

Animal Study

Investigation of the Combination of Pregabalin with Duloxetine or Amitriptyline on the Pharmacokinetics and Antiallodynic Effect During Neuropathic Pain in Rats

Rafaela F. Rodrigues, MSc¹, Taila Kawano, PhD¹, Rodrigo V. Placido, MSc¹, Lellis H. Costa, MSc¹, Márcia H.M.C. Podestá, PhD², Rafaela S. Santos, MSc³, Giovane Galdino, PhD³, Carlos M. Barros, MD⁴, and Vanessa B. Boralli, PhD¹

From: ¹Department of Clinical and Toxicological Analysis, Federal University of Alfenas, MG, Brazil; ²Department of Pharmaceutical Sciences, Federal University of Alfenas, MG, Brazil; ³Sciences of Motricity Institute, Federal University of Alfenas, MG, Brazil; ⁴Federal University of Alfenas, MG, Brazil

Address Correspondence:
Vanessa B. Boralli, PhD
Department of Clinical and Toxicological Analysis, Federal University of Alfenas
Rua Gabriel Monteiro da Silva 700
Alfenas - MG, Brazil, CEP: 37130-001
E-mail:
vanessa.marques@unifal-mg.edu.br

Disclaimer: This study was supported by Coordenação de Aperfeiçoamento de Pessoal de Nível Superior -- Brasil (CAPES) [Finance Code 001].

Conflict of interest: Each author certifies that he or she, or a member of his or her immediate family, has no commercial association (i.e., consultancies, stock ownership, equity interest, patent/licensing arrangements, etc.) that might pose a conflict of interest in connection with the submitted manuscript.

Manuscript received: 06-16-2020
Revised manuscript received: 11-14-2020
Accepted for publication: 11-20-2020

Free full manuscript:
www.painphysicianjournal.com

Background: Amitriptyline, duloxetine, and pregabalin are among the most pharmacotherapeutic, effective treatments for neuropathic pain control. However, the evaluation of synergism by combining these treatments is still poorly investigated.

Objectives: To evaluate the pharmacokinetics of the combination of pregabalin plus duloxetine and pregabalin plus amitriptyline, as well as the effect of these on neuropathic pain on rodent model.

Study Design: The experimental study.

Setting: The research took place in the research laboratories at the Federal University of Alfenas after ethics committee approval.

Methods: This study used male Wistar rats weighing between 220 and 250 g. The animals were randomly divided into the following groups: monotherapy (pregabalin, amitriptyline, duloxetine), combined therapy (pregabalin + amitriptyline, pregabalin + duloxetine), and vehicle (ultrapure water). Pharmacokinetic analysis of pregabalin or combination (pregabalin + amitriptyline or pregabalin + duloxetine) in the plasma were performed by ultraperformance liquid chromatography tandem mass spectrometry. Neuropathic pain was induced by sciatic nerve constriction (chronic constriction injury [CCI]) model, and nociceptive threshold was measured by von Frey filaments test. In addition, to evaluate the influence of the treatments on the motor coordination, the rotarod test was used.

Results: The pharmacokinetic disposition of pregabalin was changed in the association with amitriptyline, presenting a clearance reduction and consequently an increase in bioavailability. Furthermore, after the 14th day of CCI, pregabalin was administered orally and induced antiallodynic effect after 1, 2:15, 4, and 8 hours of its administration and showed the greatest antiallodynic effect after 4 hours of its administration. Moreover, this effect was prolonged (up to 8 hours) by combination with amitriptyline. Additionally, pregabalin and pregabalin + duloxetine showed a hypoalgesic effect in sham rats. In addition, the rotarod test results showed that drugs did not influence the motor coordination of the rats.

Limitations: Potential competition mechanisms during the excretion of pregabalin, when pregabalin was combined with amitriptyline, were not investigated in this study.

Conclusions: The data demonstrated that combined therapy of pregabalin plus amitriptyline improved the bioavailability of pregabalin and potentiated the efficacy of the antiallodynic effect of pregabalin alone, proving to be advantageous for the treatment of sciatic neuropathic pain.

Key words: Neuropathic pain, pregabalin, duloxetine, amitriptyline, pharmacokinetic, antiallodynic effect, combined therapy, rats

Pain Physician 2021; 24:E511-E520

Neuropathic pain is defined by the International Association for the Study of Pain as “pain caused by a lesion or disease of the somatosensory nervous system” (1). In addition, it is estimated that 8% to 10% of the global population suffers from neuropathic pain (2-3). Thus it is important to recognize that neuropathy affects many aspects of patients’ daily lives and is associated with general health problems, poor sleep, reduced quality of life, and increased anxiety and depression (2).

In addition to being a chronic condition, neuropathic pain is difficult to treat, mainly because it responds poorly to treatment with common analgesics. Therefore the pharmacotherapeutic treatment recommended for neuropathic pain includes antidepressants, such as tricyclic antidepressants (TCAs) (e.g., amitriptyline), noradrenaline and serotonin reuptake inhibitors (e.g., duloxetine), and anticonvulsants (e.g., gabapentin and pregabalin) (4). These drugs are US Food and Drug Administration (FDA) approved for treatment of neuropathy, such as for diabetic peripheral neuropathy, post-herpetic neuralgia, and chronic pain (5).

Pregabalin has anticonvulsant, anxiolytic, and analgesic properties (6). Its mechanism of action occurs by binding with great affinity and specificity to alpha-2-delta-1 for the calcium channel voltage-dependent proteins in the central nervous system (CNS), preventing the release of excitatory neurotransmitters (7). Moreover, pregabalin can also increase the activity of the transporter of neuronal glutamate reuptake type 3 and open the potassium channels ATP-sensitive (KATP) (8), producing antinociception (9).

Additionally, amitriptyline and duloxetine are antidepressant drugs that act by inhibiting serotonin and norepinephrine reuptake in the CNS, leading to higher levels of these neurotransmitters in the synaptic clefts, which may activate the descending pain inhibitory pathway (10). Moreover, amitriptyline is also able to block channels of sodium, calcium, and potassium, reducing the conduction of nociceptive impulse (11), and duloxetine can block sodium channels voltage-dependent in the CNS and peripheral nervous system (PNS) (12).

However, these drugs have side effects that negatively impact the patient’s quality of life and continuity of treatment. Pregabalin may have cognitive effects including somnolence, difficulty focusing, and short-term memory loss, as well as peripheral edema, dizziness, and weight gain as the main side effects. The side effects of amitriptyline include somnolence, dizziness,

dry mouth, blurred vision, confusion, weight gain, urinary retention, and tachycardia. Duloxetine mainly presents nausea, somnolence, dizziness, and anorexia as side effects (4-5).

For a better management of this type of pain, a combination of 2 or more drugs with different mechanisms of action can improve analgesic efficacy because different pain control pathways can be activated (13). In addition, this type of therapeutic regimen is widely used to improve the effectiveness of drugs, reduce toxicity, or treat coexisting diseases (14). The pain management is often clinically based on titration on polypharmacy focused on each patient (15). An extra effect of gabapentinoids combined with TCAs, duloxetine, or opioids as compared with monotherapy has been reported (16-20). However, this synergistic effect for pain control is often done without concern for clinically significant pharmacokinetic interactions (14). Pharmacokinetic drug interactions resulting from polypharmacy can interfere with drug absorption, distribution, metabolism, or excretion, changing the magnitude and duration of the effect, but the final response of the drug is preserved (21-22).

It is known that rats are biologically and behaviorally similar to humans, and many human symptoms can be applied to small rodents. However, it is important to recognize that humans differ from animals mainly on drug-metabolizing enzymes. Therefore animal models are fundamental for understanding the mechanisms involved in neuropathic pain and for the development of effective and appropriate therapies for its management in humans, always considering these genetic differences in metabolism to make interspecies extrapolation (23-26).

Thus the present study aimed to investigate and compare the use of pregabalin alone and combined with duloxetine or amitriptyline in healthy rats after oral single dose, and evaluate the correlation of pharmacokinetic characteristics with antiallodynic effect in animals with neuropathic pain.

METHODS

Experimental Animals

This study used conventional heterogeneous male Wistar rats (*Rattus norvegicus albinus*) weighing 220 to 250 g, aged 7 weeks, which were housed in groups of 5 per cage, containing shavings as bedding material, under 12-hour light/dark cycle at constant room temperature (22°C ± 2°C) and humidity (60%). Water and

standard food were available ad libitum. All experiments were carried out within the animals' circadian cycle, respecting the light cycle, between 7 and 19 hours.

All procedures followed the committee for research and ethical issues of the International Association for the Study of Pain (27), and the study was approved by the ethics committee on the use of animals of Federal University of Alfenas, Brazil (protocol number 670/2015).

Drugs and Grouping

Pregabalin was purchased from Pfizer (Karlsruhe, Germany), and amitriptyline purchased from Merck (Kenilworth, NJ). Both drugs were dissolved in distilled water. Duloxetine was purchased from Libbs (São Paulo, Brazil) and prepared as suspension in water. All drugs or vehicles were orally administrated by gavage (2.5 mL/kg) in single dose. The dose selection was done based on previous studies, briefly pregabalin 10 mg/kg, amitriptyline 1 mg/kg, and duloxetine 30 mg/kg (28-30).

All animals were randomized in a simple blind manner and allocated to an experimental group, according to the treatment received orally: vehicle (animals treated with ultrapure water control), pregabalin (animals treated with pregabalin), amitriptyline (animals treated with amitriptyline), duloxetine (animals treated with duloxetine), pregabalin + amitriptyline (animals treated with pregabalin and amitriptyline), pregabalin + duloxetine (animals treated with pregabalin and duloxetine).

Outcomes

The primary outcome studied was pharmacokinetics parameters of pregabalin in healthy animals, comparing the combined treatment (pregabalin + antidepressants) with pregabalin alone. The secondary outcome studied was the measurement of neuropathic pain invoked by chronic constriction injury (CCI) of sciatic nerve (by measuring mechanical allodynia with von Frey hair) and how effective are the pharmacologic treatments, correlating with pharmacokinetics parameters. Also, analyses of the impact of pharmacologic treatment on motor coordination and balance.

Pharmacokinetic Study

This study used 33 rats (33/129), divided into 3 groups (n = 11): pregabalin, pregabalin + amitriptyline, and pregabalin + duloxetine. Before treatment, each

rat was cannulated in the jugular vein for blood collection (31). Twelve hours after cannulation, drugs were given (in combined treatment, the animals first received pregabalin and after 5 minutes they received one antidepressant) and after 0.083, 0.16, 0.25, 0.5, 0.75, 1, 1.5, 2, 4, 6, 8, 10, 16, and 24 hours, 500 µL of blood samples were collected; the volume was reposed with sterile saline solution. Also, during the experiment, the animals had water ad libitum and were fasting by the time of the procedure up to 2 hours after drug administration, to avoid interference with absorption.

Samples were collected in heparinized tubes and centrifuged; plasma was separated and stored at -70°C until pharmacokinetics assays.

The pregabalin and diazepam (internal standard) were analyzed by ultraperformance liquid chromatography mass spectrometry in positive electrospray ionization, with monitoring of the following mass transitions: 158.00 > 140.80, 158.00 > 123.00, and 158.00 > 95.20 for pregabalin; and 285.00 > 154.05, 285.00 > 192.90, and 285.00 > 222.10 for diazepam. For analysis, a sample preparation with protein precipitation (with methanol) followed by high-speed centrifugation was done.

Mobile phase was performed with methanol: ammonium hydroxide solution pH 7.0 (90:10% v/v), the pH was adjusted with acetic acid and stationary phase was C18 column (pre-column and column). The method was validated in accordance with the FDA validation guideline (32), and the level of detection was 0.29 ng/mL of plasma. The method was accurate and linear from 10 to 6,250 ng/mL of plasma.

Pharmacokinetic parameters were calculated based on plasma concentrations. To evaluate differences between the groups, the apparent total clearance (CIT/f) and area under the curve (AUC) parameters were used. The pharmacokinetic analysis was performed using the WinNonlin 4.0 software (Pharsight Corp, Mountain View, CA).

Neuropathic Pain Model

The CCI of sciatic nerve was used as a model for the induction of neuropathic pain (33). Briefly, animals were anesthetized, and the sciatic nerve was exposed and loosely ligated with 4-0 chronic gut thread at 4 sites with an interval of 1 mm.

The 72 (72/129) rats were divided in the following 5 groups: vehicle, pregabalin, amitriptyline, duloxetine, pregabalin + amitriptyline, and pregabalin + duloxetine. Each group had CCI animals (n = 6): animals that underwent CCI and sham animals (n = 6): rats that had

the sciatic nerve exposed but not ligated. In combined treatment, the animals first received pregabalin; after 5 minutes they received the other drug.

Experimental Protocol of Mechanical Allodynia Assessment

First, rats were placed in the cages with a metal mesh floor and allowed to acclimatize for at least 30 minutes. Mechanical allodynia was determined by measuring paw withdraw in response to increasing stimulation with a series of calibrated von Frey filaments, ranging from 0.16 to 180 g. Each filament was pressed over the plantar region of the hind paw for 6 seconds and repeated 3 times, with an interval of at least 3 minutes (34).

Based on a pilot experiment performed by our group (Fig. 1A and Supplementary Fig. S1), the evaluation of treatments on the neuropathic pain was performed on the 14th day of CCI. The drugs were administered in a single dose. To avoid interference with absorption, animals were fasting by 6 hours before the administration and up to 2 hours after drug administration. To evaluate the effect in hours, each drug, or combination, the nociceptive threshold measurement

was assessed before drug administration and 1, 2:15, 4, 8, and 24 hours after administration (Fig. 1B). Measurements always occurred at the 12-hour light cycle.

Motor Coordination

The 24 healthy rats (24/129) were randomly and in simple blind fashion divided in the following groups ($n = 6/\text{group}$): vehicle, pregabalin, pregabalin + amitriptyline, and pregabalin + duloxetine. In combined treatment, the animals first received pregabalin and after 5 minutes they received another drug.

Motor coordination and balance was assessed using a rotarod apparatus (Insight, Ribeirao Preto, Brazil). All rats underwent a 2-day training program during which a steady baseline level of performance was attained (35). During that period, rats were trained to walk against the motion of a rotating drum at a constant speed of 16 rotations per minute (rpm) for a maximum of 2 minutes. Following the training days, a 1-day test was performed using accelerating speed levels (5–16 rpm) mode of the apparatus over 2 minutes. First, the baseline latency (BL) was measured, and new tests were performed after 30, 60, and 120 minutes after drugs or vehicle administration. Then, the mean latency to fall off the rotarod was recorded.

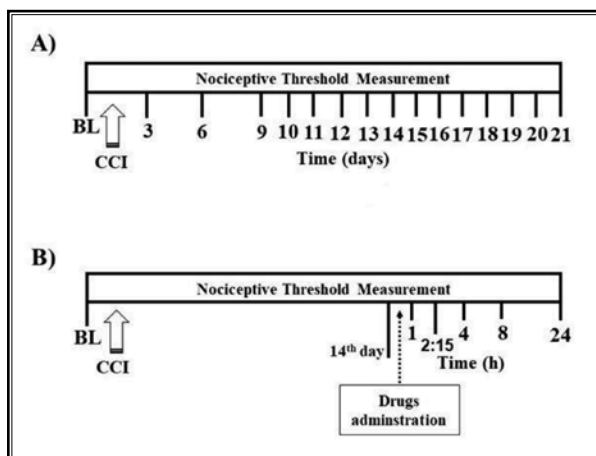


Fig. 1. Schematic representation of experimental protocol of nociceptive threshold measurements. (A) Pilot experiment: first, the BL of the nociceptive threshold in each rat was obtained. Then, neuropathic pain was performed by chronic constriction injury (CCI) or sham procedure was performed, and new measures of nociceptive threshold were done at the 3rd, 6th, and from the 9th to 21st day after CCI. (B) Evaluations of antiallodynic effect of each drug alone or in combination was assessed at the BL, before drug administration, and at 1, 2:15, 4, 8, and 24 hours after drug administration on the 14th day following CCI.

Statistical Analysis

Data are presented as mean \pm standard error of mean (SEM) to behavior experiments and as median for pharmacokinetic parameters. Two-way analysis of variance (ANOVA) with repeated measures followed by Bonferroni test were used for comparisons between-groups of mechanical allodynia and motor test. For pharmacokinetic data, the Mann-Whitney 2-tailed test was applied. Statistical tests were performed with GraphPad InStat (GraphPad, San Diego, CA); the level of significance was set at 5%.

RESULTS

Pharmacokinetics of Pregabalin and Combinations

When then evaluated the median of each pharmacokinetic parameter (AUC and clearance); Table 1 shows that in both parameters a significant difference of pregabalin + amitriptyline ($P < 0.05$) occurred compared with pregabalin. There was no difference ($P > 0.05$) of pregabalin + duloxetine compared with pregabalin. The group that received pregabalin + amitriptyline increased AUC by 4.8 times compared with

Table 1. Estimated pharmacokinetic parameters of pregabalin alone or combined.

Parameter	Pregabalin (10 mg/kg)	Pregabalin (10 mg/kg) + Amitriptyline (1 mg/kg)	Pregabalin (10 mg/kg) + Duloxetine (30 mg/kg)
AUC (h.ng/mL)	1717.0 (1049.5–2926.3)	8190.0* (3654.9–12348.0)	3550.8 (897.3–7800.1)
CIT/f (L/h/kg)	5854.0 (3882.7–7395.5)	1304.0* (353.24–4664.4)	3145.0 (1067.9–6070.6)

CIT/f: total clearance of formation; AUC: area under the curve; h.ng/mL: nanogram per milliliter multiplied per hour (unit of AUC); L/h/Kg: liter per hour per kilogram (unit of clearance).

* $P < 0.05$ indicates a statistical difference between pregabalin and other groups, Mann-Whitney U test.

the pregabalin group, and consequently reduced by 4.5 times the total clearance of formation of the group that received pregabalin.

Pharmacokinetics profiles are represented in Fig. 2; compartmental profiles show that pregabalin combined with amitriptyline reached higher plasma concentrations than pregabalin alone or pregabalin combined with duloxetine.

Evaluation of Pregabalin Alone or Combined in CCI-Induced Mechanical Allodynia

CCI-induced mechanical allodynia in all groups at the 14th day before drug administration ($P < 0.05$, $F_{7,35} = 7,507$) when compared with sham animals (Fig. 3). The mechanical allodynia induced by CCI continued during the 24 hours tested in the vehicle, amitriptyline, and duloxetine groups (Fig. 3).

We then evaluated the effect of drugs on CCI animals after hours of their administrations; we found that only the groups of rats that received pregabalin, or pregabalin combined (pregabalin + amitriptyline or pregabalin + duloxetine), reduced the mechanical allodynia, when compared with the CCI + vehicle group (Fig. 3). This effect was found after 1 ($P < 0.05$, $F_{7,35} = 7,507$), 2:15 ($P < 0.001$, $F_{7,35} = 7,507$), 4 ($P < 0.001$, $F_{7,35} = 7,507$), and 8 hours ($P < 0.05$, $F_{7,35} = 7,507$) of pregabalin administration; 2:15 ($P < 0.001$, $F_{7,35} = 7,507$), 4 ($P < 0.001$, $F_{7,35} = 7,507$), and 8 hours ($P < 0.001$, $F_{7,35} = 7,507$) of pregabalin + amitriptyline; and 4 ($P < 0.001$, $F_{7,35} = 7,507$) and 8 hours ($P < 0.01$, $F_{7,35} = 7,507$) of pregabalin + duloxetine (Fig. 3). When we compared pregabalin with pregabalin + amitriptyline and pregabalin + duloxetine, pregabalin showed higher antiallodynic effect ($P < 0.05$, $F_{7,35} = 7,507$, $P < 0.001$, $F_{7,35} = 7,507$, respectively) at the fourth hour. However, at the eighth hour, only the pregabalin + amitriptyline combination presented the antiallodynic effect higher than pregabalin ($P < 0.001$, $F_{7,35} = 7,507$). It was possible to observe that the pregabalin + duloxetine combination presented a tendency in an antiallodynic effect higher

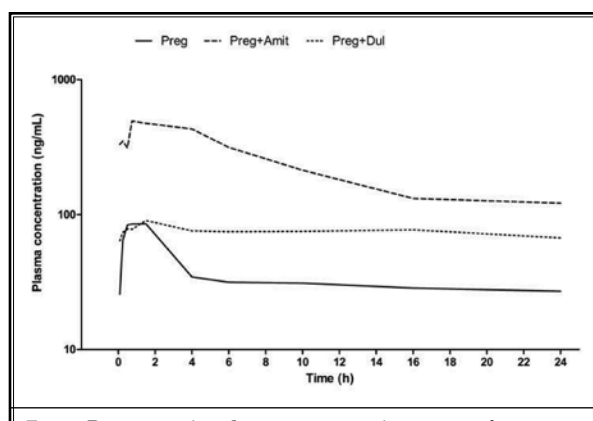


Fig. 2. Representative plasma concentration curves of pregabalin, pregabalin + duloxetine, and pregabalin + amitriptyline. Data are presented as the mean of 6 pharmacokinetic profiles obtained per group. Pharmacokinetic tests were performed in healthy rats. Plasma samples were quantified by ultraperformance liquid chromatography with tandem mass spectrometry, and the data obtained were modeled by the compartmental model.

than pregabalin at the eighth hour, however, it was not significant ($P > 0.05$, $F_{7,35} = 7,507$) (Fig. 3).

The nociceptive threshold in the sham groups that received drugs was not changed, except for animals treated with pregabalin after 1 ($P < 0.05$, $F_{6,30} = 3,502$) and 2:15 hours ($P < 0.01$, $F_{6,30} = 3,502$), and with pregabalin + duloxetine, which was increased after 2:15 ($P < 0.01$, $F_{6,30} = 3,502$), 4 ($P < 0.001$, $F_{6,30} = 3,502$), and 8 hours ($P < 0.001$, $F_{6,30} = 3,502$) compared with the vehicle group (Fig. 4). These results suggest a hypoalgesic effect of pregabalin and pregabalin + duloxetine. Also, the prolongation of the hypoalgesic effect of pregabalin was observed after 4 and 8 hours ($P < 0.001$, $F_{6,30} = 3,502$ for both) after pregabalin + duloxetine administration (Fig. 4). Nevertheless, a tendency of hypoalgesic effect was observed in pregabalin + amitriptyline after 1 and 2:15 hours; in duloxetine group at the first hour, however, it was not significant ($P > 0.05$, $F_{6,30} = 3,502$).

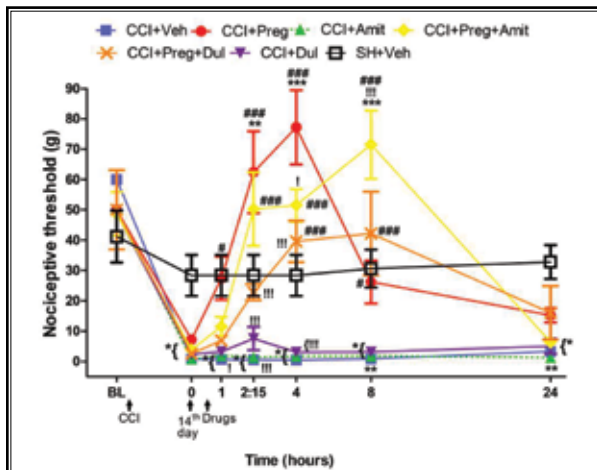


Fig. 3. Effect in hours of pregabalin, duloxetine, amitriptyline, pregabalin plus duloxetine, and pregabalin plus amitriptyline at the 14th day of neuropathic pain. After BL, rats were undergoing the neuropathic pain model, and at the 14th day they were pretreated with oral administration of each drug or combination. Data are expressed as the mean \pm SEM of the nociceptive threshold of 6 animals per group.

*** $P < 0.001$, ** $P < 0.01$, and * $P < 0.05$ indicates a statistical difference between animals with neuropathic pain and sham animals. ! $P < 0.001$, and ! $P < 0.05$ indicates a statistical difference between CCI + Preg and other CCI groups. ### $P < 0.001$, and # $P < 0.05$ indicates a statistical difference between CCI + Veh and other CCI groups. Sham + Veh, sham rats that received vehicle; CCI + Veh, animals underwent chronic constriction surgery and then received vehicle; CCI + Preg, animals underwent chronic constriction surgery and then received pregabalin; CCI + Amit, animals underwent chronic constriction surgery and then received amitriptyline; CCI + Dul, animals underwent chronic constriction surgery and then received duloxetine; CCI + Preg + Dul, animals underwent chronic constriction surgery and then received pregabalin plus duloxetine; CCI + Preg + Amit, animals underwent chronic constriction surgery and then received pregabalin plus amitriptyline; 2-way ANOVA with repeated measures followed by Bonferroni test.

Evaluation of Pregabalin Alone or Combined on the Motor Coordination and Balance of Rats

The rotarod results demonstrated that the drugs in single dose did not alter ($P > 0.05$) the time of stay of animals in the test after 30, 60, and 120 minutes of drug administration (Fig. 5). This result concludes that the antiallodynic effect found by pregabalin alone and combined was only sensory and not motor.

DISCUSSION

The present study demonstrated, for the first time to our knowledge, a reduction of total apparent clearance and an increase of pregabalin bioavailability when pregabalin was combined to amitriptyline.

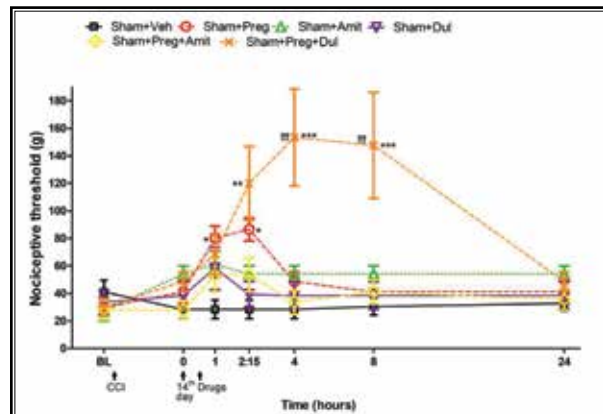


Fig. 4. Effect of pregabalin, duloxetine, amitriptyline, pregabalin plus duloxetine, and pregabalin plus amitriptyline after 14 days sham rats. After 14 days of BL, sham rats were pretreated with oral administration of each drug or combination. Data are expressed as the mean \pm SEM of the nociceptive threshold of 6 animals per group. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ indicates a statistical difference between sham rats that received vehicle (Sham + Veh) and other sham groups. !!! $P < 0.001$ indicates a statistical difference between sham rats that received pregabalin (Sham + Preg) and other sham groups. Sham + Veh, sham rats that received vehicle; Sham + Preg, sham rats that received pregabalin; Sham + Amit, sham rats that received amitriptyline; Sham + Preg + Amit, sham rats that received pregabalin plus amitriptyline; Sham + Preg + Dul, sham rats that received pregabalin plus duloxetine; 2-way ANOVA with repeated measures followed by Bonferroni test.

Pregabalin is not metabolized *in vivo* by cytochromes (CYP) enzymes (36), being excreted practically unchanged in the urine (37). Moreover, its absorption occurs by the L-transporter system and by the sodium channels located in the apical membrane of the intestine (38-39), not being a substrate the P-glycoprotein (P-gp) (40). Conversely, amitriptyline is metabolized by several CYP isoforms, mainly CYP2C19 and CYP2D6 (41), which is a substrate of P-gp (42) and is excreted as inactive metabolites in the urine (43). Also, duloxetine is metabolized by several CYP isoforms, mainly CYP1A2 and CYP2D6 (15), which is a P-gp inhibitor (44), and most part is excreted in the urine mainly as conjugated metabolites (45). Therefore neither amitriptyline or duloxetine have absorption, distribution, and metabolic pharmacokinetic interactions described with pregabalin. Therefore it is important to note that for amitriptyline and duloxetine, which have enzymes involved in their metabolisms with polymorphisms that stratify the population from slow to ultrafast metabolizers, there will be differences for these drugs between humans and animal models (23). Thus the increased bioavail-

ability of pregabalin combined with amitriptyline may be associated with a competition during the pregabalin excretion because the renal drug–drug interaction involving the inhibition of tubular secretion mediated by transporters most commonly manifests as an increase in one of the substances in the plasma (46), but more studies need to be performed to evaluate this process.

In addition, the present study also demonstrated that pregabalin, pregabalin plus amitriptyline, or pregabalin plus duloxetine were efficient in reduce mechanical allodynia induced by neuropathic pain. Furthermore, we found that combined with amitriptyline, the pregabalin-induced antiallodynic effect was prolonged and this response was associated with pharmacokinetics findings, showing this correlation for the first time, to our knowledge. Therefore pregabalin combined with amitriptyline has been demonstrated to be a great treatment strategy for neuropathic pain control because our study has shown that only one administration of pregabalin + amitriptyline induced antiallodynic effects for 8 hours, prolonging the antiallodynic effect of pregabalin in monotherapy, and these findings corroborate with a study that investigated the effect of combination of pregabalin and amitriptyline in 1269 patients with severe neuropathic pain (47). The authors found a reduction of Visual Analog Scale score in just 7 days after treatment (47). Moreover, another study investigated the safety and the efficacy of pregabalin and amitriptyline as monotherapy or as low-dose combination in 92 patients showing similar pain reduction profile for evaluated therapies, and the low-dose combination had less adverse effects than monotherapy (20).

Our study also showed that the antiallodynic effect of pregabalin combined with duloxetine had no significant prolonged effect when compared with pregabalin treatment alone, and pregabalin alone was more effective in reducing neuropathic pain than the combination with duloxetine (Fig. 3). This agrees with a multicentric study on diabetic peripheral neuropathic pain, in which the patients demonstrated that combined pregabalin + duloxetine was less effective than monotherapy; however, the monotherapy dose used was higher (19). Nonetheless, a reduction of the neuropathic pain induced by paclitaxel in women treated for ovarian cancer was found after duloxetine + pregabalin therapy (48). These findings reinforcing that this combined therapy may be effective, safe, and well tolerated, but the effects may vary according to the type of neuropathic pain and/or the dose used.

In addition, when monotherapies were compared,

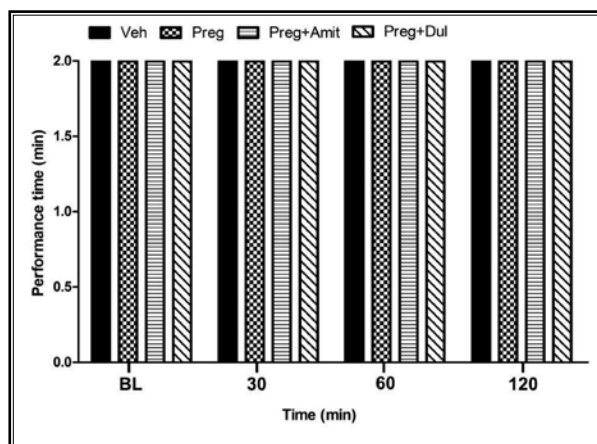


Fig. 5. Motor behavior was performed by rotarod test. After BL, healthy rats were pretreated with oral administration of vehicle, pregabalin, or pregabalin and combinations. New measurements of the motor behavior were performed after drug administration (30, 60, and 120 min). Data are expressed as the mean \pm SEM. No statistical difference was observed; 2-way ANOVA with repeated measures followed by Bonferroni test.

amitriptyline and duloxetine did not show antiallodynic effect after single-dose administration, and only pregabalin reduced mechanical allodynia. To achieve the analgesic effect, the antidepressants amitriptyline and duloxetine need to recruit secondary downstream mechanisms and long-term neuroplasticity, exerting action only when administered repeatedly. However, gabapentinoids are effective for neuropathic pain with acute or chronic administration (13). This profile of pain reduction is also demonstrated in a study with patients presenting with postherpetic neuropathic pain, which found better results at pain relief with pregabalin compared with amitriptyline (49).

Moreover, we found a hypoalgesic effect of pregabalin and pregabalin + duloxetine. This effect may be related to the inhibition of the development of central sensitization (13), mainly by binding to the voltage-gated calcium-channels, that leads to the reduction of the release of excitatory neurotransmitters that mediate pain, such as substance P, calcitonin gene-related peptide, and glutamate (7), and consequently triggering nociceptive impulses even beyond the baseline threshold. Thus we hypothesize that the prolonged hypoalgesic effect of pregabalin + duloxetine group occur as a result of synergism between these 2 drugs with hypoalgesic effect. In accordance with our findings, other studies also demonstrated a tendency for hypoalgesia in sham rats treated with pregabalin (50-51).

The hypoalgesic effect of pregabalin and pregabalin + duloxetine demonstrated in this study may improve the drug choice for other painful conditions involving both the CNS, such as other types of neuropathic pain, and the PNS, such as postoperative pain. Studies show that pregabalin was effective to reduce postoperative pain after eyelid surgery (52), reduce consumption of opioids and analgesics postoperatively (53-54), and reduce preoperative anxiety (55).

By the rotarod test, the present study demonstrated that pregabalin administered alone or in combination, did not alter motor coordination or balance in rats, validating the obtained antiallostatic effect. A study performed with 65 patients with chronic diabetic peripheral neuropathic pain had already shown that none of these treatments affected patients' motor coordination (56). In addition, the authors have shown that both treatments increased the performance on sensory motor tasks (56).

Thus combination of pregabalin with either TCAs or serotonin-norepinephrine reuptake inhibitors is described as a treatment option if a patient cannot tolerate high-dose monotherapy (57), mainly when using 2 drugs with different mechanisms of action.

Limitations

In this study, it was not possible to verify alteration

in pregabalin excretion when it was combined with antidepressants, and if this pathway is related to the increase of pregabalin bioavailability when combined to amitriptyline.

CONCLUSIONS

Taken together, the present study concludes that combination of pregabalin + amitriptyline may be a safe, effective, and advantageous strategy to neuropathic pain compared with pregabalin alone because this combination showed a better pharmacokinetic profile, with increased pregabalin bioavailability, and proved to be the most effective treatment for neuropathic pain induced by sciatic nerve injury because of its greater efficacy and higher duration of the effect. Furthermore, the combination pregabalin + amitriptyline, despite the side effects of amitriptyline, is inexpensive in relation to the pregabalin + duloxetine combination. In addition, we conclude that the hypoalgesic effect shown by pregabalin and pregabalin + duloxetine could help the physicians in the drug choice as a safe, effective, and advantageous strategy for other peripheral pain conditions, such as reducing postoperative pain. Therefore the findings of this study provide scientific information on animals, which can serve as a basis for more effective treatments in humans.

REFERENCES

- Merskey H, Albe Fessard D, Bonica JJ, et al. Pain terms: A list with definitions and notes on usage. Recommended by the IASP subcommittee on taxonomy. *Pain* 1979; 6:249-252.
- Zilliox LA. Neuropathic pain. *Continuum* 2017; 23:512-532.
- Bouhassira D. Neuropathic pain: definition, assessment and epidemiology. *Rev Neurol* 2019; 175:16-25.
- Dworkin RH, O'Connor AB, Backonja M, et al. Pharmacologic management of neuropathic pain: Evidence-based recommendations. *Pain* 2007; 132:237-251.
- Vinik A, Casellini C, Nevoret ML. Diabetic neuropathies. [Updated 2018 Feb 5]. In: Feingold KR, Anawalt B, Boyce A, et al., (eds). *Endotext* [Internet]. South Dartmouth, MA: MDText.com Inc; 2000-. Available from: www.ncbi.nlm.nih.gov/books/NBK279175/. Accessed 05/30/2020.
- White PF, Tufanogullari B, Taylor J, Klein K. The effect of pregabalin on preoperative anxiety and sedation levels: A dose-ranging study. *Anesth Analg* 2009; 108:1140-1145.
- Patel R, Dickenson AH. Mechanisms of the gabapentinoids and $\alpha 2 \delta$ -1 calcium channel subunit in neuropathic pain. *Pharmacol Res Perspect* 2016; 4:e00205.
- Ryu JH, Lee PB, Kim JH, Do SH, Kim CS. Effects of pregabalin on the activity of glutamate transporter type 3. *Br J Anaesth* 2012; 109:234-239.
- Kweon TD, Kim JY, Kwon IW, Choi JB, Lee YW. Participation of K(ATP) channels in the antinociceptive effect of pregabalin in rat formalin test. *Korean J Pain* 2011; 24:131-136.
- Obata H. Analgesic mechanisms of antidepressants for neuropathic pain. *Int J Mol Sci* 2017; 18:2483.
- Lawson K. A brief review of the pharmacology of amitriptyline and clinical outcomes in treating fibromyalgia. *Biomedicine* 2017; 5:24.
- Wang SY, Calderon J, Kuo Wang G. Block of neuronal Na⁺ channels by antidepressant duloxetine in a state-dependent manner. *Anesthesiology* 2010; 113:655-665.
- Kremer M, Salvat E, Muller A, Yalcin I, Barrot M. Antidepressants and gabapentinoids in neuropathic pain: Mechanistic insights. *Neuroscience* 2016; 338:183-206.
- Senderovich H, Jeyapragasan G. Is there a role for combined use of gabapentin and pregabalin in pain control? To good to be true? *Curr Med Res Opin* 2018; 34:677-682.
- Jay GW, Barkin RL. Neuropathic pain: Etiology, pathophysiology, mechanisms, and evaluations. *Dis Mon* 2014; 60:6-47.
- Gilron I, Bailey JM, Tu D, Holden RR, Jackson AC, Houlden RL.

- Nortriptyline and gabapentin, alone and in combination for neuropathic pain: A double-blind, randomised controlled crossover trial. *Lancet* 2009; 374:1252-1261.
17. Gilron I, Jensen TS, Dickenson AH. Combination pharmacotherapy for management of chronic pain: From bench to bedside. *Lancet Neurol* 2013; 12:1084.
 18. Gilron I, Tu D, Holden RR, Jackson AC, DuMerton-Shore D. Combination of morphine with nortriptyline for neuropathic pain. *Pain* 2015; 156:1440-1448.
 19. Tesfaye S, Wilhelm S, Lledo A, et al. Duloxetine and pregabalin: High-dose monotherapy or their combination? The "COMBO-DN study" a multinational, randomized, double-blind, parallel-group study in patients with diabetic peripheral neuropathic pain. *Pain* 2013; 154:2616-2625.
 20. Chakrabarty S, Biswas S, Maiti T, Das A, Mandal A, Banerjee P. Pregabalin and amitriptyline as monotherapy or as low-dose combination in patients of neuropathic pain: A randomized, controlled trial to evaluate efficacy and safety in an eastern India teaching hospital. *Ann Indian Acad Neurol* 2019; 22:437-441.
 21. Rang H, Dale M, Ritter J, Flower R. *Rang & Dale's Pharmacology*. 6th ed. London: Churchill Livingstone; 2007.
 22. Secoli SR. Drug interactions: Fundamental aspects for clinical practice nursing. *Rev Esc Enferm USP* 2001; 35:28-34.
 23. Martignoni M, Groothuis GMM, Kanter R. Species differences between mouse, rat, dog, monkey and human CYP-mediated drug metabolism, inhibition and induction. *Expert Opin Drug Metab Toxicol* 2006; 2:875-894.
 24. Jaggi AS, Jain V, Singh N. Animal models of neuropathic pain. *Fundam Clin Pharmacol* 2011, 25:1-28.
 25. Sousa AM, Lages GV, Pereira CL, Slullitel A. Experimental models for the study of neuropathic pain. *Rev Dor* 2016; 17:527-530.
 26. Sheahan T. Pain 101—Neuropathic pain: One size does not fit all. Available at: <https://relief.news/pain-101-neuropathic-pain-one-size-does-not-fit-all/>. Accessed 06/10/2020.
 27. Zimmermann M. Ethical guidelines for investigations of experimental pain in conscious animals. *Pain* 1983; 16:109-110.
 28. Okazaki R, Namba H, Yoshida H, Okai H, Taquchi K, Kawamura M. Combined antiallodynic effect of Neutropin® and pregabalin in rats with L5-spinal nerve ligation. *Life Sci* 2013; 92:259-265.
 29. Yamamoto H, Shimoshige Y, Yamaji T, Murai N, Aoki T, Matsuoka N. Pharmacological characterization of standard analgesics on mechanical allodynia in streptozotocin-induced diabetic rats. *Neuropharmacology* 2009; 57:403-408.
 30. Iyengar S, Webster AA, Hemrick-Luecke SK, Xu JY, Simmons RMA. Efficacy of duloxetine, a potent and balanced serotonin-norepinephrine reuptake inhibitor in persistent pain models in rats. *J Pharmacol Exp Ther* 2004; 311:576-584.
 31. Burvin R, Zloczower M, Karnielli E. Double-vein jugular/inferior vena cava clamp technique for long-term in vivo studies in rats. *Physiol Behav* 1998; 3:511-515.
 32. US Food and Drug Administration. Guidance for industry: Bioanalytical method Validation, 2013. **{AU: Please provide web address and accessed date for reference 32}**
 33. Bennet GJ, Xie YK. A peripheral mononeuropathy in rat that produces disorders of pain sensation like those seen in man. *Pain* 1988; 33:87-107.
 34. Chaplan SR, Bach FW, Pogrel JW, Chung JM, Yaksh TL. Quantitative assessment of tactile allodynia in the rat paw. *J Neurosci Methods* 1994; 53:55-63.
 35. Dunham NW, Miya TS. A note of a simple apparatus for detecting neurological deficit in rats and mice. *J Am Pharm Assoc* 1957; 46:208-209.
 36. Einarsdottir S, Bjornsson E. Pregabalin as a probable cause of acute liver injury. *Eur J Gastroenterol Hepatol* 2008; 20:1049.
 37. Ranitidis EJ, Posvar EL, Alvey CH, Sedman AJ, Cook JA, Bockbrader HN. Pharmacokinetics of pregabalin in subjects with various degrees of renal function. *J Clin Pharmacol* 2003; 43:277-283.
 38. Piyapolrungron N, Li C, Bockbrader H, Liu G, Fleisher D. Mucosal uptake of gabapentin (neurontin) vs. pregabalin in the small intestine. *Pharm Res* 2001; 18:1126-1130.
 39. Bockbrader HN, Wesche D, Miller R, Chapel S, Janiczek N, Burger P. A comparison of the pharmacokinetics and pharmacodynamics of pregabalin and gabapentin. *Clin Pharmacokinet* 2010; 49:661-669.
 40. Chan PS, Zhang C, Zuo Z, Kwan P, Baum L. In vitro transport assays of rufinamide, pregabalin, and zonisamide by human P-glycoprotein. *Epilepsy Res* 2014; 108:359-366.
 41. Shen Y, Zhu RH, Li HD, Xu P. Validated LC-MS (ESI) assay for the simultaneous determination of amitriptyline and its metabolite nortriptyline in rat plasma: Application to a pharmacokinetic comparison. *J Pharm Biomed Anal* 2010; 53:735-739.
 42. De Klerk OL, Nolte IM, Bet PM, et al. ABCB1 gene variants influence tolerance to selective serotonin reuptake inhibitors in a large sample of Dutch casa with major depressive disorder. *Pharmacogenomics J* 2013; 13:349-353.
 43. Muñoz FL, Alamo C. Monoaminergic neurotransmission: The history of the discovery of antidepressants from 1950s until today. *Curr Pharm Des* 2009; 15:1563-1586.
 44. Ruike Z, Junhua C, Wenxing P. In vitro and in vivo evaluation of the effects of duloxetine on P-gp function. *Hum Psychopharmacol Clin Exp* 2010; 25:553-559.
 45. Lobo ED, Heathman M, Kuan HY, et al. Effects of varying degrees of renal impairment on the pharmacokinetics of duloxetine. *Clin Pharmacokinet* 2010; 49:311-321.
 46. Feng B, Varma MV. Evaluation and quantitative prediction of renal transporter-mediated drug-drug interactions. *J Clin Pharmacol* 2016; 58:S110-S121.
 47. Kiran MD, Pawaskar L, Sheikn SN. Safety and efficacy of the combination of amitriptyline and pregabalin SR in severe neuropathic pain: Data of logic trial. *Int J Inn Res Med Sci* 2019; 4:81-86.
 48. Daniele A, Olivero F, Biglia N, Menato G. Combination of duloxetine and pregabalin for peripheral neuropathy induced by paclitaxel in patients treated for ovarian cancer. *Eur J Obstet Gynecol Reprod Biol* 2016; 206:e31.
 49. Achar A, Chakraborty PP, Bisai S, Biswas A, Guharay T. Comparative study of clinical efficacy of amitriptyline and pregabalin in postherpetic neuralgia. *Acta Dermatoneol Croat* 2012; 20:89-94.
 50. Cao Y, Wang H, Chiang CY, Dostrovsky JO, Sessle BJ. Pregabalin suppresses nociceptive behavior and central sensitization in a rat trigeminal neuropathic pain model. *J Pain* 2013; 14:193-204.
 51. Lau W, Dykstra C, Thevarkunnel S, et al. A back translation of pregabalin and

- carbamazepine against evoked and non-evoked endpoints in the rat spared nerve injury model of neuropathic pain. *Neuropharmacology* 2013; 73:204-215.
52. Wei LA, Davies BW, Hink EM, Durairaj VD. Perioperative pregabalin for attenuation of postoperative pain after eyelid surgery. *Ophtal Plast Reconstr Sur* 2015; 31:132-135.
53. Popa G, Tartau LM, Stoleriu I, Lupusoru RV, Lupusoru CE, Ochiuz L. The effects of pregabalin-codeine combination on partial sciatic nerve ligation-induced peripheral mononeuropathy in rats. *J Physiol Pharmacol* 2016; 67:465-469.
54. Bornemann-Ciment H, Lederer AJ, Wejbor M, et al. Preoperative pregabalin administration significantly reduces postoperative opioid consumption and mechanical hyperalgesia after transperitoneal nephrectomy. *Br J Anaesth* 2012; 108:845-849.
55. Spreng UJ, Dahl V, Raeder J. Effect of a single dose of pregabalin on postoperative pain and pre-operative anxiety in patients undergoing discectomy. *Acta Anaesthesiol Scand* 2011; 55:571-576.
56. Boyle J, Eriksson ME, Gribble L, et al. Randomized, placebo-controlled comparison of amitriptyline, duloxetine, and pregabalin in patients with chronic diabetic peripheral neuropathic pain: Impact on pain, polysomnographic sleep, daytime functioning, and quality of life. *Diabetes Care* 2012; 35:2451-2458.
57. Finnerup NB, Attal N, Haroutounian S, et al. Pharmacotherapy for neuropathic pain in adults: A systematic review and meta-analysis. *Lancet Neurol* 2015; 14:162-173.

Supplementary Table S1. Baseline data of all animals (before any procedure).

Baseline Data			
Group	Mean Weight	Microbiological Status	Drug Prior Treatment
PK Preg	237.8	Healthy	Anesthes
PK Preg + Amit	238.5	Healthy	Anesthes
PK Preg + Dul	237.3	Healthy	Anesthes
Pilot CCI	235.6	Healthy	Anesthes
Pilot SH	235.2	Healthy	Anesthes
Veh CCI	237	Healthy	Anesthes
Veh SH	237.6	Healthy	Anesthes
Preg CCI	232.2	Healthy	Anesthes
Preg SH	237.2	Healthy	Anesthes
Preg + Amit CCI	234.5	Healthy	Anesthes
Preg + Amit SH	233.2	Healthy	Anesthes
Preg + Dul CCI	234	Healthy	Anesthes
Preg + Dul SH	234	Healthy	Anesthes
Amit CCI	232	Healthy	Anesthes
Amit SH	234.2	Healthy	Anesthes
Dul CCI	233.8	Healthy	Anesthes
Dul SH	234.8	Healthy	Anesthes
Rotarod Veh	237.8	Healthy	None
Rotarod Preg	235.6	Healthy	None
Rotarod Preg + Amit	236	Healthy	None
Rotarod Preg + Dul	232.7	Healthy	None

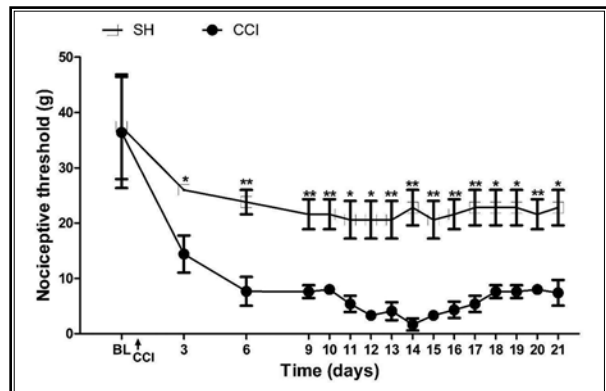


Fig. S1. Pilot experiment: first, the BL of the nociceptive threshold in each rat was obtained. After that, neuropathic pain was performed by CCI, or sham procedure was performed and new measures of nociceptive threshold were done at the third, sixth, and from ninth to 21st day after CCI. The animals did not receive any drug. Data are expressed as the mean \pm SEM of the nociceptive threshold of 5 animals per group. ** $P < 0.01$ and * $P < 0.05$ indicates a statistical difference between animals with neuropathic pain (CCI) and sham animals (SH), Mann-Whitney test.