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Effect of nicotine withdrawal on pain sensitivity in rats to mechanical stimulation and thermal stimulation

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Abstract. – OBJECTIVE: To establish an improved rat model of nicotine withdrawal and dependence by subcutaneous injection of pure nicotine, and observe the effect of nicotine withdrawal on the pain sensitivity in rats.

MATERIALS AND METHODS: 30 SD rats were randomly divided into 5 groups with 6 rats in each group, including the control group, normal saline group (NS group), nicotine group of 3 mg/kg/d (NT3 group), nicotine group of 9 mg/kg/d (NT9 group) and nicotine group of 18 mg/kg/d (NT18 group). The 5 groups were respectively subcutaneously injected with nothing, normal saline, 1 mg/kg nicotine, 3 mg/kg nicotine and 6 mg/kg nicotine with 3 times per day for 7 consecutive days. 60 min after last injection in the 7th d, 1 mg/kg mecamylamine was subcutaneously injected. The body weight change, survival and nicotine withdrawal score of rats were observed during injection of nicotine and after withdrawal. Mechanical withdrawal threshold (MWT) and Thermal withdrawal latency (TWL) in the right hind sole of another 18 rats selected from the control group, NS group and NT9 group (6 rats from each group) were respectively tested in 7d after injection of normal saline or nicotine.

RESULTS: Compared with the NT3 group, the body weight of rats in the NT9 group and NT18 group were slowly increased in 7d after injection of nicotine ($p < 0.05$), but were rapidly increased in 1d and 2d after withdrawal ($p < 0.01$). Rats in the NT9 group and NT18 group had more withdrawal symptoms after stimulation with mecamylamine ($p < 0.01$), but the mortality of rats in the NT18 group reached 17%. Compared with the control group, MWT in the rats of the NT9 group were significantly decreased in 1-7d after nicotine withdrawal ($p < 0.01$), and were particularly significantly decreased in 1d and 2d ($p < 0.01$); TWL was also significantly decreased ($p < 0.01$), and was most significantly decreased in 4d ($p < 0.01$).

CONCLUSIONS: An improved rat model of nicotine dependence and withdrawal can be successfully established by intermittent subcutaneous injection of nicotine at 9 mg/kg/d for 7 days, and the pain sensitivity in rats is increased after nicotine withdrawal.

Key Words:
Nicotine, Mecamylamine, Dependence, Withdrawal, Pain.

Introduction

The harm of tobacco is a global public health problem, and it is estimated that 1/3 of adults smoke in the world. Clinically, long-term smokers have increased risks of suffering from lumbago and back pain; 48h after coronary bypass, smokers need opioid drugs 33% more than non-smokers; after gynecological operation, smokers need more opioid drugs than non-smokers[1]. It is very necessary to establish an animal model of nicotine dependence and withdrawal to study its specific mechanism. In the 1990s, Malin et al[2,3] established a rat model of nicotine dependence and withdrawal through continuous infusion of nicotine at 9 mg/kg/d with a subcutaneously embedded automatic infusion pump. But this model is questioned and restricted due to two reasons: one is that stable plasma concentration of nicotine can be achieved through the automatic infusion pump, but in reality there are no uninterrupted smokers; the other is that the embedded automatic infusion pump is very expensive. Accordingly, this study is provided to establish an improved rat model of nicotine dependence and withdrawal through intermittent subcutaneous injection of nicotine for 7d and then withdrawal, and observe the change of pain sensitivity in rats to plantar mechanical stimulation and thermal stimulation, so as to lay the foundation for further study on the mechanism of pain sensitivity changes after nicotine withdrawal.

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Materials and Methods

Animal Selection

Healthy male Sprague Dawley (SD) rats of clean grade with the body weight of 150-200 g (provided by Shandong Institute of Pharmaceutical Industry) and license number of SCXK (Shandong) 20080002. The animals were cultured at room temperature of 24±2°C and humidity of 50%, and with circadian rhythm alternation, free food intake and water intake. This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The animal use protocol has been reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) of Liaocheng People's Hospital, and the operation followed the requirements of laboratory animal ethics.

Establishment of an Improved Rat Model of Nicotine Dependence and Withdrawal

30 healthy male SD rats were randomly divided into 5 groups using a random number table. (1) Control group (n = 6): The rats were cultured under the above conditions without any treatment, followed by subcutaneous injection of 1 mg/kg mecamylamine (produced by Sigma Company, St Louis, MO, USA) 7d later; (2) Normal saline group (NS group, n = 6): The rats were cultured under the above conditions, and were respectively subcutaneously injected with normal saline at 5:00, 13:00 and 21:00 every day with 0.1 ml each time and 3 times/day for 7 consecutive days. 60 min after last injection in 7 d, 1 mg/kg of mecamylamine was subcutaneously injected; (3) Nicotine group of 3 mg/kg/d (NT3 group, n = 6): Rats were cultured under the above conditions, and were respectively subcutaneously injected with nicotine (Sigma Company, St Louis, MO, USA) at the above 3 time every day with 1 mg each time and 3 times/day for 7 consecutive days. 60 min after last injection in 7 d, 1 mg/kg of mecamylamine was subcutaneously injected; (4) Nicotine group of 9 mg/kg/d (NT9 group, n = 6): Rats were cultured under the above conditions, and were respectively subcutaneously injected with nicotine at the above 3 time every day with 1 mg each time and 3 times/day for 7 consecutive days. 60 min after last injection in 7 d, 1 mg/kg of mecamylamine was subcutaneously injected; (5) Nicotine group of 18 mg/kg/d (NT18 group, n = 6): Rats were cultured under the above conditions, and were respectively subcutaneously injected with nicotine at the above 3 time every day with 6 mg each time and 3 times/day for 7 consecutive days. 60 min after last injection in 7 d, 1 mg/kg of mecamylamine was subcutaneously injected.

Behavioral Observation and Scoring

Mecamylamine was injected with blinding method without knowing the group of the rat. The withdrawal symptoms of rats in each group after stimulation with mecamylamine were improved according to the scoring standard of Malin et al[2], and then respectively scored. Observers count the frequency of following symptoms within 15 min, including chewing/interlocking tooth; writhing/pant; trembling/tremor; and other symptoms (licking foot, scratching and yawning), the frequency of which was respectively divided by 10 to get a score; mild, medium and heavy blepharoptosis was respectively scored as 1, 2 and 3.

All rats were respectively weighed after scoring of the withdrawal symptoms every day. The difference between their body weight every day and that last day was recorded, and this difference represented their body weight changes. Meanwhile, their behavioral performance was observed after injection of nicotine each time.

Determination of Pain behavior in Rats of Nicotine Withdrawal

18 additional rats were selected from the control group, NS group and NT9 group with 6 rats from each group. Since the next day after stopping injection of nicotine, the mechanical withdrawal threshold (MWT) and thermal withdrawal latency (TWL) were determined in 8:00AM-12:00AM every day for 7 consecutive days. Rats were placed in the test environment 30min before test every day. The MWT was first tested, and then TWL was tested. The blind method was used in all behavioral tests.

The determination of MWT: BME-404 electronic mechanical stimulator (produced by Institute of Biomedical Engineering, Chinese Academy of Medical Science at Tianjin, China) was used with the main technical parameters as follows: end face diameter of the test probe: 0.6 mm, force measuring range: 0.1-50 g, force resolution: 0.05 g. An organic glass box (26 cmx20 cmx14 cm) was placed on the sieve of a metal frame. After the rats were adapted to the organic glass box for 30 min, the test probe on the measuring handle was pointed at the right hind sole of rats, until they had avoidance behavior. Each time the record was kept for 1 min, and the recorded data were auto-

matically saved. Each rat was tested 5 times with the time interval of at least 5 min. The arithmetic mean of the measured values was the measured MWT.

The determination of TWL: BME-410C full-automatic thermal pain stimulator (produced by Institute of Biomedical Engineering, Chinese Academy of Medical Science at Tianjin, China) was used with the main technical parameters as follows: 12V/10W halogen lamp with the stimulating light area of less than 20 mm^2, timing accuracy of 10ms and stimulating temperature of 45-65°C (adjustable). Main measuring unit was placed on the desktop, and the lamp was placed under the experimental bench. The organic glass box was placed on a glass plate of 3 mm thick. The rats were respectively placed in spaced glass boxes and were adapted for 30 min. The calibrated light was pointed at the skin of the right hind sole in rats, and then the button on the control handle was pressed down. The timing was started since the lamp gave off strong stimulating light, and was stopped until the animals had avoidance behavior. The time thus obtained was the latent period. The test was repeated 5 times with the time interval of at least 5 min. The average value thereof was the measured TWL.

Statistical Analysis

SPSS13.0 statistical software (SPSS Inc., Chicago, IL, USA) was used for analysis, and the measurement data were expressed as the mean± standard deviation (±*s*). Repeated measures analysis of variance was used for intragroup comparison, and multivariate analysis of variance was used for intergroup comparison. $p < 0.05$ represented statistically significant difference.

Results

Behavioral Performance of Rats after Subcutaneous Injection of Nicotine

Rats in the NS group and NT3 group did not have behavioral changes after respective subcutaneous injection of normal saline and nicotine at 3 mg/kg/d; all rats in the NT18 group had nicotine poisoning symptoms after subcutaneous injection of nicotine at 18 mg/kg/d, such as convulsion and shortness of breath, and 1 rat died in 7 days after continuous injection with the mortality of 17%. Rats in the NT9 group showed transient mild shortness of breath after subcutaneous injection, but they can soon walk freely with free food intake and water intake.

Body Weight Changes of Rats During Nicotine Injection and in 2d after Withdrawal

During nicotine injection and in 1d and 2d after nicotine withdrawal, the body weight of rats in the control group and normal saline group was increased by about 8-10 g every day. Compared with the NT3 group, the body weight of rats in the NT9 group and NT18 group was obviously slowly increased in 7 d after nicotine injection ($p < 0.05$), and was rapidly increased in 1 d and 2 d after withdrawal ($p < 0.01$) (Figure 1).

Comparison of Nicotine Withdrawal Symptoms in Rats after Stimulation with Mecamylamine

Compared with the rats in the NT3 group, rats in the NT9 group and surviving rats in the NT18 group showed more behaviors and symptoms as follows after stimulation with mecamylamine: chewing/inter-

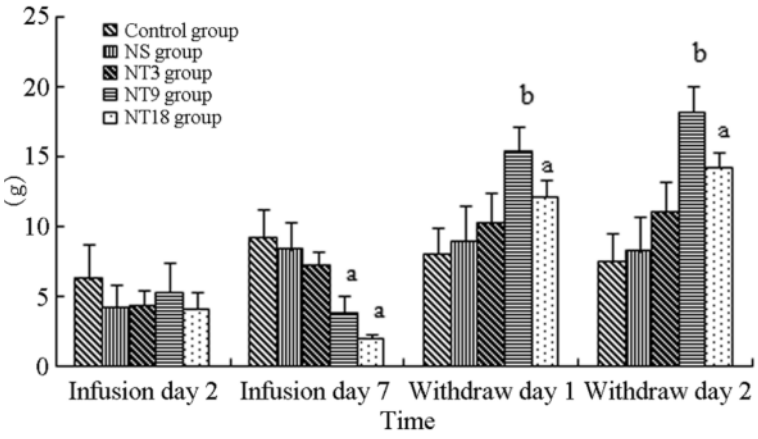

Figure 1. Body weight changes of rats during continuous injection of nicotine and after withdrawal. Note: compared with NT3 group, $^{a}p < 0.05$ $^{b}p < 0.01$.

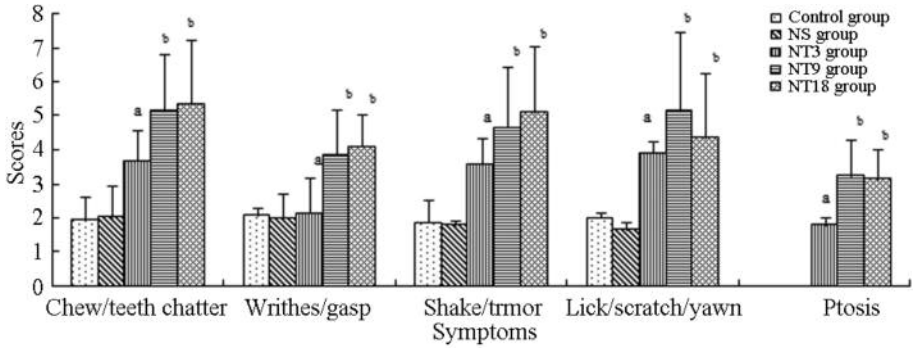

Figure 2. Comparison of scoring of nicotine withdrawal symptoms in rats after stimulation with mecamylamine. Note: compared with the control group, $^{a}p < 0.05$; compared with the NT3 group, $^{b}p < 0.05$.

locking tooth ($p < 0.01$); writhing/pant ($p < 0.01$); trembling/tremor ($p < 0.01$); licking feet, scratching and yawning symptoms ($p < 0.01$); and all rats in the NT9 group and NT18 group showed severe blepharoptosis, which was significantly more severe than that in the NT3 group ($p < 0.01$). The above withdrawal symptoms in the NT9 group were not different from those in the NT18 group ($p > 0.05$), and the above withdrawal symptoms in the rats of the NT3 group were not different from those in the rats of the control group and NS group ($p > 0.05$) (Figure 2).

Changes of MWT in Rats after Nicotine Withdrawal

Compared with the control group, MWT at the sole of the rats in the NT9 group was significantly decreased in 1-7 d after nicotine withdrawal ($p < 0.01$). The intragroup comparison showed that MWT in the rats of the NT9 group was more significantly decreased in 1 d and 2 d after withdrawal ($p < 0.01$) (Figure 3).

Changes of TWL in Rats after Nicotine Withdrawal

Compared with the control group, TWL at the sole of the rats in the NT9 group was significantly decreased in 1-7 d after nicotine withdrawal ($p < 0.01$). The intragroup comparison showed that TWL in the rats of the NT9 group was more significantly decreased in 4 d after withdrawal ($p < 0.01$) (Figure 4).

Discussion

This study found that an improved rat model of nicotine dependence and withdrawal can be successfully established by subcutaneous injection of nicotine at 9 mg/kg/d with 3 times per day for 7 consecutive days. If the dose was increased to 18 mg/kg/d, the rat mortality would be increased, while if the dose was reduced to 3 mg/kg/d, it would not be enough to cause obvious symptoms of nicotine withdrawal. This study also found that MWT in rats was significantly decreased in 1 d and 2 d after nicotine withdrawal, and TWL in rats was significantly decreased in 4 d after nicotine withdrawal.

At present, the harm of tobacco is one of the most serious public health problems in the world. In all dead female British smokers, 2/3 died due to smoking, and the life of smokers was shortened by at least 10 years[4]. Smoking not on-

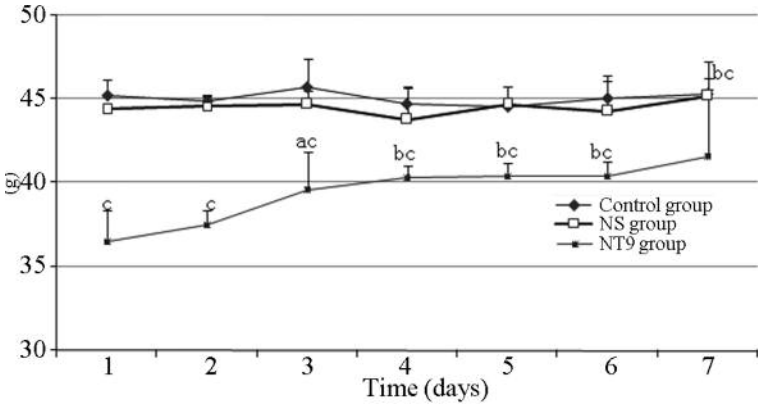

Figure 3. MWT changes in rats after nicotine withdrawal. Note: compared with 1d after withdrawal $^{a}p < 0.05$ $^{b}p < 0.01$, compared with the control group, $^{c}p < 0.01$.

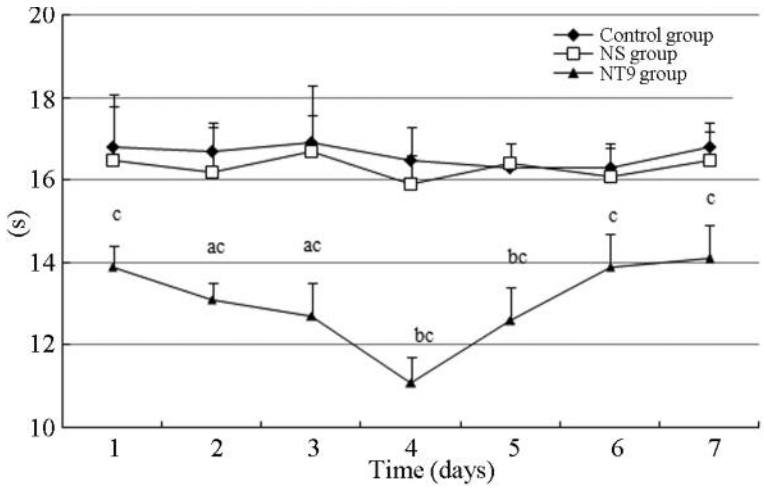

Figure 4. Plantar TWL changes in rats after nicotine withdrawal. Note: compared with 1d after withdrawal $^{a}p < 0.05$ $^{b}p < 0.01$, compared with the control group, $^{c}p < 0.01$.

ly is related to the occurrence of severe perioperative complications, but also increases the occurrence of chronic pain and needs more postoperative analgesic opioids[5-7]. Establishment of the animal model of nicotine dependence and withdrawal is of great significance for studies on the physiological function changes after nicotine withdrawal. There are many kinds of animal models of nicotine dependence and withdrawal[8]. The model used for the studies of chronic pain under the conditions of nicotine dependence and withdrawal was proposed by Malin et al[3]. Nicotine was infused at 3 mg/kg/d and 9 mg/kg/d for 7 consecutive days through a subcutaneously embedded automatic infusion pump. After withdrawal, rats in the nicotine group of 9 mg/kg/d showed more nicotine withdrawal symptoms, while those in the group of 3 mg/kg/d did not show symptoms. Subsequent researches showed that after continuous infusion of nicotine at 9 mg/kg/d, the plasma concentration of nicotine is similar to that in a heavy smoker who smokes 40 cigarettes every day. The model can achieve stable plasma concentration, but uninterrupted smoking all day long does not exist in daily life, and the subcutaneously embedded automatic infusion pump used in the experiment is expensive. This study tries to establish an improved rat model of nicotine dependence and withdrawal through subcutaneous injection of nicotine at 3, 9 and 18 mg/kg/d with 3 times per day.

Nicotine dependence and withdrawal is judged by the symptoms of nicotine withdrawal, which are similar to opioid withdrawal syndromes, including chewing/ interlock tooth; writhing/pant; trembling/tremor; licking foot, scratching, yawning and severe blepharoptosis etc. Meanwhile, rats also showed reduced appetite and slow body weight gain during the nicotine injection, and rapid weight gain after nicotine withdrawal[9]. The above withdrawal symptoms can be stimulation by subcutaneous injection of antagonist mecamylamine, a nicotinic acetylcholine receptor (nAChR)[2]. This study found that after intermittent subcutaneous injection of nicotine in rats at 9 and 18 mg/kg/d for 7 consecutive days, the withdrawal symptoms were not different from those after stimulation by subcutaneous injection of mecamylamine at 1 mg/kg, but the mortality was as high as 17% in rats after subcutaneous injection of nicotine at 18 mg/kg/d. The death may be caused by poor appetite and malnutrition, and may also be caused by poisoning due to overdose of nicotine. However, withdrawal symptoms cannot be stimulated by mecamylamine in rats with subcutaneous injection of 3 mg/kg/d. According to the withdrawal symptoms in rats, the infusion of nicotine at constant rate of 9 mg/kg/d was changed to subcutaneous injections of 3 times/d, so that it was simple and easy to successfully establish an improved rat model of nicotine dependence and withdrawal in 7 consecutive days. The weakness of this study was that the plasma concentration of nicotine was not monitored during subcutaneous injection.

Tobacco includes more than 4000 chemical substances. Animal and human experiments showed that nicotine is the major substance for pain control[10,11]. Nicotine exposure has great influence on the central and peripheral nervous

system, and it exerts its role through combination with nAChRs, which can penetrate Na^+, Ca^{2+} and K^+. Postsynaptic activation of nAChRs has direct effect on excitatory neurons by cation channels. The presynaptic activation of nAChRs can affect the release of other neurotransmitters, including dopamine, γ-aminobutyric acid (GABA), glutamic acid, 5-hydroxytryptamine (5-HT), histamine and noradrenaline[1]. All above researches are provided to study the pain mechanism after nicotine withdrawal in the light of the anti-noxious stimulation of nicotine and mechanism of anti-noxious stimulation, and are of more clinical significance. Clinically, long-term smokers suffer from various chronic pains to a more severe extent, such as lumbago and back pain, carpal tunnel syndrome and complex regional pain syndrome, etc[12,13]. Vincler et al[14] found through studies that spinal nerve of adult male SD rats were ligated after injection of nicotine through a subcutaneously embedded micropump at the rate of 9 mg/kg/d for 7 days, and then their long-term exposure to nicotine can increase the mechanical pain sensitivity after peripheral nerve injury, which coincided with the increase of cAMP responsive element binding protein (CREB) after spinal cord phosphorylation. Further studies found that long-term exposure to nicotine increases the production of sciatic nerve IL-1 β and activates spinal microglia[15]. The increase of such mechanical stimulation on pain sensitivity is also related with the increase of the spinal dynorphin level[16]. It was reported that smokers who underwent an operation after quitting smoking would suffer from more serious postoperative pain, or need more postoperative painkillers than non-smokers[17,18]. This phenomenon may be related to the fact that nicotine has anti-noxious stimulation and long-term smokers have increased pain sensitivity after quitting smoking, but so far the specific mechanism is not reported yet. This study tested the plantar MWT and TWL on one side of rats of nicotine withdrawal, and found that in 7d after withdrawal, both MWT and TWL were increased compared with those in the control group and were particularly significantly increased respectively in 1 d, 2 d and 4 d after withdrawal. The above clinical phenomena are partially explained from the animal behavior. If corresponding pain model is established on this basis, it will lay the foundation for further study on the mechanism of occurrence of pain sensitivity changes after nicotine withdrawal.

Conclusions

An improved rat model of nicotine dependence and withdrawal can be successfully established by intermittent subcutaneous injection of nicotine at 9 mg/kg/d for 7 consecutive days. Rats have increased pain sensitivity to mechanical stimulation and thermal stimulation after nicotine withdrawal, while the mechanism of occurrence thereof still needs to be further studied.

Conflict of Interest

The Authors declare that there are no conflicts of interest.

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