

Effect of Rohypnol on pain threshold and acoustic startle reflex in Wistar rats

ABSTRACT

Some physical and psychological factors resulting in heterogeneous pain have been implicated in either increasing or decreasing pain thresholds. Rohypnol, which is a benzodiazepine, produces both sedative and muscle relaxant effects; hence its use as a pre-anesthetic and also in the treatment of insomnia. This study investigated the effect Rohypnol on pain threshold and acoustic startle reflex in Wistar rats.

Twenty five (25) female Wistar rats (160 – 180g) were randomly grouped into five groups (A-E) of five rats each, sequel to their two acclimatization under standard ethical and environmental conditions. Rats in group A served as the control and received 0.5mL distilled water once daily. Rats in group B received 2mg/kg b.w. Diclophenac Sodium (DIC) once daily; while rats in groups C, D and E received 3mg/kg b.w. Rohypnol, once, twice and thrice daily, respectively. Treatment to experimental rats in all groups lasted for eight days, while all animals received standard rat feed and water ad libitum throughout the study. Pain threshold was recorded using an analgesy-meter and tail clip tests; while the acoustic startle reflex (ASR) response time was assessed using an acoustic bell.

The results showed that Rohypnol administration significantly increased ($P<0.05$) the pain threshold of Wistar rats, while a non-significant delay in ASR response time was observed when compared to the control. From the results of the study, Rohypnol caused an increase in the pain threshold of experimental rats to comparable values elicited by DIC.

This effect may be attributed to the muscle tone depression, lack of muscle control and alertness, and the seemingly delayed acoustic startle reflex response associated with the drug. Although Rohypnol was seen to possess antinociceptive properties comparable to DIC, however caution should be applied in its use as a pain reliever due to its sedating and nervous system depressing effects.

Keywords: Rohypnol, Benzodiazepines, Pain threshold, Acoustic startle reflex, Diclophenac.

BACKGROUND OF STUDY

Pain is defined as an unpleasant sensory and emotional experience that is associated with actual or potential tissue damage or that is described in terms of such damage¹. While assessments of both the associated sensations and emotions are important for understanding pain, these methods are limited because perception of pain differs among individuals and is affected by environmental and psychological factors at different times. In clinical practice, such traits related to pain make diagnosis and treatment difficult and contribute to the development of refractory and chronic pain^{2,3}.

The point at which pain starts to be felt on a curve of increasing perception of a stimulus is known as the threshold of pain. It is wholly a subjective phenomenon and is defined as the lowest intensity of a stimulus that is considered as painful¹. Factors that have been reported to be associated with low pain thresholds include physical variables, such as the severity and duration of the pain, and decreased autonomic function^{4,5}. On the other hand, pain thresholds have been reported to increase in patients with depression^{6,7,8,9}. According to these reports, physical and psychological factors which result in heterogeneous chronic pain is strongly associated with increasing or decreasing the pain thresholds; thus complicating the central sensitization¹⁰.

The acoustic startle reaction (ASR) is a reflexive muscle contraction brought on by an abrupt loud sound. The ASR is evolutionarily conserved across mammals¹¹, but is measured in various

ways, depending on the species. While it is frequently measured in humans by the intensity of the eye-blink response to a sound pulse, it is typically quantified in non-human animals by the whole-body response to a brief high-level sound pulse. The ASR and measurement paradigms based on the ASR (such as those measuring pre-pulse inhibition) have been a mainstay of studies on a range of brain-based disorders in human and non-human animals^{12,13,14,15,16,17,18,19}.

ASR is a motor reaction to a certain class of stimuli of different modalities. Behaviorally, the startle response consists of rapid contraction of head, neck, trunk and legs muscles²⁰ in addition to the arrest of ongoing activity²¹. Auditory, visual and several types of tactile stimuli were successfully used for eliciting startle^{22,23,24,25,26}. In laboratory practice most widely used are intense auditory signals eliciting so called acoustic startle response (ASR). Sensitivity to the ASR in a variety of experimental treatments has made it an important research tool in studies of brain mechanisms of learning, memory, emotions and movement control^{27,28}.

Flunitrazepam, which is a trade name for Rohypnol, is a central nervous system depressant in a class of drugs called benzodiazepines. It is used in some countries to treat severe cases of insomnia²⁹. Rohypnol, also referred to as a “date rape drug”, produces sedative, anxiolytic, hypnotic, anticonvulsant, and muscle relaxant effects in humans and rodents^{29,30}.

In most countries where the drug is legally approved, it is administered as a pre-anesthesia³¹ and also in the treatment of insomnia; wherein it is administered just as other hypnotic drugs, strictly in a short-term basis, or occasionally with caution in cases of chronic insomnia³². An abrupt withdrawal of Rohypnol therapy may present a clinical condition referred to as “benzodiazepine withdrawal syndrome” characterized by the following symptoms: Insomnia, psychosis, seizures, and anxiety³³.

The abuse of the drug by individuals has been the bane of legalizing the drug for “over the counter” purchase and administration/usage in countries such as Nigeria. Rohypnol got its street name “Date Rape Drug” because the drug has been used illicitly to facilitate sexual assault, as the victims are unable to recall the incidence of their sexual encounter/assault. Other street names for Rohypnol include Roofies, Rophies, Rope, Roach, Roach-2, Roche, Roopies, Robutal, Row-shay, Ruffles, Wolfies, La Rocha, Lunch Money Drug, R-2, Mexican Valium, Circles, Pingus, Forget Me Pill³⁴. The major pharmacological effect of Rohypnol as a lipophilic drug is its enhancement of Gamma-aminobutyric acid (GABA) at the localized GABA receptors³⁵. As such, people under the impact of Rohypnol abuse often experience a state of dissociation or automatism which makes it difficult for the person to remember what occurred while under the influence of the drug, even after the effect of the drug wears off³⁶.

The impact of Rohypnol consumption may leave its abusers or patients with the following clinical conditions; sluggish and uncoordinated movement of the limbs, hang-over, anterograde amnesia, woozy feelings, stomach upset, dizziness, and confusion³⁶. In a study involving healthy volunteers, Rohypnol induced significant increases in some pleasurable but relative subjective effects (such as; feeling of likeness, feeling good effects, and feeling of a high) that may be related to its abuse potential³⁷. The pharmacological basis that may explain the preference for this drug by drug abusers are unknown. However, it has been suggested that the fast onset of the effect of the drug may be a factor contributing to its high abuse potential³⁸, which may be as a result of the lipophilic nature of the drug; thus enabling the drug to enter rapidly into the central nervous system³⁹.

Exposure to Rohypnol at high doses has been shown to have effects such as lack of muscle control and tone, alertness, correct response, etc.³⁶. Due to its high lipophilic nature, it is a

common choice for drug abusers and addicts, who believe that the consumption of this drug makes them unaware of their environment, helps them forget their pain and worries, makes them calm, and has the tendency to suppress the body systems. Hence, these effects leads them to the excessive consumption and overdose of Rohypnol. It is important to note that the effect of Rohypnol has been scarcely assessed in experimental studies relating to pain and startle reflex; as such this study is aimed at investigating the effect Rohypnol on pain threshold and acoustic startle reflex in Wistar rats.

UNDER PEER REVIEW

MATERIALS AND METHODS

Animal Model

Twenty five (25) apparently healthy female Wistar rats (160 – 180g), locally sourced from the animal house of the Department of Human Physiology, were used for the study. The animals were housed in standard rat cages under hygienic animal husbandry conditions: temperature, 25 - 28°C; humidity 40 – 60%, while maintaining a 12hr light/dark cycle. The animals were allowed to acclimatize for two (2) weeks to their new environment before the commencement of the study. During this period, they were allowed a standard rat chow and water ad libitum.

Drugs

Flunitrazepam (Rohypnol) (produced and marketed by SWISS Pharma Nigeria Ltd. under the license of Global Healthcare Ltd, Basel Switzerland) and Diclofenac Sodium (manufactured by Laborate Pharmaceuticals Ltd, India, and marketed in Nigeria by EMBASSY Pharmaceuticals and Chemical Ltd, Lagos, Nigeria) were purchased from a local pharmacy using ethically approved drug prescription for the study.

Other Materials

Other materials used for the study include the following; Analgesy-meter (Ugo Basile), Acoustic bell, distilled water, measuring beakers, syringes and tail clip (a metal plier with rubber hand grip).

Experimental Design

The twenty five (25) female Wistar rats were randomly grouped into five groups (A-E) of five rats each. Rats in group A served as the control and received 0.5mL distilled water once daily. Rats in group B received 2mg/kg b.w. Diclophenac Sodium (DIC) once daily; while rats in groups C, D and E received 3mg/kg b.w. Rohypnol, once (x1), twice (x2) and thrice (x3) daily, respectively. Treatment to experimental rats in all groups lasted for eight days. The rats were subjected to the various experimental tests before the start of the study, and at every two days interval until the eighth day.

Dosage preparation

A suitable amount of DIC (1gram) was weighed and dissolved in distilled water to produce a 1 mg/ml solution, from which 2mg/kg DIC was administered daily by gavage to the rats in group B. Same procedure was used in the dosage preparation of Rohypnol, and the rats were administered the respective dosages as stated above.

Pain Threshold Test

- Using Analgesy-meter

The pain threshold test using an Analgesy-meter machine was used to determine the pain threshold of the rats in each group. The test was carried out by placing the sharp and pointed part of the analgesy-meter on the paw of the rat. The plinth increases at a constant rate, thereby enabling reproducible measurements to be made. The machine stops running immediately the pedal is released at the point of paw withdrawal, and the pain threshold on the analgesy-meter was recorded. Each record was measured in seconds which is the time it took for the rat to withdraw its paw due to pain. After each test, the slide is returned to its starting point by lifting and pushing to the left. This test was used in determining the anti-nociceptive activity of the drug administered.

- Using Tail Clip

The pain threshold test and sensitivity was also investigated using the tail clip test. The tail clip is regarded as a stressful activating stimulus and can influence the rat's cognitive decisions and actions. The essence of tail-clip was to increase mechanical pressure on the tail of the rat and determine how long it would take each rat to attempt to elicit a response such as head bending towards tail, shouting and/or attempt to remove clip. The reaction time (in seconds) was recorded for each rat. The tail-clip was padded with a soft material to avoid injury to the rat.

Startle Reflex Test

This startle reflex test was done using an acoustic bell. The rats were placed in a free space (one at a time), then the acoustic bell was rung to create noise causing fear in the rats, after which their response reaction time (in seconds) was observed. The response sought out for were; running towards the bell, running away from bell or no movement. These responses were observed and recorded for each rat.

Ethical Considerations

Animals used for the study were housed and handled in compliance with standard guidelines and care of the use of laboratory animals^{40,41}. The research design and protocol were approved by the intuitional research ethics committee.

Statistical Analysis

Data obtained from laboratory investigations in the study were analyzed using IBM Statistical Product and Service Solutions (SPSS version 25). The mean and standard error of the mean were calculated for each parameter. The mean values obtained for the experimental groups (II & III) were compared to the control (Group I) using the analysis of variance (ANOVA) followed by a

least significant difference (LSD) posthoc analysis (ANOVA). A p-value less than 0.05 ($p < 0.05$) was considered statistically significant.

RESULTS

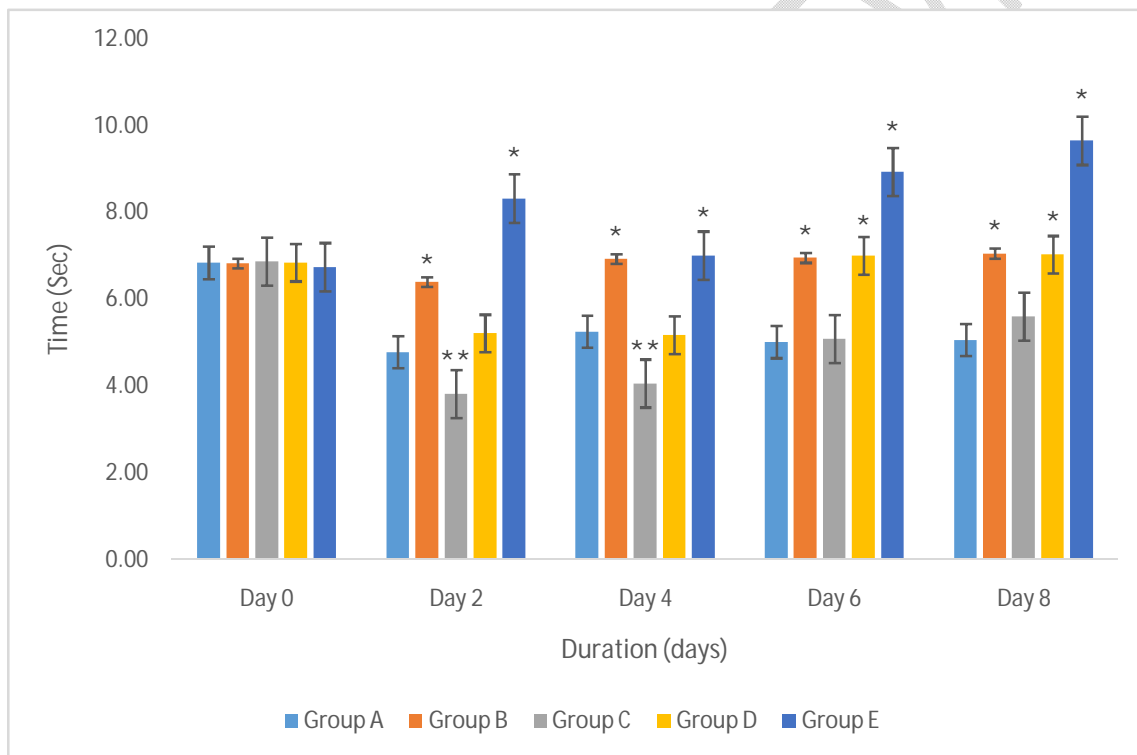


Figure 1: Bar Chart showing the pain threshold (in seconds) of Wistar rats on various days of the study using an Analgesy-meter.

*Indicates that the pain threshold time (in seconds) for that rat group is significantly higher ($P < 0.05$) when compared to Group A (control group) for the same day of the study.

**Indicates that the pain threshold time (in seconds) for that rat group is significantly lower ($P < 0.05$) when compared to Group A (control group) for the same day of the study.

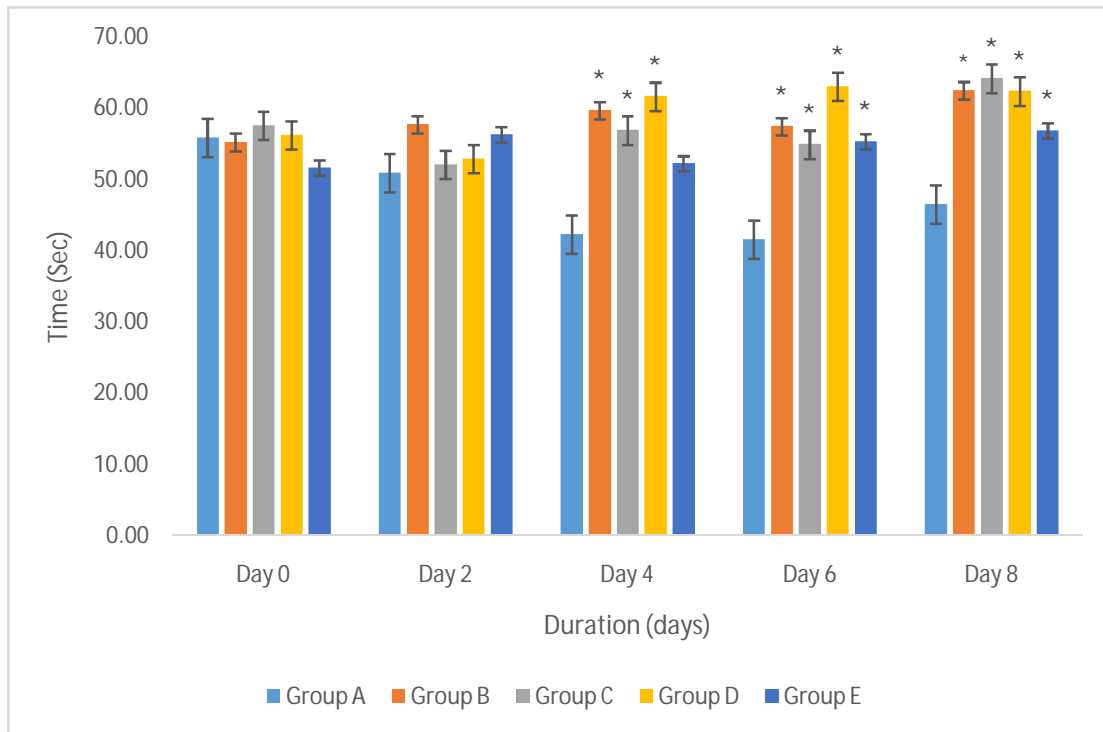


Figure 2: Bar chart showing the Tail Twitch Reaction Time (in seconds) of Wistar rats on various days of the study using a Tail Clip.

*Indicates that the tail twitch reaction time for that rat group is significantly higher ($P < 0.05$) when compared to Group A (control group) for the same day of the study.

Table 1: Table showing the Reaction Time (in seconds) of Wistar rats using an Acoustic Bell on various days of the study.

Rat Groups	N	Reaction Time (in seconds) for DAY 0	Reaction Time (in seconds) for DAY 2	Reaction Time (in seconds) for DAY 4	Reaction Time (in seconds) for DAY 6	Reaction Time (in seconds) for DAY 8
GROUP A (Control) Administered 0.5mL distilled water once daily	5	1.87 ± 0.81 (1.67 / 2.00)	2.07 ± 0.16 (1.67 / 2.67)	1.80 ± 0.13 (1.33 / 2.00)	2.00 ± 0.24 (1.33 / 2.67)	2.00 ± 0.15 (1.67 / 2.33)
GROUP B: Administered 2mg/kg Diclofenac once daily for 8 days	5	2.00 ± 0.15 (1.67 / 2.33)	2.00 ± 0.21 (1.67 / 2.67)	2.07 ± 0.07 (2.00 / 2.33)	1.93 ± 0.16 (1.67 / 2.33)	2.13 ± 0.08 (2.00 / 2.33)
GROUP C: Administered 3mg/kg Roprenol once daily for 8 days	5	2.07 ± 0.22 (1.33 / 2.67)	2.00 ± 0.10 (1.67 / 2.33)	2.13 ± 0.33 (1.00 / 3.00)	1.87 ± 0.23 (1.33 / 2.67)	2.07 ± 0.22 (1.33 / 2.67)
GROUP D: Administered 3mg/kg Roprenol twice daily for 8 days	5	2.20 ± 0.23 (1.67 / 3.00)	2.20 ± 0.17 (1.67 / 3.00)	2.47 ± 0.08* (2.33 / 2.67)	2.47 ± 0.23 (2.00 / 3.00)	2.20 ± 0.23 (1.67 / 3.00)
GROUP E: Administered 3mg/kg Roprenol thrice daily for 8 days	5	2.27 ± 0.13 (2.00 / 2.67)	2.20 ± 0.17 (1.67 / 2.67)	2.00 ± 0.10 (1.67 / 2.33)	2.07 ± 0.07 (2.00 / 2.33)	2.13 ± 0.08 (2.00 / 2.33)

Values are expressed as Mean ± SEM

N = No. of rats per group.

Values in bracket indicate the minimum value and maximum reaction time for each age group.

Significant level set at 95% confidence interval ($P < 0.05$)

*Indicates that the reaction time (in seconds) for that rat group is significantly higher ($P < 0.05$) when compared to Group A (control group) for the same day of the study.

DISCUSSION

The present study evaluated the effect of Rohypnol on pain threshold and acoustic startle reflex in female Wistar rats. The ability of the rat to withstand pain after being treated with Rohypnol in varying durations per day for 8 days was observed using an analgesy-meter and tail clip tests; while the acoustic startle reflex (ASR) response time of the rats was assessed using an acoustic bell. The study also compared the antinociceptive effect of Rohypnol with that of the standard drug for pain, Diclophenac sodium.

Effect on pain threshold

The Analgesy-meter test was used to assess the pain threshold in Wistar rats (Figure 1). On day 0, the result showed that there was no significant difference ($P > 0.05$) in pain threshold in the drug treated groups when compared to the control group (group A). However, the rats in group B (2mg/kg b.w. DIC) and group E (3mg/kg b.w. Rohypnol x3 daily) showed a significant increase ($P < 0.05$) in pain threshold on days 2, 4, 6 and 8 (6.40 ± 0.48 and 8.32 ± 0.20 ; 6.93 ± 0.32 and 7.00 ± 0.20 ; 6.95 ± 0.31 and 8.93 ± 1.30 ; 7.05 ± 0.29 and 9.66 ± 0.71 seconds, respectively) when compared to the control group (4.7 ± 0.23 ; 5.26 ± 0.44 ; 5.01 ± 0.38 ; 5.06 ± 0.30 seconds). This indicates that the rats in groups B and E were hyposensitive to pain and had a high pain threshold. Likewise, the result showed a significant increase ($P < 0.05$) in the pain threshold of rats in group D (3mg/kg b.w. Rohypnol x2 daily) on days 6 and 8 (7.00 ± 0.12 and 7.03 ± 0.34 seconds, respectively) when compared to the control group (5.01 ± 0.38 and 5.06 ± 0.30 seconds).

However, rats in group C (3mg/kg b.w. Rohypnol x1 daily) had a significant decrease ($P < 0.05$) in their pain threshold on days 2 and 4 (3.82 ± 0.31 and 4.06 ± 0.24 seconds, respectively) when compared to the control group (4.7 ± 0.23 and 5.26 ± 0.44 seconds); but no significant difference ($P > 0.05$) was observed in their pain threshold on days 6 and 8 when compared to the control group. This possibly indicates that moderate dosage administration of Rohypnol may be unable to abate pain and as such ineffective in increasing pain threshold, while an excessive dosage administration of Rohypnol as seen in group D (3mg/kg b.w. Rohypnol x2 daily) and group E (3mg/kg b.w. Rohypnol x3 daily) significantly increased the pain threshold of the rats. This increased pain threshold may not be unconnected with the feeling of numbness, lack of muscle control and tone, alertness, and correct response that has been attributed to excessive Rohypnol administration³⁶. The result also showed that only rats in group E had a significant increase in ($P < 0.05$) in pain threshold when compared to the rats in group B (2mg/kg b.w. DIC) treated with the standard drug for pain (Diclophenac sodium). This increased pain threshold can be attributed to the muscle tone depressing effect of Rohypnol^{35, 36}.

Similarly, the tail clip test revealed a significant increase ($P < 0.05$) in the pain threshold of the treated rats on days 4, 6, and 8 when compared to the control (Figure 2). The tail twitch test is a valuable tool for evaluating the analgesic effects of drugs and for studying the mechanisms of pain and analgesia. The increased pain threshold and reaction time of the treated rats in groups B, C, D and E, indicates that the rats could endure the pain for a longer period of time. This finding highlights the analgesic effect of Rohypnol in reducing the pain hypersensitivity of rats, as the pain threshold of the Rohypnol treated rat groups were relative to that of the rat group treated with Diclophenac sodium.

The progression increase in mechanical sensory threshold with each day, especially as seen in group E (3mg/kg b.w. Rohypnol x3 daily), indicate that high doses Rohypnol leads to increased hyposensitivity to pain in Wistar rats mainly to its sedating effects. By acting on the GABA-A receptor, a brain neurotransmitter receptor implicated in the control of anxiety, sleep, and pain perception, Rohypnol exerts its analgesic effects⁴². The principal inhibitory neurotransmitter in the central nervous system, GABA, is more readily bound when Rohypnol binds to the benzodiazepine site on the GABA-A receptor. Rohypnol's sedative and anxiolytic effects result from this interaction's overall increase in the activity of GABAergic neurons. However, this same interaction also results in the suppression of pain signalling pathways in the brain and spinal cord, leading to the drug's analgesic effects⁴³. Rohypnol's ability to block pain signalling is presumed to be due to a combination of actions on the GABA-A receptor and other pain-modulating systems in the brain and spinal cord, although the precise mechanism by which this happens is still unclear⁴⁴.

Acoustic startle reflex (reaction time) test

The acoustic startle reflex (ASR) which a survival mechanism of alarm, rapidly alerts and arouses organisms to a sudden loud auditory stimulus. Behaviourally, the ASR involves a rapid and sequential activation of muscles along the length of the body as well as an autonomic physiological response⁴⁵. The acoustic bell test is a valuable tool for studying the startle reflex and for evaluating the functioning of the nervous system. The startle reflex test evaluates an individual's involuntary motor response to a sudden, unexpected stimuli. This test is frequently used in clinical and research settings to evaluate how well the nervous system is functioning and to investigate how different medicines affect physiology and behaviour.

The result obtained from this test showed that there was no significant difference ($P>0.05$) in the startle reflex reaction time in the treated groups when compared to the control group, except on day 4 where the reaction time of the rats in group D (3mg/kg b.w. Rohypnol x2 daily) was significantly increased compared to the control group (2.47 ± 0.08 vs. 1.80 ± 0.13 seconds). The general result obtained from this test indicates that Rohypnol has no significant effect on the startle reflex and alertness of the rats. However Rohypnol did cause a non-significant delay in the startle response in the treated rat groups when compared to the control group, as the treated rats experienced some milliseconds to seconds delay in their acoustic startle reflex reaction time. This study also suggests that the effects of Rohypnol on the startle reflex in rats may be dose-dependent. This finding correlates with Swerdlow, et al., (2000)⁴⁶ who reported that Rohypnol had no significant effect on the startle response or prepulse inhibition at doses up to 3 mg/kg; except at higher doses (5 and 10 mg/kg).

CONCLUSION

From the results of the study, it can be deduced that administration of Rohypnol caused an increase in the pain threshold of experimental rats to comparable values elicited by the standard pain drug, Diclophenac sodium. This effect may be attributed to the feeling of numbness, muscle tone depression, lack of muscle control and alertness, and the seemingly delayed acoustic startle reflex response associated with the drug. Although Rohypnol was seen to possess antinociceptive properties as seen from the results of the study, however caution should be applied in its use as a pain reliever due to its sedating and nervous system depressing effects.

REFERENCES

1. IASP. "IASP Pain Terminology". IASP Press. Archived from the Threshold of pain 2022. In Wikipedia. https://en.wikipedia.org/wiki/Threshold_of_pain
2. Kouyanou, K., Pither, C.E. and Wessely, S.. Latrogenic factors and chronic pain. *Psychosom Med*; 1997;59(6):597–604.3.
3. Strong J. Pain: A text book for therapists, Japanese edition. Nagoya: The University of Nagoya press 2002.
4. Raj PP, Chado HN, Angst M, Heavner J, Dotson R, Brandstater ME, Johnson B, Parris W, Finch P, Shahani B, Dhand U, Mekhail N, Daoud E, Hendler N, Somerville J, Wallace M, Panchal S, Glusman S, Jay GW, Palliyath S, Longton W, Irving G. Painless electrodiagnostic current perception threshold and pain tolerance threshold values in CRPS subjects and healthy controls: a multicenter study. *Pain Pract*. 2001;1:53–60.
5. Wallin M, Liedberg G, Börsbo B, Gerdle B. Thermal detection and pain thresholds but not pressure pain thresholds are correlated with psychological factors in women with chronic whiplash-associated pain. *Clin. J. Pain*. 2012;28:211–21.
6. Bär KJ, Brehm S, Boettger MK, Boettger S, Wagner G, Sauer H. Pain perception in major depression depends on pain modality. *Pain*. 2005;117:97–103.
7. Jiang ZC, Qi WJ, Wang JY, Luo F. Chronic administration of 5-HT1A receptor agonist relieves depression and depression-induced hypoalgesia. *ScientificWorldJournal*. 2014;2014:405736.
8. Dickens C, McGowan L, Dale S. Impact of depression on experimental pain perception: a systematic review of the literature with meta-analysis. *Psychosom Med*. 2003;65:369–75.

9. Sheng J, Liu S, Wang Y, Cui R, Zhang X. The Link between Depression and Chronic Pain: Neural Mechanisms in the Brain. *Neural Plast.* 2017;2017:9724371.
10. Crosbie TW, Packman W, Packman S. Psychological aspects of patients with Fabry disease. *J Inherit Metab Dis.* 2009;32:745–53. doi: 10.1007/s10545-009-1254-1.
11. Braff DL, Geyer MA, Swerdlow NR. Human studies of prepulse inhibition of startle: normal subjects, patient groups, and pharmacological studies. *Psychopharm (Berlin)* 2001;156:234–258.
12. Geyer MA and Braff DL. Startle habituation and sensorimotor gating in schizophrenia and related animal models. *Schizophr Bull.* 1987;13:643–668.
13. Davis M, Falls WA, Campeau S, and Kim M. Fear-potentiated startle: a neural and pharmacological analysis. *Behav Brain Res.* 1993;58:175–198.
14. Grillon C. Startle reactivity and anxiety disorders: aversive conditioning, context, and neurobiology. *Biol Psychiatry.* 2002;52:958–975.
15. Grillon C, Morgan CA, Southwick SM, Davis M, and Charney S. Baseline startle amplitude and prepulse inhibition in Vietnam veterans with posttraumatic stress disorder. *Psychiatry Res.* 1996;64:169–178.
16. Meincke U, Light GA, Geyer MA, Braff DL, and Gouzoulis-Mayfrank E. Sensitization and habituation of the acoustic startle reflex in patients with schizophrenia. *Psychiatry Res.* 2004;126:51–61.
17. Ludewig S, Geyer MA, Ramseier M, Vollenweider FX, Rechsteiner E, and Cattapan-Ludewig K. Information-processing deficits and cognitive dysfunction in panic disorder. *J Psychiatry Neurosci.* 2005;30:37–43.

18. Gallo FJ, Klein-Tasman BP, Gaffrey MS, and Curran P. Expecting the worst: observations of reactivity to sound in young children with Williams syndrome. *Res Dev Disabil.* 2008;29:567–581.
19. Madsen GF, Bilenberg N, Cantio C, and Oranje B. Increased prepulse inhibition and sensitization of the startle reflex in autistic children. *Autism Res.* 2014;7:94–103.
20. Szabo I. Analysis of the muscular action potentials accompanying the acoustic startle reaction. *Acta Physiol Hung* 1964;27: 167-178.
21. Graham FK. Distinguishing among orienting, defense, and startle reflexes. In: *The orienting reflex in humans* (Eds. H.D. Kimmel, E.H. van Olst and J.F.Orlebeke). Lawrence Erlbaum Associates Publishers, Hillsdale, New Jersey, 1979; 137-167.
22. Hoffman HS and Ison JR. Reflex modification in the domain of startle: I. Some empirical findings and their implication for how the nervous system processes sensory input. *Psychol Rev* 1980; 87: 175-189.
23. Ison JR and Russo JM. Enhancement and depression of tactile and acoustic startle reflex with variation in background noise level. *Psychobiology* 1990; 18: 96-100.
24. Seaman RL, Beblo DA, Raslear TG. Modification of acoustic and tactile startle by single microwave pulses. *Physiol Behav* 1994; 55: 587-595.
25. Stitt CL, Hoffman HS, Marsh RR, Schwartz GM. Modification of the pigeon's visual startle reaction by sensory environment. *J Comp Physiol Psychol* 1976;90: 601-619.
26. Woodworth CH, Johnson AK. Isolation, tactile startle and resting blood pressure in Long-Evans rats. *Physiol. Behav* 1988;43: 609-616.

27. Davis M. Animals models of anxiety based on classical conditionig: The conditioned emotional response (CER) and the fear-potentiated startle effect. *Pharmacol Ther.* 1990; 47: 147-165.
28. Koch M. The neurobiology of startle. *Prog Neurobiol.* 1999; 59: 107-128.
29. Geller A. Neurological effects. In: Graham, A.W., and Wilford, B.B., eds. *Principles of Addiction Medicine*. 2d ed. Chevy Chase, MD: American Society of Addiction Medicine, 1998. pp. 775–792.
30. File S. The history of benzodiazepam dependence: a review of animal studies. *Neuroscience and Biobehavioral Review.* 1990;14, 135-146.
31. Fish EW, Mckenzie-Quirk SD, Bannai M, and Miczek KA. 5-HT [1B] receptor inhibition of alcohol-heightened aggression in mice: comparison to drinking and running. *Psychopharmacology [Berl].* 2008; 197:145-156.
32. Mattila MAK and Larni HM. Flunitrazepa m : a review of itspharmacological properties and therapeutic use. *Drugs* 1980; 20 :353-374.
33. Kales A, Scharf MB, Kales JD, Soldatos CR. Rebound Insomnia. A potential hazard following withdrawal of certain benzodiazepines. *Journal of the American Medical Association*, 1979; Vol.241, No.16, 1692–5.
34. Druid H, Holmgren P, and Ahlner J. Flunitrazepam: an evaluation of use, abuse and toxicity. *Forensic Sci Int*; 2001; 122 [2–3]:136–41.
35. Hesse ML, Venkatakrisnan K, Von Moltke LL, Shader RI, and Greenblatt DJ. CYP3A4 is the major CYP isoform mediating the in vitro hydroxylation and demethylation of flunitrazepam. *Drug Metabolism & Disposition*, 2001(29), No.2, 133–40.

36. Miller RL. Drugs of abuse: a reference guide to their history and use. Westport, Conn.: Greenwood Press, P.168, 2002.
37. FarrÈ M, Teran MT and Caml J. A comparison of the acute behavioral effects of flunitrazepam and triazolam in healthy vol-unteers. *Psychopharmacology* 1996; 125 : 1-12.
38. FarrÈ M and Caml J. Pharmacokinetic considerations in abuse liability evaluation. *Br J Addict* 1991; 86 : 1601-1606.
39. Arendt RM, Greenblatt DJ, deJong RH, Bonin JD, Abernathy DR, Ehrenberg BL, Giles HG, Sellers EM, Shader IR. In vitro correlates of benzodiazepine cerebrospinal fluid uptake, pharmacodynamic action and peripheral distribution. *J Pharmacol Exp Ther.* 1993; 227: 98-106.
40. Albus U. Guide for the care and use of laboratory animals (8th edn). SAGE Publications Sage UK: London, England; 2012.
41. Benjamin B. Overview of laboratory animal lifestyle, care, and management: a case study of albino rats. *Journal of Applied Sciences and Environmental Management.* 2019;23(8):1431-5.
42. Bowery, NG. GABA receptor pharmacology. In: Sieghart W. ed. *Handbook of Experimental Pharmacology.* Berlin Springer 2002 (154); 283-33.
43. Hindmarch I, ElSohly M, Gambles J, Salamone S. Pharmacological effects of the benzodiazepine receptor agonist, flunitrazepam, on cognitive and psychomotor function. *Psychopharmacology (Berl).* 1989; 98(1):65-73.
44. Ong CK, Lirk P, Seymour RA, Jenkins BJ. The efficacy of preemptive analgesia for acute postoperative pain management: a meta-analysis. *Anesth Analg.* 2005;100(3):757-773.
45. Landis, C. and Hunt, W. 1939. *The Startle Pattern.* Farrar & Rinehart; New York, NY, USA.

46. Swerdlow NR, Geyer MA, Vale WW, Koob GF. Corticotropin-releasing factor potentiates acoustic startle in rats: blockade by chlordiazepoxide. *Psychopharmacology (Berl)*. 2000; 148(3):267-274.

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