration, and then showed a rapid reverse on bilateral withdrawal thresholds from D20, when rats started to receive pregabalin. Thus, pregabalin exhibited analgesic effect.

Paw withdrawal threshold of the acid group treated with duloxetine showed significantly temporal change (ipsilateral, $\chi^2(11)=70.067$, $p<0.001$; contralateral, $\chi^2(11)=71.023$, $p<0.001$). Compared with the vehicle group, the acid group treated with duloxetine had significantly lower paw withdrawal thresholds at D7, D13, and D14 prior to duloxetine administration. The acid group treated with duloxetine showed a progressive return pattern on the withdrawal thresholds D20 and D21 starting drug administration (significant difference existed in contralateral hind limb but not in ipsilateral hind limb) followed by no difference on paw withdrawal thresholds of bilateral hind limbs compared with those of the vehicle group. Thus, duloxetine showed analgesic response in this model.

Paw withdrawal threshold of the acid group treated with diazepam showed significantly temporal change (ipsilateral, $\chi^2(11)=70.131$, $p<0.001$; contralateral, $\chi^2(11)=71.182$, $p<0.001$). The acid group treated with diazepam showed significantly lower paw withdrawal thresholds compared with the vehicle group from D7-D35. The results indicated that diazepam had no effect on acid-induced hyperalgesia.

■ Body weight

Body weights of the five groups progressively increased from 300 g to 370 g throughout the recording period (Figure S1). There were significantly difference in the factors of time (F(11,495)=283.189, $p<0.001$) and treatment×time (F(44,495) =1.426, $p=0.041$). Body weight had insignificant difference in the group factor (F(4,495)=2.113, $p=0.095$).

■ Elevated plus maze test

The percentages of open arms entry of an elevated plus maze at all measuring time points in all groups are shown in **Figure 3**. Total movement during the elevated plus maze
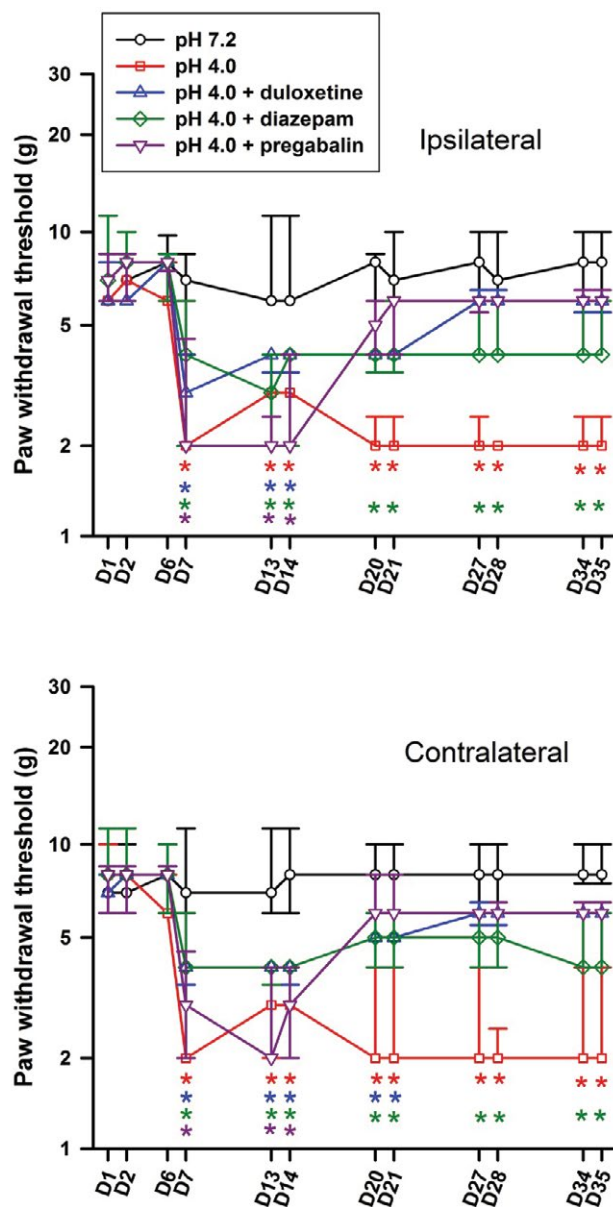
Figure 2: Changes in bilateral paw withdrawal thresholds of rats that received vehicle (pH 7.2) or acidic saline (pH 4.0) with water or drug administration at 12 time points

Von Frey filaments of varying bending forces (2, 4, 6, 8, 10, 15, and 26 g) were applied to the plantar surface of the paw to assess the withdrawal response. *$p < 0.05$ vs. pH 7.2 group. Data are presented as the median with the 25th and 75th percentile.

test was not significantly different in the five groups. The percentage of open arms entry in the open arms was significant difference in the factors of treatment ($F_{(4,135)}=6.475$, $p<0.001$), time ($F_{(3,135)}=10.344$, $p<0.001$), and treatment×time ($F_{(12,135)}=2.186$, $p=0.016$). There was no significant difference in all groups at D13 prior to drug administration, which indicated a well match at baseline among all groups. There was no significant difference on the percentage of open arms entry throughout

the recording period in the vehicle group ($F_{(3,27)}=2.236$, $p=0.107$), which indicated no alteration in repeated measures of an elevated plus maze. The percentage of open arms entry was significant difference throughout the recording in the acid group ($F_{(3,27)}=9.591$, $p<0.001$). The percentage of open arms entry was not significantly different at D13 prior to drug administration. The acid group receiving water showed significantly lower percentage of open arms entry in the open arms at D27 and
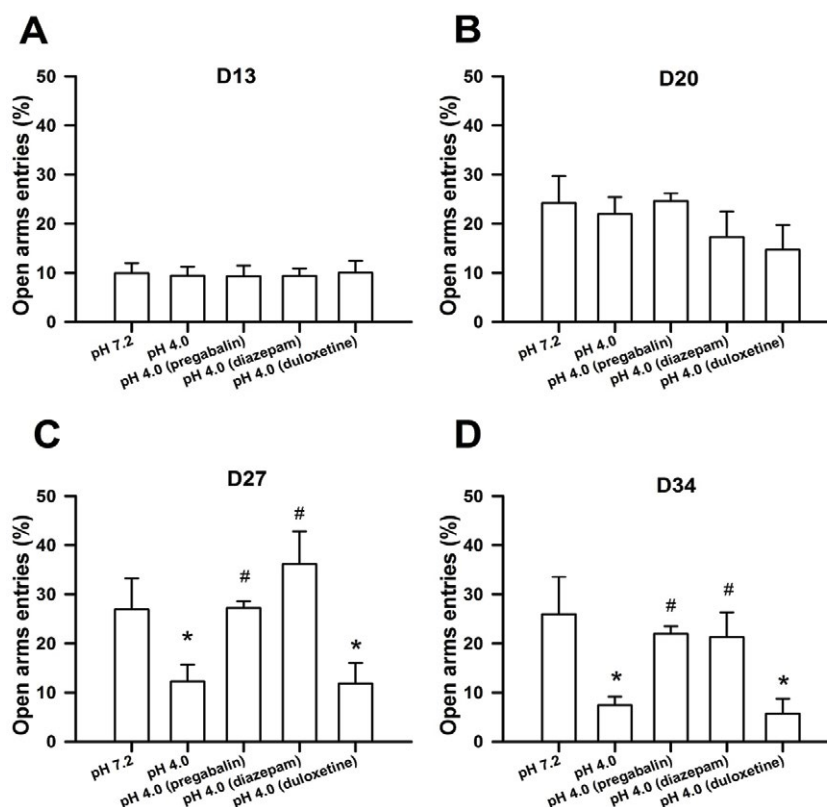
Figure 3: Percentage of open arms entry in an elevated plus maze at **(A)** D13, **(B)** D20, **(C)** D27, and **(D)** D34 in the 5 groups.

The elevated plus maze test was carried out prior to drug administration (D13) and during drug administration (D20, D27, and D34). Data are presented as the mean±SEM. *$p<0.05$ vs. pH 7.2 group; #$p<0.05$ vs. pH 4.0 group

D34 compared with the vehicle group. The acid group exhibited anxiety-like behavior.

The acid groups treated with pregabalin ($F_{(3,27)}=18.408$, $p<0.001$) or with diazepam ($F_{(3,27)}=5.041$, $p=0.007$) exhibited significant difference throughout the recording. The acid group receiving pregabalin or diazepam showed significantly higher percentage of open arms entry at D27 and D34 compared with the acid group receiving water. There was no difference between the vehicle group and the acid group treated with pregabalin or diazepam at D27 and D34. These data indicated that pregabalin or diazepam ameliorated anxiety-like behavior of the acid-induced CWP rats.

There was no significant difference on the percentage of open arms entry in the acid group treated with duloxetine ($F_{(3,27)}=0.850$, $p=0.479$). The acid group receiving duloxetine showed significantly lower percentage of open arms entry at D27 and D34 compared with the vehicle group, which indicated anxiety-like behavior in the group receiving duloxetine.

In addition to the percentage of open arms entry in an elevated plus maze, other parameters are summarized in Table S1 (see supplementary results).

■ **Sucrose preference test**

The results of the sucrose preference test at 4 time points in all groups are shown in **Figure 4**. Total liquid intake (including sucrose and water) in all groups fell in the range of 13.5-18 ml during the sucrose preference test, and there was no difference in all groups ($F_{(4,135)}=2.271$, $p=0.076$). Sucrose preference had significant differences in the factors of treatment ($F_{(4,135)}=16.199$, $p<0.001$), time ($F_{(3,135)}=3.485$, $p=0.018$), and treatment×time ($F_{(12,135)}=2.328$, $p=0.01$). There was no significant difference in all groups at D13 prior to drug administration, which indicated a well match at baseline among all groups. There was significantly progressive increase in the sucrose preference throughout the recording period in the vehicle group ($F_{(3,27)}=3.834$, $p=0.021$), which implied a hedonic preference. The acid
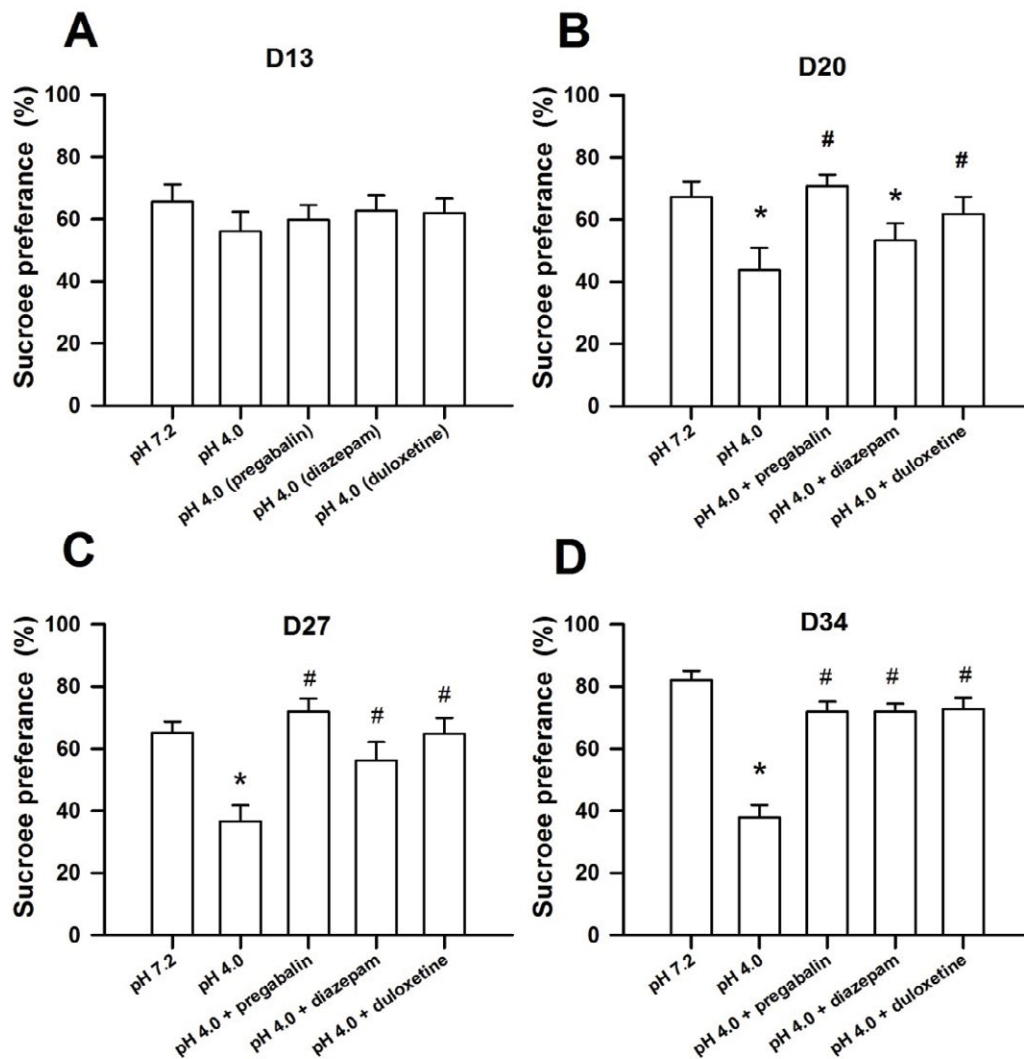
Figure 4: Preference to sucrose solution at **(A)** D13, **(B)** D20, **(C)** D27, and **(D)** D34 in the 5 groups.

The sucrose preference test was carried out prior to (D13) and during drug administration (D20, D27, and D34). Data are presented with the mean±SEM. *$p<0.05$ vs. pH 7.2 group; #$p<0.05$ vs. pH 4.0 group

group receiving water exhibited significant decrease in the sucrose preference throughout the recording ($F_{(3,27)}=3.163$, $p=0.041$), which sluggested an anhedonic pattern. The acid group receiving water exhibited significantly lower sucrose preference at D20, D27, and D34 compared with the vehicle group and had no difference with 50% chance.

The acid group receiving duloxetine exhibited no significant difference throughout the recording ($F_{(3,27)}=1.175$, $p=0.338$). The acid group receiving duloxetine had no difference at all timestamps compared with the vehicle group, but it exhibited significantly higher sucrose preference at D20, D27, and D34 compared with the acid group receiving water. The results

indicated that duloxetine reversed anhedonic response of the acid group.

The acid group receiving pregabalin exhibited no significant difference throughout the recording ($F_{(3,27)}=2.098$, $p=0.124$). The acid group receiving duloxetine had no difference at all timestamps compared with the vehicle group, but it exhibited significantly higher sucrose preference at D20, D27, and D34 compared with the acid group receiving water. The results suggested that pregabalin reduced anhedonic response of the acid group.

The acid group receiving diazepam exhibited no significant difference throughout the recording ($F_{(3,27)}=2.634$, $p=0.070$). The acid group

receiving diazepam showed significantly lower sucrose preference at D20 after starting drug administration compared with the vehicle group. Subsequently, the acid group treated with diazepam showed significantly higher sucrose preference at D27 and D34 compared with the acid group receiving water but no difference compared with the vehicle group. The data indicated that diazepam had delay reaction on anti-anhedonic response.

In addition to the percentage of sucrose preference, sucrose intake in the sucrose preference test revealed a similar trend as the sucrose preference did (Figure S2).

■ Forced swimming test

No behavioural impediment was seen during the forced swimming test. Immobility of the forced swimming test at 4 testing time points in all groups is shown in **Figure 5**. Immobility had significant difference in the factors of treatment $(F(4,135)=16.515, p<0.001)$, time $(F(3,135)=12.349, p<0.001)$, and treatment×time $(F(12,135)=2.163, p=0.017)$. There was no significant difference on the immobility throughout the recording period in the vehicle group $(F(3,27)=2.188, p=0.113)$, which indicated no alteration in repeated measures during the forced swimming test. The acid group receiving water had no significant difference throughout the recording $(F(3,27)=1.201, p=0.328)$. However, the acid group receiving water had significantly longer immobility at D14, D21, D28, and D35 compared with the vehicle group, which indicated development of despair-like behavior.

The acid group receiving duloxetine exhibited significant difference throughout the recording $(F(3,27)=6.312, p=0.002)$. The acid group treated with duloxetine showed significantly longer immobility at D21 compared with the vehicle group, and it exhibited significantly shorter immobility at D21, D28, and D35 compared with the acid group receiving water. The results indicated that duloxetine had a delay action to reduce despair-like behavior of the acid group.

The acid group receiving pregabalin exhibited significant difference throughout the recording $(F(3,27)=6.834, p=0.001)$. The acid group receiving pregabalin exhibited significantly longer immobility at D14, D21, D28, and D35 compared with the vehicle group, and it had no difference compared with the acid

group receiving water. The results indicated that pregabalin didn't modulate despair-like behavior.

The acid group receiving diazepam exhibited significant difference throughout the recording $(F(3,27)=6.332, p=0.002)$. The acid group receiving diazepam showed significantly longer immobility at D14, D21, D28, and D35 compared with the vehicle group. This group had significantly shorter immobility at D21 and D28 compared with the acid group receiving water. The data indicated that diazepam had moderate remedy on despair-like behavior at early stage of administration.

Discussion

Major findings of this study are: 1) Repeated intramuscular injections of acidic saline elicited bilateral, long-lasting mechanical hyperalgesia, and comorbid anxio-depressive behavior. 2) Pregabalin significantly increased PWTs and ameliorated comorbidity of anxiety-like and anhedonic behaviors. 3) Duloxetine significantly increased PWTs and ameliorated comorbidity of anhedonic and despair-like behavior. 4) Diazepam did not alter PWTs but ameliorated comorbidity of anxiety-like and anhedonic behavior. All results regarding various timepoints are summarized in **Table 1**. Our results indicate dissociation among these medicines in treatment of hyperalgesia and psychiatric comorbidity.

In the present study, repeated intramuscular injections of acidic solution elicited long-lasting, bilateral, and mechanical hyperalgesia in 87% of rats, which is consistent with 72%-90% of previous reports [10,12,16]. The acid-induced CWP model reveals widespread hyperalgesia with no muscle inflammation or pathology [25,26]. The present study observed acid-induced hyperalgesia accompanied by comorbidity of anxiety-like, despair-like and anhedonic behaviors [12]. The present study provides face validity of the acid-induced CWP model like clinical observations in FM patients [1,2].

Moreover, the present study systematically evaluated effects of three medicines on hyperalgesia and psychiatric comorbidity in the CWP model. Pregabalin and duloxetine, but not diazepam, significantly reduced acid-induced hyperalgesia through a chronic oral administration via gavage. The results not only
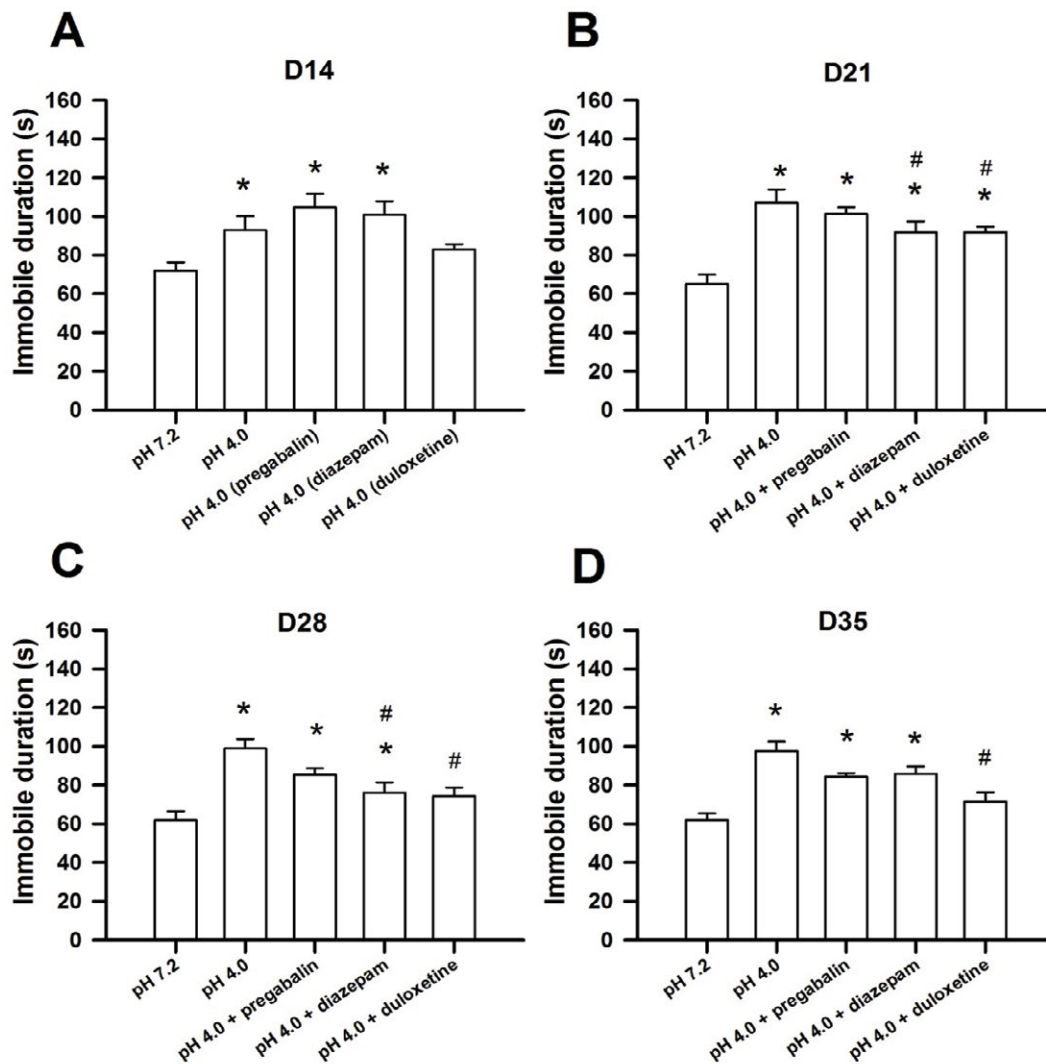
Figure 5: Immobility of the forced swimming test at **(A)** D14, **(B)** D21, **(C)** D28, and **(D)** D35 in the 5 groups.

Immobility of the forced swimming test was measured prior to (D14) and during drug administration (D21, D28, and D35). Data are presented with the mean±SEM. *$p < 0.05$ vs. pH 7.2 group; #$p < 0.05$ vs. pH 4.0 group

group Day	pH4.0 (water)	pH4.0 (pregabalin)	pH4.0 (diazepam)	pH4.0 (duloxetine)
Table 1: Changes of hyperalgesia and psychiatric comorbidity in the 4 groups.				
13-14 (W)	H+D	H+D	H+D	H
20-21 (W/M)	H+DE	D	H+DE	H[1]+D
27-28 (W/M)	H+ADE	D	H+D	A
34-35 (W/M)	H+ADE	D	H+D	A
A, anxiety; D, despair; E, anhedonia; H, hyperalgesia (bilateral); M, medicine (pregabalin, diazepam, or duloxetine); W, water				
[1]only contralateral hyperalgesia.				

extend our knowledge on short-term analgesic action of pregabalin [11] but also increase our understandings of these medicines on pain and psychiatric comorbidity in the acid-induced CWP model. Furthermore, these medicines selectively modulated anxiety-like behavior and subcategories (anhedonia or despair mood) of depression-like behavior, which is not fully reported in clinic [27,28]. Taken together, the present study strengthens face validity and predictive validity of the acid-induced CWP model as FM in humans.

Pregabalin, Duloxetine, and Diazepam Selectively Modulate Acid-Induced Hyperalgesia and Anxio-Depressive Comorbidity in Rats

Research

The present study observed analgesic effect of pregabalin in the acid-induced CWP model [11]. To our knowledge, it is the first time to present anxiolytic response of pregabalin in this animal model. Pregabalin is a selective high-affinity ligand of the $\alpha_2\delta$ subunit of the voltage-dependent Ca^{2+} channels [29]. The potent binding of pregabalin at the $\alpha_2\delta$ site reduces calcium influx at nerve terminals, resulting in reduced release of numerous neurochemicals (including glutamate, norepinephreine, and substance P) [30-32]. This mechanism of action may be responsible for the anticonvulsant, analgesic, and anxiolytic activity of pregabalin in numerous animal studies [4]. The calcium channel $\alpha_2\delta$ sub unit expresses primarily in the amygdala, hippocampus and neocortex through immunostaining technique [33]. A recent study has indicated that activation of postsynaptic extracellular-regulated kinase (ERK)-dependent component in the central amygdala is required to development of the postsynaptic potentiation in the acid-induced muscle pain [14]. Infusion of pregabalin into the amygdala exhibits comparable analgesic effect as that observed via oral administration by gavage (unpublished results). Taken together, pregabalin probably activates calcium channels in the amygdaloid related pathway to produce analgesic or anxiolytic effect.

Pregabalin ameliorated anhedonic behavior in terms of high sucrose preference and sucrose intake but exhibited no effect on despair mood as indicated by long immobility of the forced swimming test. There is no clinical report dealing with pregabalin on subcategorical items of depressive symptom in FM patients. In a neuropathic pain model by partial sciatic nerve ligation, pregabalin reduces anhedonic phenomenon but has no change in immobility of the forced swimming test [34]. Pregabalin induces development of depression and/or suicide ideation in patients with persistent neuropathic pain [35]. The pregabalin results from neuropathic pain may support our findings even though there are large differences between these two pains in etiology and treatment. It is well known that anhedonia and despair of depression-like behavior involve different neural circuits [36]. Pregabalin may have different actions in these neural circuits.

In the present study, duloxetine (3 mg/kg, p.o.) significantly improved both hyperalgesia and depression-like behaviors of the acid-induced muscle pain. In a recent study, chronic muscle hyperalgesia was induced by biogenic amine depletion using reserpine and was attenuated by high dose duloxetine (30 mg/kg, p.o.) but not in low dose (3 or 10 mg/kg, p.o.) [22]. The discrepancy of effective duloxetine dose in these two muscle pain models may arise from different cellular mechanisms. For example, reserpine specifically causes dysfunction of all aminergic neurons which have widespread influence on the brain, whereas acid-induced CWP model activates specific acid-sensing ion channel (ASIC3) which results in altered activations of several brain regions [26]. Another possibility is the different testing periods, which can result in varied hyperalgesia. The present study assessed duloxetine continuously for 2 weeks after inducing hyperalgesia, which may indicate a long-lasting stable pain. In contrast, the biogenic amine depletion model only tests up to 4 hours after reserpine injection (i.e., acute phase).

The duloxetine-treated acid group (3 mg/kg, p.o.) exhibited anxiety-like behavior at D27 and D34, and the acid group also developed anxiety-like behavior at the same timepoints **(Table 1)**. There is little guidance from clinical trials regarding the management of fibromyalgia and comorbid anxiety disorders. Both the duloxetine and pregabalin fibromyalgia trials have indicated that the reduction in pain is not related to alleviation of anxiety or depression symptom [5]. Recently, add-on therapy of pregabalin in the duloxetine treatment of fibromyalgia patients has been suggested to improve multiple clinical outcomes (including anxiety and sleep disturbance) [6]. A previous study using duloxetine (10 mg/kg, p.o.) shows no effect on the elevated plus maze test in a neuropathic pain model [37]. Duloxetine alone (10 mg/kg, i.p.) did not alter open arms entry frequency of an elevated plus maze in an anxious mice model [38]. In line with these results, duloxetine has little effect on the anxiety-like behavior.

Diazepam, a long half-life benzodiazepam-related compound, is a $GABA_A$ receptor agonist and exhibits both sedative and anxiolytic effects [39,40]. In this study, the acid group receiving diazepam still exhibited hyperalgesia, which extends understanding of acute diazepam treatment in the same model previously [16]. Bromazepam, a medium half-life benzodiazepam-related compound, exhibits negligible effect in fibromyalgia patients [41]. Taken together, these results indicate little analgesic effect of diazepam in the acid-induced CWP model and FM patients.

Diazepam is a classic anxiolytic, which has been demonstrated in numerous clinical studies [42] and various animal models with pain [40,43]. The present study also observed anxiolytic effect of diazepam in the acid-induced CWP model. Additionally, diazepam ameliorated anhedonic behavior progressively in terms of the sucrose preference test. Approaching the sucrose bottle could be a self-driven or self-motivating behavior. A previous study indicated the effect of diazepam on hedonic modulation as result of self-stimulation rather than the stimulation-escape paradigm [44]. Moreover, diazepam increases intracranial self-stimulation behavior (i.e., reward enhancing effect) [45] and high risky decision making behavior [46]. In lines with these results, diazepam may have a new application on increased hedonia or positive reward in the CWP subjects.

In the present study, all anxiety- and depression-like behaviors were repeatedly measured under the consideration of the 3R's principle on animal use. To minimize possible errors of repeated measures, we performed all measures with a long inter-measure interval of a week, which is similar to those used in previous studies [22-24]. The vehicle group had no change in PWT, open-arms entry in the elevated plus maze, and immobility in the forced swimming test. A progressive increase sucrose preference and intake was found in the vehicle group, which indicate a pleasure seeking behavior as previous observation [19,47].

These data indicate that repetitive measures are appropriate under our design. However, the within-subject experimental design may raise a concern of historical consequence. To further characterize the effect of treatment on hyperalgesia or psychiatric comorbidity, studies with between-subject design are warranted.

Conclusion

Repeated intramuscular injections of acidic saline in rats elicited bilateral, long-lasting mechanical hyperalgesia and affective comorbidity in the acid-induced CMP model. Pregabalin, duloxetine, and diazepam exerted differently on hyperalgesia and anxio-depressive comorbidity. The current study provides additional face validity and predictive validity of the acid-induced CWP model as humans with CWP syndromes. Our results also suggest valuable information of these medicines in treatment of pain and affective comorbidity.

Acknowledgements

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