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Effectiveness of Ceftriaxone and N-Acetylcysteine on Nicotine Withdrawal and Nicotine-Induced Reinstatement of Preference in Sprague–Dawley Rats

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Abstract

Objective: The research aims to compare the effectiveness of Ceftriaxone and N-acetylcysteine on nicotine withdrawal and nicotine induced reinstated preference in Sprague–Dawley rats**.**
Methodology: A total of 20 rats were used in the experiment where they were subjected to several tests such as tail immersion test, hot plate, and Conditional Place Preference (CPP) to identify the behaviors of rats when administered with the said experimental drugs during nicotine withdrawal states.
Results: There was no significant difference between the control drug, Varenicline, and the two experimental drugs. The results showed that both Ceftriaxone and N-acetylcysteine were able to eliminate the expression of somatic and nociceptic withdrawal signs in nicotine-dependent mice.
Conclusion: The results suggest that Ceftriaxone or N-acetylcysteine may be used as alternative drugs to Varenicline in nicotine cessation therapy.

Keyword/s: smoking-cessation treatment, nicotine addiction, nicotine relapse, Ceftriaxone, N-acetylcysteine, Varenicline

Introduction

The relapse rates for tobacco addiction remain high despite the proven efficacy of some current pharmacotherapies, thus indicating the need for novel or more efficacious medication approaches. Relapse is a major concern in smokers aiming to quit smoking, as most people who attempt to quit smoking experience withdrawal symptoms [1].

The primary drug used in the Philippines to address nicotine withdrawal is Varenicline. This drug is indicated for smoking-cessation treatment in adults due to its partial agonist activity at the α4β2 nicotinic acetylcholine receptor (i.e., agonist activity to a lesser degree than nicotine), while simultaneously preventing nicotine binding (i.e., antagonist activity). However, the benefits of the drug are limited by reports of serious neuropsychiatric symptoms in patients being treated with Varenicline tartrate, including depressed mood, agitation, aggression, hostility, changes in behavior, suicide related events,

including ideation, behavior, attempted suicide and suicide, as well as worsening of pre-existing psychiatric disorder. These events have occurred in patients with and without pre-existing psychiatric disorders. During treatment, alcohol intake may increase the risk of experiencing psychiatric adverse events [2].

Another drug, N-Acetylcysteine (NAC), has been shown to significantly attenuate perceived reward from smoking following an ad-lib smoking period among abstinent smokers. NAC may be most effective in treating frontostriatal circuitry function under conditions of abstinence, and thus may help to prevent relapse of nicotine use [1]. NAC is a molecule derived from the amino acid cysteine and is commonly used in the treatment of respiratory diseases, in paracetamol poisoning, and in the prevention of contrast-induced nephropathy [3]. NAC is a precursor of cysteine and of glutathione, and works as a prodrug of these molecules [4]. As a prodrug of cysteine, NAC produces interesting effects in the glutamatergic system. Extracellular cysteine generated from NAC is transported into the cell, while intracellular glutamate is transported out of the cell through the cysteine/glutamate transporter [5]. These receptors are mostly presynaptic, and inhibit glutamatergic neurotransmission and excitotoxicity. This effect of NAC and its implications for drug addiction were demonstrated in animal models, wherein the reestablishment of normal levels of extracellular glutamate has reduced relapse of drug-seeking behavior in rats chronically treated with cocaine and heroin [6].

Recently, Ceftriaxone was also reported to enhance nicotine-induced antinociception in the tail flick test and to attenuate the development of chronic nicotine tolerance in mice [7]. Ceftriaxone is a ß-lactam antibiotic drug that has a property to attenuate the reinstatement of nicotine by increasing the synthesis and membrane insertion of glial glutamate transporter-1 (GLT-1), a sodium-dependent transporter found on astrocytes that is responsible for the removal of at least 90 % of extrasynaptic glutamate. It may have something to do with alterations in the glutamate balance in the nucleus accumbens, which counteracted addiction-related disorders such as substance-induced locomotory behavior [8].

To date, the comparative effects of Ceftriaxone and NAC have not yet been established. The effectiveness of these two drugs in comparison to Varenicline also lacks sufficient scientific evidence [9]. Hence, this research aimed to compare the effectiveness of Ceftriaxone and N-acetylcysteine on nicotine withdrawal and nicotine induced reinstated preference in Sprague–Dawley rats and to assess nicotine reward using the conditioned place preference (CPP) paradigm and the physical (somatic and hyperalgesia) and affective (anxiety-related behaviors) nicotine withdrawal signs in rats.

Methodology

Experimental Animals

The researchers used Sprague–Dawley rats obtained from Benguet State University, School of Veterinary Medicine. Sprague–Dawley rats are an outbred multipurpose breed of albino rat used extensively in medical research. Aside from being easily accessible and cost effective, the rats are known for their calmness and ease of handling. Other benefits of using the rats are their genetic standardization, which assures experimental reproducibility [9].

A total of twenty (20) Sprague-Dawley rats were used and grouped by fives (5) in metal cages with a bed of wood shavings and paper shreds. The subjects were naive 8- to 10-week-old male rats that weighed between 180g and 200g at the beginning of the experiment. Only a single gender (male) was utilized in the study to avoid unnecessary reproduction and pregnancies that may interfere with the experiment results. The colony was subjected to a 12:12-hour light–dark cycle. They were placed in a controlled environment with proper ventilation and a heat source that aided in maintaining a temperature of 23 to 27C. Food and water were available *ad libitum*.

Drugs, chemicals, and administration

Varenicline (Champix®) 0.5mg tablet; Ceftriaxone (Forgram®) 1g powder; and N-acetylcysteine (NAC) (Fluimucil®) IV solution for injection were all obtained from a local drugstore.

Varencline and Ceftriaxone were separately dissolved and freshly prepared as needed, in physiological saline (0.9 % sodium chloride) in accordance with their respective titer volumes (Jenkins, 1998). 0.9% sodium chloride as a solvent have been found suitable in most instances and does not greatly affect drug action because of its inert property and inherent physiologic property upon subcutaneous injection. Although distilled water can be used under certain conditions, saline is preferable because water *ad injectionem* injected subcutaneously causes pain, and intravenously may cause hemolysis [10]. Test substances, solutions and equipment were all prepared aseptically and kept free from pyrogens.

Ceftriaxone and NAC were the experimental control, while Varenicline served as the positive control. As a negative control, 0.9% sodium chloride solution was used. All drugs including 0.9% sodium chloride were subcutaneously (s.c) injected [11]. Subcutaneous injection is the best option when a relatively long period of absorption is desired [12].

Overall, subcutaneous injection is the preferred method for the administration of substances into rats. This is due to the simplicity of the injection technique, greater choice of injection sites and possibility of depositing large volumes. The maximum volume at each site should be 1mL per 100mg body weight [13].

Withdrawal studies were conducted as described by Jackson, 2016. All rats received nicotine (2 mg/kg/day) at a consistently set time of day. Nicotine was administered with increasing dose every five (5) days for a maximum of fifteen (15) days to achieve the desired effect. Experimental variables Ceftriaxone and NAC, were separately injected in a respective cohort of rats (double-blind study) with the same dose of 90mg/kg s.c.; 90mg/kg 0.9% sodium chloride s.c. was also injected in another respective group. The control Varenicline was administered at a dose of 0.03mg/kg s.c. which is its established effective dose [3]. The said variables were injected once a day at a consistently set time of day starting on the 15thday for 7 days.

Nicotine Controlled Place Preference Studies

Using the unbiased mouse CPP paradigm, two chambers consisted of two distinct compartments separated by a smaller intermediate compartment with openings that allowed access to either side of the chamber. On day 1, animals were confined to the intermediate compartment for a 5-minute habituation period and then allowed to move freely between compartments for 15 minutes. Time spent in each compartment was recorded. These data were used to separate the animals into groups of approximately equal bias [14].

Days 2–14 were conditioning days during which the saline group receives saline in both sides of the boxes and with the drug groups receiving nicotine on one of the sides and saline on the opposite side. Drug-paired sides were randomized among all groups. Conditioning lasted for 14 days, with animals in the drug group receiving nicotine each day.

Nicotine-paired compartments were randomized among all groups. Day 15 was the drug-free test day and the procedure was done in the same way as day 1. Time spent on each side was measured and the preference score was expressed as time spent on the nicotine-paired side post-conditioning minus time spent on the nicotine-paired side preconditioning. A positive number indicates a preference for the nicotine-paired side, whereas a negative number indicates an aversion to the nicotine-paired side. A number at or near zero signifies no side preference.

Nicotine withdrawal assessment

Withdrawal studies were conducted. Specified rats received nicotine for 15 consecutive days. On day 37, specified rats were injected with Varenicline, Ceftriaxone, N-acetylcysteine, or saline solution for 15 consecutive days. Every after administration of the said drugs, rats injected with Varenicline, Ceftriaxone, NAC and saline solution, testing were initiated 15 minutes later. The rats were first evaluated for 5 minutes in the elevated plus maze test for anxiety-related behavior, followed by a 20-minutes observation of somatic signs measured as paw and body tremors, head shakes, backing, jumps, curls, and ptosis [15].

Hyperalgesia was evaluated by the hot plate test and tail flick test immediately following the somatic sign observation period. The testing sequence was chosen based on the study by Jackson et al. (2008) that this order of testing

reduced within-group variability and produced the most consistent results. An observer blinded to the experimental treatments of Varenicline, Ceftriaxone, or N-acetylcysteine scored behavior and analyzed the data [15].

Tail Immersion Test

The tail immersion measures the latency for the rat to remove its tail away from a heated liquid [15]. The rat was restrained in an opaque cylinder. Ventilation was made on top and in front of the tube. The rat's tail protruded from the back of the tube and was placed in a liquid that was heated to a specified temperature of 48 °C. The protruding 2/3 end of its tail was dipped into the water bath and the chronometer was started. Stopping of the chronometer occurred as soon as the mouse withdrew its tail from the hot water. The latency time was recorded in seconds. In the absence of any nociceptive reaction, a 25 second cut-off was used to prevent tissue damage.

Hot Plate Analgesia Assessment

The hot-plate method is a test of the pain response in animals and measures a rat's latency to lick a paw or jump. Rats were confined to the hot plate's surface in a chamber with a clear Plexiglas lid. Two observers, blind to the rats' group condition, recorded the time, to the nearest hundredth of a second, each rat's latency to either lick a paw or jump, whichever came first. The response latency is the mean of the two observations. Animals that neither licked a paw nor jumped after 45 second were removed from the apparatus to prevent tissue damage [14].

Evaluation of Somatic Signs Withdrawal

Twenty minutes after injection of both saline and nicotine, each rat was placed in a transparent plastic container where the rat could move around freely. For the observation phase, a single blind set up was applied in which the observer had no knowledge of the substances injected to the rats being studied. The frequency and time of occurrence of the following signs were recorded: body shakes, chews, escape attempts, foot licks, genital licks, head shakes, scratches, and teeth chattering derived from a checklist of nicotine abstinence signs. Multiple successive counts of any signs require a distinct pause between episodes indicates nicotine addiction. The total number of somatic signs per ten-minute observation period was defined as the sum of the individual occurrences of the above mentioned withdrawal signs. Subjects were habituated to the observation room and containers for ten minutes over three days before the first antagonist injection [16].

Nicotine-primed reinstatement of nicotine CPP

A nicotine CPP reinstatement paradigm was utilized. Three individual cohorts of five rats were tested in the CPP paradigm and classified into group A, group B, and group C. On day 23, 1 day after test day, which was previously described, the initial test day procedure was repeated in the groups for six additional days to measure extinction of nicotine CPP. All rats received saline injections prior to being placed in the chambers on these days. [14].

During the six days of extinction, animals received Varenicline, Ceftriaxone, or NAC 10 minutes before the saline injection. On the reinstatement test session on day 30; rats received an injection of nicotine and were immediately placed into the middle chamber for the evaluation of nicotine CPP reinstatement [7].

Pre-habituation phase

After a week of accommodation to the colony room, rats were weighed once daily for 3 days, weighed twice daily for three more days, and then weighed and injected with saline daily for 8 days. All of these procedures took place in the colony room and were intended to reduce the discriminative salience and stress-inducing effects of injections and handling procedures [7].

Allocating animals' experimental group

Using the unbiased mouse CPP paradigm, two chambers consisted of two distinct compartments separated by a smaller intermediate compartment with openings that allowed access to either side of the chamber. On day 1, animals were confined to the intermediate compartment for a 5-minute habituation period and then allowed to move

freely between compartments for 15 minutes. Time spent in each compartment was recorded. These data were used to separate the animals into groups of approximately equal bias [7].

Experimental outcomes

Nicotine withdrawal assessment

Withdrawal studies were conducted. Specified rats have received nicotine for 15 consecutive days. On day 37, specified rats were injected with Varenicline, Ceftriaxone, N-acetylcysteine, or saline solution for 15 consecutive days. Every after administration of Varenicline, Ceftriaxone, NAC and saline solution in the respective rat cohorts, testing was initiated 15 minutes later. The rats were first evaluated for 5 minutes in the elevated plus maze test for anxiety-related behavior, followed by a 20-minutes observation of somatic signs measured as paw and body tremors, head shakes, backing, jumps, curls, and ptosis [15].

Hyperalgesia was evaluated in the hot plate test and tail flick test immediately following the somatic sign observation period. The testing sequence was based on the study by Jackson et al. (2008) which was shown to reduce within-group variability and produced the most consistent results. An observer blinded to the experimental treatments of Varenicline, Ceftriaxone, or N-acetylcysteine scored behavior and analyzed the data [15].

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Data Analysis

Descriptive analysis was used to describe changes in the behavior of the rats. Inferential analysis of all behavioral studies was then done through independent *t*-test and an analysis of variance (ANOVA) with the post hoc Newman-Keuls test when appropriate. p values of <0.05 were considered statistically significant [7].

Results

Phase 1: Addiction Phase

Nicotine addiction was established in the rats exposed to nicotine injections. Table 1 shows that, compared to the saline group, the nicotine group was effectively conditioned to produce a robust and significant CPP. A *t*-test was conducted to compare the average time the subjects from the saline group stayed in the nicotine box as opposed to the nicotine-exposed group. There was a significant difference in the times for the saline group (M = 148; SD = 31.161) and the nicotine group (M = 238; SD = 48.19); t(18) = 3.876, p = 0.001.

Table 1. Mean time of the rats in the nicotine/ intervention box (N=20)

Group	N	Average Time (seconds)
Saline	5	148*
Nicotine	15	238*
*p = 0.001		

Tests on nociception were found to be similar among all groups during induction of nicotine addiction. Table 2 shows the average reaction time each group had for each test. An independent *t*-test was performed to compare the two groups and no significant difference was found for the tail immersion test for the saline group (M = 10.68; SD = 2.22) and the nicotine group (M = 10.94; SD = 1.84); t(18) = -0.238, p = 0.815, as well as for the hot plate test for the saline group (M = 22.21; SD = 3.04) and the nicotine group (M = 23.71; SD = 5.72); t(18) = -0.763, p = 0.455.

Table 2. Average reaction times for the Tail Immersion and Hot Plate tests (N=20)

Group	n	Tail Immersion Test (Seconds)	Hot Plate Test (Seconds)
Saline	5	10.68	22.21
Nicotine	15	10.94	23.71
		p = 0.815	*p = 0.455*

Phase 2: Treatment & Withdrawal Phase

The experimental groups showed similar behaviors on the elevated plus maze test compared to the control group. There was no significant difference between the open arm times of all experimental groups and the control group at the $p<0.05$ level [F(3, 16) = 1.999, p = 0.155]. Also, there were no significant differences between all groups for their first anxiety-related response on the elevated plus maze [F(3, 16) = 0.474; p = 0.705] and the average number of somatic signs [F(3, 16) = 1.793; p = 0.189].

Table 3. Average behaviors on the elevated plus maze (N=20)

Group	n	Open Arm Time (Seconds)	Time until first response (Seconds)	Number of Somatic Signs (Average)
Saline	5	43.27	77.01	17
Varenicline	5	28.36	60.98	22
Ceftriaxone	5	30.61	55.70	28
N-acetylcysteine	5	24.59	66.04	32
		p = 0.155	*p = 0.705*	*p = 0.189*

Tests on nociception were found to be similar among all groups during the treatment phase. No difference was found in the nociceptive responses between the control group and experimental groups as seen in the one-way ANOVA for the tail immersion test [F(3, 16) = 2.472; p = 0.099] and the hot plate test [F(3, 16) = 1.380; p = 0.285].

Table 4. Average reaction times for the Tail Immersion and Hot Plate tests (N=20)

Group	n	Tail Immersion Test (Seconds)	Hot Plate Test (Seconds)
Saline	5	10.67	17.46
Varenicline	5	8.74	25.93
Ceftriaxone	5	8.05	18.08
N-acetylcysteine	5	8.30	25.96
		p = 0.099	*p = 0.285*

Phase 3: Relapse Phase

The drugs given to the experimental groups were able to block relapse behavior in the nicotine-primed CPP. Relapse, seen as a form of drug-seeking behavior, was elicited through a nicotine-primed CPP. Table 5 shows that there was no significant difference seen in the average times within the nicotine box when the saline group and the experimental groups were compared using a one-way ANOVA [F(3, 16) = 0.737; p = 0.545].

Table 5. Mean time of the rats in the nicotine/ intervention box post-treatment (N=20)

Group	N	Average Time (seconds)
Saline	5	310.40
Varenicline	5	204.40
Ceftriaxone	5	167.40
N-acetylcysteine	5	264.20

**p = 0.545*

During the course of the study, nicotine administration brought about excessive stimulation of the CNS, which resulted in seizure-like episodes, and temporary loss of gross motor control, seen as inability to move hind limbs, which the rats recovered from within 5-10 minutes after nicotine administration. These adverse effects required the researchers to make adjustments to the routine of the experiment to allow the research animals to regain motor function before testing could resume for the day. No adverse effects were seen during the administration of Varenicline, Ceftriaxone, or N-acetylcysteine.

Discussion

The present study investigated the comparative effects of Ceftriaxone and N-acetylcysteine to Varenicline on the behavioral aspects of nicotine addiction induced in rats, mimicking the characteristics of nicotine dependence in humans.

The study shows that Ceftriaxone was able to eliminate the expression of somatic and nociceptic withdrawal signs in nicotine-dependent rats. Furthermore, it was noted that the drug attenuates the relapse of nicotine preference in rats. These findings were similar to the study conducted by Alajaji et al. in 2013, which postulated two possible hypotheses, particularly cysteine–glutamate-exchanger and the GLT-1 in the brain. Since xCT and GLT-1 are down-regulated in the nucleus accumbens by nicotine, the present data support their potential relevance in the development and/or expression of dependence. System xc- and glutamate transport via GLT-1 occur predominantly in glia [17]; these proteins may be co-regulated [18].

In the study conducted by Sondheimer and Knackstedt in 2011, the attenuation was due to the restoration of GLT-1 expression in the nucleus accumbens of rats although the study was conducted with the use of cocaine as inducer of addiction. The same results were seen in the study conducted by Sari in 2014 using alcohol as the inducer of addiction. Additionally, in 2009, Knackstedt et al. posited that nicotine administration reduces the expression of GLT-1, a sodium-dependent transporter found on astrocytes that is responsible for the removal of extrasynaptic glutamate [19]; thus, with the restoration of this pathway, the prolonged effect of glutamate in nicotine addiction is decreased [20]. Nicotine stimulates the release of glutamate through activation of nAChRs located on glutamatergic terminals in several brain regions including the ventral tegmentum area [19]. In turn, glutamate is the major excitatory neurotransmitter in the brain that plays a critical

role in the acute and long-term effects of nicotine [21], which explains the attenuation behavior observed in this study. Another potential pathway is the cysteine–glutamate exchanger also known as the *system xC*−, which is responsible for the exchange of extracellular cystine for intracellular glutamate, the rate-limiting step in glutathione synthesis and the main source of extracellular glutamate [20].

In the study, the effect of NAC has been found to be comparable to that of Varenicline, too. No significant difference was noted in the results of the different tests to assess nicotine withdrawal syndrome. This result corroborated the findings of Froeliger et al. in 2015 on the effects of N-acetylcysteine compared with that of a placebo drug in healthy adult smokers. The NAC group reported less nicotine-withdrawal symptoms and maintained abstinence [22].

The study also shows that Ceftriaxone and NAC prevented nicotine relapse as evidenced by the absence of any significant difference in the average times within the nicotine box when the saline group and the experimental groups were compared using a one-way ANOVA. This finding corroborates the study of Schmaal et al. in 2011 where heavy smokers served as subjects and were randomized to receive either placebo or NAC. The subjects were asked to stop smoking and report on nicotine craving and withdrawal symptoms. After 3.5 days of withdrawal, the NAC group reported that their cigarette smoking experience after an abstinence period was less rewarding. This finding led them to conclude that NAC might be a promising new treatment option in preventing nicotine relapse [19].

The effect of NAC on rats could be related to its capacity to inhibit dopamine release and reduce synaptic release of glutamate by increasing extracellular levels of glutamate. NAC has been shown to inhibit dopamine release, therefore reducing reward behavior, as dopamine is one of the neuromodulators that has been shown to be involved in the reward system [24].

NAC inhibits the glutamate homeostasis involved in the xc- system, which allows an increase in extracellular glutamate levels. This increase appears to reduce the synaptic release of glutamate, thus normalizing extracellular glutamate dysregulation. NAC is a GSH precursor which is an anti-oxidant allowing potentiation of brain NMDA receptor to respond to glutamate in rats. This condition therefore causes reduced cravings and reward behavior [17, 23]

There are some limitations to the findings of this study though. First, the short amount of time utilized to induce nicotine exposure might not be representative of an adult who is a moderate to heavy smoker. Second, the length of NAC and Ceftriaxone treatments were relatively short; longer periods of treatment might be needed to reach steady-state levels for both drugs. Finally, generalization of the results of this study must be limited to Sprague-Dawley rats, as further investigations are needed to establish the effects of Ceftriaxone and N-acetylcysteine in humans.

In conclusion, the effectiveness of Ceftriaxone and N-acetylcysteine were comparable based on nicotine withdrawal using Varenicline and nicotine-induced reinstated preference in Sprague–Dawley rats. Altogether, N-acetylcysteine was found to be successful in inhibiting the relapse potential of nicotine addiction. Likewise, it was able to reverse nicotine signs measured in the rats by inhibiting dopamine release, resulting in reduced cravings and reward behavior seen in the rats.

The effect of N-acetylcysteine is comparable to Ceftriaxone in addressing nicotine withdrawal. There is no significant difference between the effects of Ceftriaxone or N-acetylcysteine on nicotine withdrawal and induced preference. Moreover, the results showed that both Ceftriaxone and N-acetylcysteine were able to eliminate the expression of somatic and nociceptic withdrawal signs in nicotine-dependent mice. However, Ceftriaxone as a beta-lactam antibiotic presents a more problematic choice in mitigating nicotine withdrawal due to the risk of drug resistance. Thus, the researchers recommend further clinical trials using N-acetylcysteine since it shows promise as a cheaper and safer drug in managing nicotine addiction.

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