Coll. Antropol. **32** (2008) Suppl. 1: 93–97
Original scientific paper

The Effect of Repeated Daily Measurements on Paw Withdrawal Latencies in Hargreaves Test

Dragana Kocevski and Ante Tvrdeić

Department of Pharmacology, School of Medicine, University »Josip Juraj Strossmayer«, Osijek, Croatia

A B S T R A C T

The hypothesis that repeated measurements during 4 subsequent days affect withdrawal latencies in Hargreaves test was investigated. Paw withdrawal latencies to radiant heat were determined in the control, tramadol or saline group of male Wistar rats. The control group (N=10) had no treatment. Tramadol group (N=7) and saline group (N=7) received one daily intraperitoneal injection of tramadol (15 mg/kg) or saline (0.9% NaCl), respectively. A significant decline in withdrawal latencies was observed in the control group on the day 2 to day 4, when compared to day 1 ($p<0.05$ Bonferroni test). In the saline and tramadol groups, latencies remained stable from day 1 to day 4. During the entire testing period withdrawal latencies were 27–50% longer in tramadol group ($p<0.05$ ANOVA) compared with the saline group. When compared to the control group, the effect of tramadol, was noted from the second to forth day ($p<0.01$ Bonferroni test), but not on the first day. Finally, a tendency to decrement in withdrawal latencies existed on day 1 in the saline group compared with control group, but this difference does not reach significance. We conclude that one day of training affect withdrawal latencies in the Hargreaves test.

***Key words**: pain, rats, training, Hargreaves test*

Introduction

Hargreaves test is widely used for assessing tolerance to thermally induced pain in rats[1]. The pharmacologists successfully use this test for revealing analgesic drug action and for predicting their analgesic effect in humans. It has proved to be a sensitive method for detecting hyperalgesic as well as analgesic responses. Hargreaves test is comparable to the tail flick[2] and hot plate tests[3]. These tests are also based on the use of thermal stimuli. Hargreaves test is quite different from another popular test, a Randall Selitto test[4]. Randall Selitto test is based on the use of mechanical nociceptive stimuli applied to the paw or tail. Randall Sellito test, the tail flick test and the hot plate test are all sensitive to the training phenomenon – decrease in the pain response with repeated exposure of animals to experimental conditions[5]. For example, four to five training sessions during one week or one month are sufficient to halve the reaction time in the hot plate test[6,7]. Using Randall Selitto test, Taiwo et al. reported 40% decrease in pressure threshold with repeated measurements during three days and increase in the sensitivity for detection of hyperalgesic effect of bradykinin[8]. Training sessions are commonly used in the Randall Sellito and tail flick tests. In these tests animals are restrained during assay. Restrain is stressful procedure for both animals and human being. Stress is known to produce antialgesic effect in laboratory animals[9]. One obvious advantage of Hargreaves test and hot plate test is that pain stimulus is applied to the foot of freely moving animal. There is no need for animal restrain and restrain stress is absent. Consequently, in the hot plate test and Hargreaves test, training period, before start of the experiment, is used only occasionally. However, rodents, unfamiliar with experimental procedure, exhibit strong emotional reaction to the new environment[10] which is manifested in modification of behavior (exploratory behavior, defecation, urination). This emotional reaction is considered as the form of anxiety to the new environment. The facilitator effect of anxiety on the action of morphine is known for a long time[11] in both human and laboratory animals. Emotional/anxiety reactions to the new environment should be more intensive in freely moving rodents. Therefore, in attempt to optimize condi-

Received for publication June 1, 2007.

tions for our research based on Hargreaves method, we investigated the effect of repeated measurement (training) on the paw withdrawal latencies in Hargreaves test. We are not aware of any published study aimed to investigate the effect of training on withdrawal latencies in Hargreaves test. In addition, we investigated the effect of training on sensitivity of Hargreaves test to detect analgesic effect of tramadol. Tramadol is centrally acting analgesic structurally related to morphine. It has a weak affinity for opioid receptors, but inhibits neuronal uptake and causes a release of monoamine neurotransmitters noradrenalin and serotonine[12]. Therefore, tramadol acts on both mechanisms involved in the inhibition of painful signal: the opioid and the descending monoaminergic system. Tramadol induces analgesic and antihyperalgesic effects in laboratory animals[13,14]. It is effective in different behavioral methods related to pain, including method of Hargreaves.

Materials and Methods

Animals

Twenty four male Wistar rats obtained from the Charles River Laboratory (Italy) were used to collect all data. The rat weight was 0.30–0.37 kg at the time of testing. Animals were lodged in the group of four in clear plastic cages with wire mash roof and floor lined with sawdust. Standard laboratory rodent chow and tap water were available *ad libitum*. Animals were housed in a room with constant temperature under a 12/12 hours light/dark cycle (6:00–18:00). Rats were handled several times on the daily base throughout the testing period. The experiments reported here were approved by the Ministry of Agriculture, Forestry and Water Management and by the Animal Care and Ethics Committee of J. J. Strossmayer University of Osijek, Medical Faculty Osijek. The guidelines on ethical standards for investigations of experimental pain in animals were followed[15]. All efforts were made to minimize animal pain and stress. Animals were not restrained and could easily remove the paw from the source of radiant heat. With intensity of radiant heat set on 65 units, we produced the flux of energy of 235 mW/cm^2 and temperature of approximately 50 °C on the floor of the testing device. Besides, the heat stimuli produced by device were tested on the operator hands. Operator experienced only brief sense of pain and mild discomfort.

Drugs

Tramadol (Lumidol, Belupo, Croatia) was provided by the University Hospital Osijek Pharmacy. Saline was prepared as fresh solution of 0.9% NaCl in distilled water. Tramadol was dissolved in saline. Both, saline and tramadol were injected by intraperitoneal route in a volume of 1 mL per each 100 g of rat body weight.

Hargreaves test

Paw withdrawal latencies to radiant heat were measured using the method originally described by Hargreaves[1]. All measurements were done using commercially available Plantar test device (Ugo Basile, Italy). Briefly, each rat was placed individually in a clear Perspex enclosure situated on an elevated glass floor. The rat was allowed to acclimatize to the new environment until the cessation of exploratory behavior (usually 5–10 minutes for male rats). A movable radiant heat generator was then placed by the operator beneath the glass floor, directly bellow one of hind paws. To begin the test, the operator switched on the start key. Radiant heat source and digital timer were activated. When the rat feels pain and withdraws its paw, a sensor switch off both, the heat source and the timer. The timer was stopped, determining the withdrawal latency time in 0.1 seconds. If the animal did not react, heat source was automatically switched off after 33 s to prevent the potential tissue damage. As behavioral end point, a simple protective flexion movement of irradiated hind limb was used. Other behavioral responses related or not related to pain stimuli were not counted. Three latency values were obtained alternatively from each paw 3 minutes apart. To ensure that intensity of the heat stimulus remains constant throughout entire testing session, test device was calibrated using Heat flux I.R. Radiometer (Ugo Basile, Italy).

Experimental design

All experiments presented here were done for the period of three weeks in the winter season, always from the Tuesday to Saturday. Testing took place in the light phase of the day, always between 10 to 17 hours. Only one person operated with Plantar instrument during entire test session. Animals were randomly assigned to the control (N=10), tramadol (N=7) or saline (N=7) group. In all experimental groups withdrawal latencies were measured once a day during 4 consecutive days. The control group had no treatment, tramadol group had one daily intraperitoneal injection of tramadol (15 mg/kg) and the saline group had one daily intraperitoneal injection of saline (0.9% NaCl). Paw withdrawal latencies were determined 30 minutes after injection of tramadol or saline. To avoid the effect of testing order, the testing/injecting order was changed each day during the testing session. Interval between preceding dose and the next dose for the same animal was approximately 24 hours.

Statistical analysis

Paw withdrawal latencies means for 3 treatments during 4 days were analyzed using repeated measures Analysis of variance (ANOVA), with treatment as between factor and day as repeated measures factor. When the ANOVA detected significant treatment effect or effect over time, Bonferroni *post hoc* multiple comparison test was used to determine which treatment or testing day differed significantly from other treatments or testing days. The significance was set at $p<0.05$. We observed no consistent left versus right differences throughout the study. The results were expressed as the mean ± standard error of the mean (SEM) for right paw.

Results

The effect of repeated measurement (training)

Paw withdrawal latencies were shortened from 12.8± 0.4 seconds (X±SEM) on the first day of testing period, to 10.2±0.5 seconds (s) on the 2nd day, 9.1±0.5 s on the 3rd day and 9.7±0.5 s on the 4th day in the control group (Figure 1). As indicated by repeated measures Analysis of variance (ANOVA), a decline in paw withdrawal latencies observed on the day 2 to day 4, when compared to day 1 reach the statistical significance ($p<0.05$ ANOVA, $F(3,27) = 17.80$, $p<0.05$ Bonferroni). From second to forth day, there was no significant change in withdrawal latencies within control group. In the group of rats treated with one daily injection of saline there was no change in paw withdrawal latencies from the day 1 to day 4. The effect over time on withdrawal latencies was also missed in the group of rats treated with one daily dose of 15 mg/kg tramadol.

Tramadol treatment versus saline treatment

Comparison of paw withdrawal latencies between tramadol and saline group revealed significant increase in the latency values for tramadol group on day 1 ($p<0.01$ ANOVA, $F(2,21)=6.70$, $p<0.01$ Bonferoni), day 2 ($p<0.01$ ANOVA, $F(2,21)=13.36$, $p<0.01$ Bonferroni), day 3 ($p<0.01$ ANOVA, $F(2,21) = 16.70$, $p<0.01$ Bonferroni) and day 4 ($p<0.01$ ANOVA, $F(2,21)=17.89$, $p<0.01$ Bonferroni). The results imply that between factor (treatment) was significantly different between tramadol group and saline group throughout the testing period. Increase in withdrawal latencies, clearly suggests analgesic effect of tramadol (Figure 1).

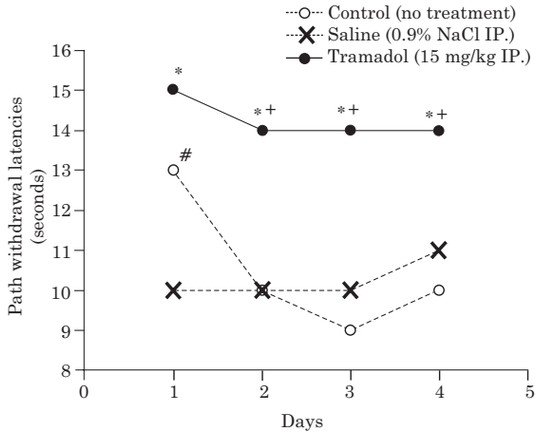

*Fig. 1. Paw withdrawal latencies collected during four day testing period in Wistar rats without any treatment, treated once a day with tramadol or with saline. Data collection using Hargreaves test was started at day 1 and ended by day 4. Paw withdrawal latencies were determined 30 minutes after intraperitoneal (IP.) injection of tramadol or saline. Each point represents the mean of three measurements for right hind paw within a daily session in control (○, N=10), saline (×, N=7) or tramadol group (●, N=7). *p<0.01 tramadol versus saline, +p<0.01 tramadol versus control, #p<0.05 day 1 versus days 2–4 within control group.*

Tramadol treatment versus no treatment

The effect of tramadol, when compared to the control group, was noted from the second to forth day ($p<0.05$ ANOVA and $p<0.01$ Bonferroni). A tendency for increase in withdrawal latencies of rats treated with tramadol when compared to control rats, was monitored on the first day of treatment period, but this difference did not reach statistical significance (14.7±1.3 seconds and 12.8± 0.4 seconds, respectively).

Saline treatment versus no treatment

A tendency for decrease in withdrawal latencies of rats treated with saline compared to control rats existed on the first day of the test period, only. However, ANOVA failed to detect statistical significance (10.3±0.8 and 12.8±0.4 seconds, respectively).

Discussion

Significant increase in withdrawal latencies was observed with tramadol throughout the study, with exception of the abrupt appearance of tramadol effect compared to control on the first day of testing period (Figure 1). Increase in latencies suggests analgesic effect of tramadol. The effect tramadol vs. saline observed in our study is in accordance with literature data[13,14]. Dose of 15 mg/kg used in our study is comparable to dose used in work of Bianchi[14] and lower than dose used in work of Rojas-Corrales[13]. In dose used in our study tramadol had no significant effect on general motor activity, as jugged by the activity cage test (results were not shown).

The main finding of this study was that only one day of repeated measurement (training) had a significant outcome on paw withdrawal latencies in Hargreaves test. As far as we know, this is the first report suggesting the effect of training on Hargreaves test results. Significant decrease in the baseline latencies (the control group of rats) stabilized after a single training session (Figure 1). Our results with baseline latencies are in agreement with results published by Anseloni[16] and coworkers, in spite of the fact that pain assay used in this study were Randall Sellito test. Moreover, decrease in the baseline pressure threshold reported in Anseloni study, also stabilized after only a single day of training. The results presented here also support those published by Taiwo et al[8]. In this study, decrease in the baseline pressure threshold was detected after three days of training. A slower stabilization of baseline threshold seen in this study may be the result of use of very stressful restrain procedure. Another similarity between the study of Taiwo and our study is »hyperalgesic« effect of saline. In the work of Taiwo, significant, dose dependent hyperalgesic effect of saline appeared on the first day and disappeared on the second day to the end of testing period. In our study, almost 30% decreases in withdrawal latencies for saline group versus control group was monitored on the first day of testing period. However, this difference did not reach the statistical significance.

It is well known that common experimental conditions, i.e. animal genotype, testing season, cage density, time of day, animal gender and order of testing might have a significant impact on the results of pain studies in rodents[17–19]. Moreover, these common and »controlled« experimental conditions are very often unspecified in pain literature. All experiments in our study were done on the same 24 male Wistar rats, during three weeks of the winter season, always from the Tuesday to Saturday. Animals were tested in the same experimental room with constant temperature during testing period (23±0.5 °C), constant ventilation and constant light conditions. Testing took place in the light phase of the day, always between 10 to17 hours. Only one person operated with Plantar instrument during entire test session. Animals were not restrained in Hargreaves test. Two persons were involved in drug administration throughout the study – one for grasping and restrain of a rat and another for injection of drug. To avoid the effect of testing/injecting order, the order of animals was alternated each day all through the testing session. In spite of this, some influence of the season or biological rhythms with frequency of week or longer, could not be ruled out. But, if there is any influence of these factors on our results, all experimental groups of rat were equally exposed. Hargreaves test is susceptible to the peripheral sensitization phenomenon – a reduction in the pain response with repetitive stimulation[5]. Sensitization increases with shortening of the interval between painful stimuli and with intensity of stimuli. As first, we used the radiant heat set on 65 IR units throughout the testing period. We know that 65 IR units produced the constant intensity of infrared radiation, constant flux of energy of 235 mW/cm^2 and temperature of approximately 50 °C on the floor of the testing device. To ensure that intensity of the heat stimulus remains constant throughout entire testing session, we calibrate our heat generator. As second, paw withdrawal latencies were measured once a day, with interval between daily sessions of approximately 24 hours. Within daily sessions, three latency values were obtained alternatively from each paw 3 minutes apart. We observed no consistent differences in latencies from the first to the last measurement within daily sessions. Thus, it is hard to believe that the sensitization phenomenon has significant influence on results of our study.

Learning is non-noxious phenomenon that is known to interfere with the second presentation of stimulus in the hot plate and Randall Sellito test[6–8]. It is known that gradual application of heat to the skin activates thermo receptors, before nociceptors are activated[5]. As the result, sensation of hot appears before sensation of pain. Thus, sensation of pain could be anticipated and learned. Rodents are known to learn very rapidly. Significant decrease in the baseline latencies detected with Hargreaves test and stabilized after a single training session could be explained by learning phenomenon. Another non noxious phenomenon which could be of importance for explanation of findings presented in this paper is adaptation response to the new environment. From day one to the end of testing period, it was obvious that exploratory behavior of rats decreases, as jugged by movements within enclosure of testing device. Besides, during the 4 day period, rats defecate and urinate much less and, finally, became indifferent to the operator and to the testing procedure. It seems that time course of adaptation response to the new environment is interrelated with stabilization of withdrawal latencies in our study.

In conclusion, our results demonstrate that one day of training significantly affect paw withdrawal latencies in the Hargreaves test. Consequently, with only one presentation of heat stimulus before the start of experiment, Hargreaves test would become more trustworthy instrument for pain research on laboratory animals.

Acknowledgements

This work is supported by Croatian Ministry of Science, Education and Sport research grant number 219-2192382-2369.

REFERENCES

1. HARGREAVES K, DUBNER R, BROWN F, FLORES C, JORIS J, Pain, 32 (1988) 77. — 2. D'AMOUR FE, SMITH DL, J Pharmacol Exp Ther, 72 (1941) 74. — 3. WOOLFE G, MACDONALD AL, J Pharmacol Exp Ther, 80 (1944) 300. — 4. RANDALL LO, SELITTO JJ, Arch Int Pharmacodyn Ther, 111 (1957) 409. — 5. LE BARS D, GOZARIU M, CADDEN SW, Pharmacol Rev, 53 (2001) 597. — 6. SANDKÜHLER J, TREIER AC, LIU XG, OHNIMUS M, Neuroscience, 73 (1996) 657. — 7. LAI YY, CHAN SHH, Physiol Behav, 28 (1982) 1111. — 8. TAIWO YO, CODERRE TJ, LEVINE JD, Brain Res, 487 (1989) 148. — 9. TERMAN GW, SHAVIT Y, LEWIS JW, CANNON JT, LIEBESKIND JC, Science, 226 (1984) 1270. — 10. LEUSSIS MP, BOLIVAR VJ, Neurosci Biobehav Rev, 30 (2006) 1045. — 11. HILL EH, BELLEVILLE RE, WIKLER A, Proc Soc Exp Biol Med, 186 (1954) 881. — 12. BAMIGBADE TA, LANGFORD RM, Pain Rev, 5 (1998) 155. — 13. ROJAS-CORRALES MO, ORTEGA-ALVARO A, GIBERT-RAHOLA J, ROCA-VINARDELL A, MICÓ JA, Pain, 88 (2000) 119. — 14. BIANCHI M, PANERAI AE, Brain Res, 797 (1998) 163. — 15. COVINO BJ, DUBNER R, GYBELS J, LIEBESKIND JC, STERNBACH RA, VYKLICKI L, YAMAMURA H, ZIMMERMANN M, Pain, 9 (1980) 141. — 16. ANSELONI VCZ, ENNIS M, LIDOW MS, J Neurosci Meth, 131 (2003) 93. — 17. CHESLER EJ, WILSON SG, LARIVIERE WR, RODRIGESZAS SL, MOGIL JS, Neurosci Biobehav Rev, 26 (2002) 907. — 18. MOGIL JS, CHESLER EJ, WILSON SG, JURASKA JM, STERNBERG WF, Neurosci Biobehav Rev, 24 (2000) 375. — 19. TVRDEIĆ A, ĐURKOVIĆ M, BIRUŠ I, Period Biol, 106 (2004) 427.

A. Tvrdeić

Faculty of Medicine, Department of Pharmacology, University »Josip Juraj Strossmayer«, J. Huttlera 4, 31000 Osijek, Croatia
e-mail: tvrdeic@mefos.hr

UČINAK UZASTOPNIH MJERENJA NA LATENCIJE ODMICANJA ŠAPE U HARGREAVES TESTU

S A Ž E T A K

Testirana je hipoteza o učinku opetovanih mjerenja tijekom 4 uzastopna dana na vrijeme latencije do odmicanja šape u Hargreaves testu. Latencije su mjerene u mužjaka Wistar štakora podijeljenih u kontrolnu (N=10), fiziološku (N=7) skupinu i skupinu tretiranu tramadolom. Kontrola nije bila izložena nikakvom tretmanu. Skupine tramadol i fiziološka su primile jedanput dnevno intraperitonealnu injekciju tramadola (15 mg/kg; skupina tramadol) ili fiziološke otopine (0.9% NaC; skupina fiziološka). U kontrolnoj skupini zabilježen je značajan pad latencija od drugog do četvrtog dana u usporedbi s prvim danom testiranja ($p<0.05$, Bonferroni). U skupinama fiziološka i tramadol, latencije su ostale stabilne od prvog do četvrtog dana. Tijekom čitavog perioda testiranja vrijednosti latencija bile su za 27–50% duže u skupini koja je primila tramadol ($p<0.05$, ANOVA) u usporedbi sa skupinom koja je primila fiziološku otopinu. U odnosu na kontrolnu skupinu, učinak tramadola zabilježen je od drugog do četvrtog dana ($p<0.01$, Bonferroni), ali ne i prvi dan testiranja. Konačno, prvog dana testiranja uočena je tendencija prema nižim vrijednostima latencija u fiziološkoj skupini u odnosu na kontrolnu skupinu, ali ta razlika nije dosegla statističku značajnost. Iz svega navedenog, zaključili smo da jedan dan treninga utječe na latencije u Hargreaves testu.