

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 to either increase or decrease pain thresholds. Rohypnol which produces both sedative and muscle relaxant effects, is a choice drug for abusers and addicts. This study investigated the effect of 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 acclimatization under standard ethical conditions. Rats in group A served as the

control and received 0.5mL distilled water once daily. Rats in group B received 2mg/kg Diclophenac Sodium (DIC) once daily; while rats in groups C, D and E received 3mg/kg Rohypnol, once, twice and thrice daily, respectively. Treatment to rats in all groups lasted eight days. All rats received standard feed and water ad libitum throughout the study. Pain threshold was assessed using analgesy-meter and tail-clip tests; while acoustic startle reflex (ASR) response time was assessed using an acoustic bell.

The results revealed a significant increase in pain threshold in groups B and E 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) compared to the control (4.7±0.23; 5.26±0.44; 5.01±0.38; 5.06±0.30 seconds). Pain threshold in group D significantly increased on days 6 and 8 (7.00±0.12 and 7.03±0.34 seconds, respectively). An increase in ASR response time was observed in the treated rat groups compared to the control, except on day 4 where the response time in group D was significantly increased compared to the control (2.47±0.08 vs. 1.80±0.13 seconds).

This finding highlights the analgesic effect of Rohypnol in reducing pain hypersensitivity, which may be attributed to the muscle tone depression, lack of muscle alertness, and the increase in ASR response associated with the drug.

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

INTRODUCTION

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 damage [1]. 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 painful [1].

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 [6-9]. 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 sensitization [10].

The acoustic startle reaction (ASR) is a reflexive muscle contraction brought on by an abrupt loud sound. The ASR is evolutionarily conserved across mammals [11], 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 [12-19].

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 muscles [20], in addition to the arrest of ongoing activity [21]. Auditory, visual and several types of tactile stimuli were successfully used for eliciting startle [22-26]. 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 insomnia [29]. 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-anesthesia [31] and also used 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 insomnia [32]. An abrupt withdrawal of Rohypnol therapy may present a clinical condition referred to as “benzodiazepine withdrawal syndrome” characterized by Insomnia, psychosis, seizures, and anxiety [33-35].

Studies have indicated that Rohypnol consumption may leave its abusers or patients with symptoms of sluggish and uncoordinated movement of the limbs, hang-over, anterograde amnesia, woozy feelings, stomach upset, dizziness, and confusion [36]. 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 potential [37]. Although the pharmacological basis that may explain the preference for this drug by drug abusers may be 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 potential [38], which may be as a result of the lipophilic nature of the drug; thus enabling the drug to enter rapidly into the central nervous system [39].

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 drug for 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 unnecessarily calm, and has the tendency to suppress body system functions. These effects drive abusers and addicts to the excessive consumption and overdose of Rohypnol; and also serves as a choice ‘date rape drug’ for some male rapists against their vulnerable female victims; hence the choice of female rats for this study. 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.

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 and Other Materials

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 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 the 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 anxiety and/or 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 institutional 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 (Groups B – E), were compared to the

control group (Group A) using analysis of variance (ANOVA). A p-value less than 0.05 ($p < 0.05$) was considered statistically significant.

RESULTS

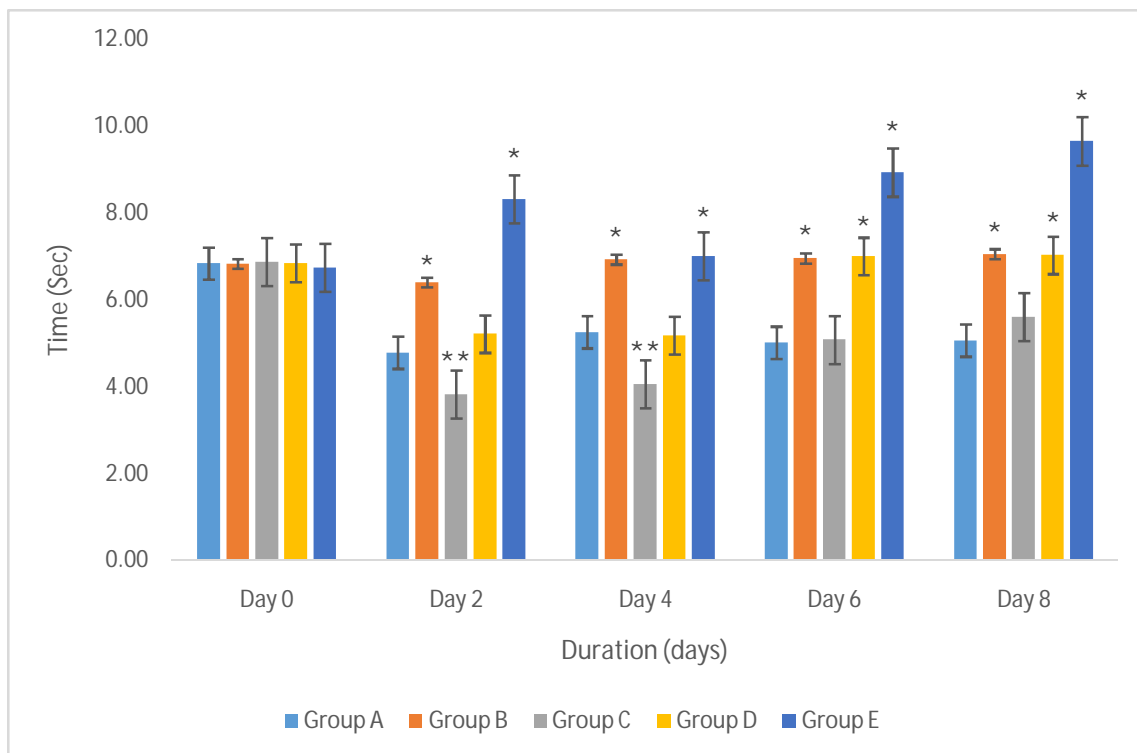


Figure 4.1: Bar Chart showing the pain threshold (in seconds) of Wistar rats on various days of the study using an Analgesy-meter.

*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.

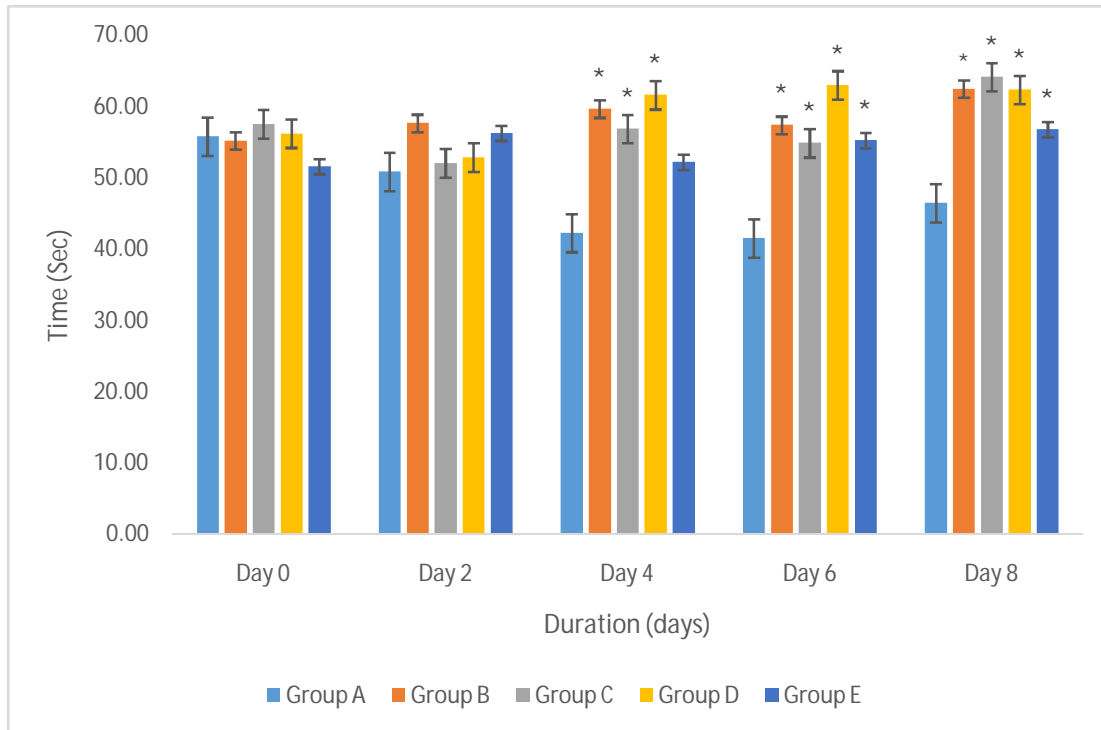


Figure 2: Bar chart showing the Tail Twitch Reaction Time (in seconds) of Wistar rats on various days of the study using a Tail Clip.

*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 result from the analgesy-meter test was used to assess the pain threshold in Wistar rats (Figure 1). The result from the study indicates that the rats in groups D and E were hyposensitive to pain and had a high pain threshold in a dose dependent manner. This possibly indicates that lower dosage administrations of Rohypnol may moderately abate pain and increase pain threshold, while higher dosage administrations of Rohypnol can significantly increase pain threshold. 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 administration [36]. The result also showed that only rats in group E had a significant increase in pain threshold when compared to the rats in group B which were treated with the standard drug (Diclophenac sodium). This increased pain threshold may be attributed to the muscle tone depressing effect of Rohypnol [35,36].

Similarly, the tail clip test revealed a significant increase 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, indicate that high doses Rohypnol leads to increased hyposensitivity to pain mainly due 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 effects [42]. 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 effects [43]. 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 unclear [44].

Effect on Acoustic Startle Reflex (reaction time)

The acoustic startle reflex (ASR) which is 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 response [45]. 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 was significantly increased when compared to the control group. 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. The impact of Rohypnol consumption on ASR as seen in this study correlates with documented clinical conditions of sluggish and uncoordinated movement of the limbs, hang-over, anterograde amnesia, woozy feelings, stomach upset, dizziness, and confusion [36], that is often seen in abusers or patients taking the drug.

These findings also confirms its effect when used as a “date rape drug” for sexual assault on women as the victims are unable to recall the incidence of their sexual encounter/assault.. 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. The major pharmacological effect of Rohypnol as a lipophilic drug is its enhancement of Gamma-aminobutyric acid (GABA) at the localized GABA receptors [35]. 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 off [36].

Although Rohypnol was seen to possess antinociceptive properties as seen from the results of the study, however caution should be applied in its use to alleviate pain due to its addictive, sedating and nervous system depressing effects.

ACKNOWLEDGEMENT

Special appreciation and recognition to Late Dr. O.J. Olorunfemi, who was instrumental in the design of this research. Our appreciation also goes to Amarachukwu Amasiatu for assisting in the laboratory.

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