Behavioral Study on Pure Nicotine and Nicotine-Minor Alkaloids of Tobacco Plant Interactions in Rat.

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***Abstract*--Minor alkaloids in the plant have been suggested to participate in the biological and neuronal action of nicotine. We hypothesized that these molecules modulate the effect of nicotine on locomotor activity and anxiety.**

Effects of single injection of nicotine and alkaloids of tobacco plant at dose (i.p., 0.5 mg/kg) were investigated behaviorally on locomotor activity in the open field, and on anxiety-like status (using digging and marble burying test and the Dark/light box test). Results show that locomotor activity was significantly enhanced and reduced by nicotine and the extract, respectively. In tests addressing anxiety-like behavior, nicotine was ineffective while the extract induced opposite effects depending on the test. Thus, the extract enhanced the number of marbles buried in the sawdust in the digging and marble burying test and reduced the time spent in the lightened box compared to vehicle- and nicotine in the Dark/light box.

In conclusion, we provide behavioral evidence that the tobacco extract induces distinct effects compared to sole nicotine as it favors anxiolytic and anxiogenic-like behaviors.

Keywords— Alkaloids of tobacco plant; Nicotine; anxiety; locomotor activity.

I. INTRODUCTION

Tobacco use remains a main concern of public health for countries due to its high prevalent addiction and associated diseases. The psychoactive properties of tobacco have been attributed to nicotine, the main alkaloid fund in tobacco plant [29, 17]. It is noteworthy however that pure nicotine is never used per se. A growing array of preclinical and clinical studies has demonstrated the existence of numerous compounds contained in the plant that could contribute to the psychoactive properties of nicotine.

Nicotiana plants are rich in alkaloids (cotinine, anabasine, nornicotine, tabagisine, moysmine) in addition to nicotine (95%–97%). These minor tobacco alkaloids exhibit a similar structure to nicotine, and have pharmacological activity, albeit generally less potent than nicotine [30]. Nornicotine and cotinine also play a role as major metabolites of nicotine [7]. It has been reported that the intravenous infusion of nicotine combined with five minor alkaloids found in tobacco smoke (anabasine, nornicotine, anatabine, cotinine and myosmine) increased locomotor activity and increased behavioral sensitization following self-administration [4]. These results suggest that the minor tobacco alkaloids, particularly anatabine, cotinine and myosmine, could increase the motivation for nicotine and thus facilitate smoking behavior. . It is widely accepted that addictive properties of drugs of abuse, such as psychostimulants, opiates and possibly nicotine, are associated with an increased meso-limbic dopaminergic transmission, thus promoting locomotor hyperactivity in rodents [11]. Nicotine and other alkaloids contained in tobacco plant and smoke, in addition to cholinergic and dopaminergic transmission, can affect individually other neurochemical systems including serotonergic cells [32].

The advantage of a tobacco extract is to keep the relative concentration of the alkaloids contained in the plant compared to cocktails. It has been reported that tobacco extracts produced pronounced teratogenic effects compared to pure nicotine and induced marked effects compared on the activity of 5-HT cells [22, 23, 32]. Nevertheless, the preclinical studies using extracts compared to nicotine are limited. Based on the above mentioned data, we have postulated that the resulting effects from the injection of nicotine or the extract should differ behaviorally on locomotor activity and anxiety tests.

In the present study, we have compared the effect of pure nicotine with a tobacco extract containing alkaloids contained in the plant. Rats were tested in distinct behavioral tests including open field, dark/light box test and digging marbles burying. Ease of Use

II. MATERIALS AND METHODS

A. Animals

Male Sprague-Dawley rats from the animal facility of the faculty of Sciences Semlalia, Marrakesh, Morocco weighing 300-350 g were used. Animals, housed in individual plastic

cages, were kept at constant room temperature (21 ± 2°C) and relative humidity (60 %) with a 12 hr light/dark cycle (dark from 7 P.M.) and had free access to water and food. All animals use procedures conformed to International European Ethical Standards (86/609-EEC) and the French National Committee (décret 87/848) for the care and use of laboratory animals. All efforts were made to minimize animal suffering and to reduce the number of animals used. For Every behavioral test we used 6 animals.

B. *Drugs*

The following compounds were used: nicotine (Nicotine hydrogen tartrate salt, sigma, France). Alkaloidic extract of tobacco plant contain nicotine (95-97 %), cotinine, nornicotine, anabasine, tabagisine, myosmine and anatabine and NaCl 0.9%.

C. *Open Field*

The animal was placed in the open field arena (100 X 100 X 30 cm) divided into 25 squares of equal areas. The open field test was used to evaluate the exploratory activity of the animal for 10 min. The observed parameters were number of squares crossed (locomotor activity) and occurrences of grooming (number of times the rat scratched its face with its forepaws) and rearing (number of times the rat stood completely erect on its hind legs).

D. *Light/dark box test*

The light/dark box test exploited the conflict between the animal's tendency to explore a new environment and its fear of bright light. The apparatus (length 50 cm, width 28 cm, and height 28 cm) consisted of two equal acrylic compartments, one dark and one white, illuminated by a 60-W bulb lamp and separated by a divider with a 5 - 5 cm opening at floor level. Each rat was tested by placing it in the center of the white area, facing away from the dark one, and was allowed to explore the novel environment for 5 min. The number of transfers from one compartment to the other and the time spent in the illuminated side were measured.

E. *Marble burying test*

The marble burying test is a useful model of neophobia, anxiety [19, 25] and obsessive compulsive behavior [15, 18]. It has also been proposed that the test may have predictive validity for the screening of novel antidepressants [10, 16] or anxiolytics [28, 31]. The neuronal circuitry of this behavior has not been clearly elucidated. The hippocampus and septum are likely to be important, since lesions in these areas reduce digging [9, 12].

The procedure described by Deacon (2006) [9] was employed. The cage was filled approximately 5 cm deep with wood chip bedding, lightly tamped down to make a flat, even surface. The bedding substrate can be reused if it is flattened and firmed down again between rats; reuse of bedding does not seem to affect the burying/digging performance of subsequently tested rat. A regular pattern of glass marbles was placed on the surface, evenly spaced, each about 4 cm apart. One animal was placed in each cage and left for 30 min and the number of marbles buried (to 2/3 their depth) with bedding was counted.

F. *Pharmacological treatments*

In each experimental group, animals were administered systemically either drugs or their appropriate vehicle. Alkaloids extract (0.5mg/kg, i.p.), nicotine (0.5 mg/kg, i.p.), were freshly diluted in physiological saline (NaCl 0.9%).

G. *Statistical analysis*

The number of lines crossing and rearing by rat in open field test , the number of transition and the time spent in lightened box in dark/light box test and the the number of marble burying was studied by one-way ANOVA followed, when significant, by the post hoc Tukey test, for analysis of number of transfers the analysis was studied by Kruskal-Wallis One Way Analysis of Variance on Ranks followed, when significant, by the post hoc Tukey's test was performed to determine statistical differences between groups.

III. RESULT

A. *Effect of nicotine and the extract on horizontal and vertical activity in the open field*

Fig. 1 and 2 show a single administration of nicotine and extract (0.5 mg/kg .i.p) in the open field test. One way ANOVA test revealed a significant effect of treatments for horizontal locomotor activity and vertical exploration respectively [$F(2,16) = 17.3$, $p < 0.001$, Fig 1; $F(2,16) = 31.842$; $p < 0.001$, Fig.2]. The posthoc comparisons using the Tukey's test revealed a significant difference in the number of lines crossing between nicotine vs extract and nicotine vs vehicule but no significant effect was found between extract vs vehicule. The behavioral responses were statistically different between all groups for vertical activity (Tukey's test). Both nicotine and the extract reduced vertical activity.

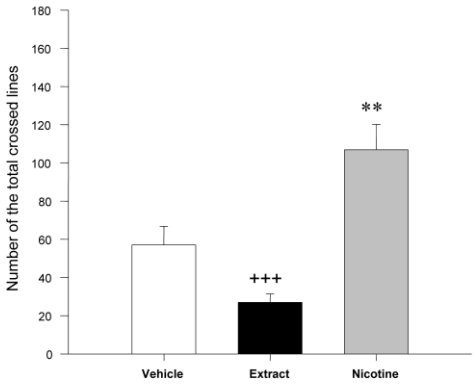

Fig.1 Mean ± S.E.M. number of lines crossed over 10 min in open field test for nicotine and extract of alkaloids rats injected intraperitoneally (0.5 mg/kg). The number of animals per group is six.

*p< .05, **p < .01, ***p < .001 extract vs vehicule group. +p< .05, ++p < .01, +++p < .001 extract vs nicotine group. (one-way ANOVA followed by tukey test),(n= 6 rats per group)

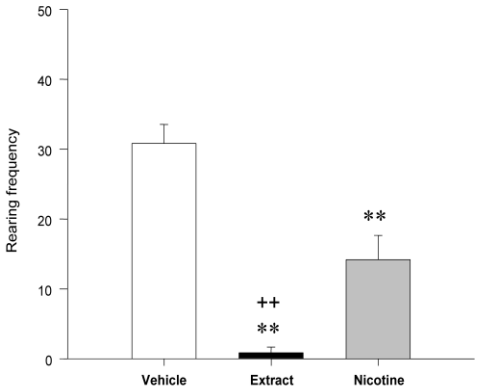

Fig 2 Mean ± S.E.M. Rearing frequency over 10 min in open field for nicotine and extract of alkaloids rats injected intraperitoneally (0.5 mg/kg) 35 min before the test. The number of animals per group is six.

*p< .05, **p < .01, ***p < .001 extract vs vehicule group. +p< .05, ++p < .01, +++p < .001 extract vs nicotine group. (one-way ANOVA followed by tukey test),(n= 6 rats per group)

B. *Effect of nicotine and the extract in the Dark/light box test*

Nicotine and extracts differed also in this behavioral test [One way ANOVA, F(2,17) = 15.14, p <0.001); H(2,17)= 10.23, P = 0.006. Nicotine at a dose of 0.5 mg/kg i.p. did not significantly alter the time spent in the lightened box compared to vehicle group (Fig 3 and 4). On the other hand, the extract (0.5 mg/kg i.p.) reduced both the time spent in the lighted compartment and the number of transfers compared to vehicle- and nicotine-treated groups (Fig 3 and 4).

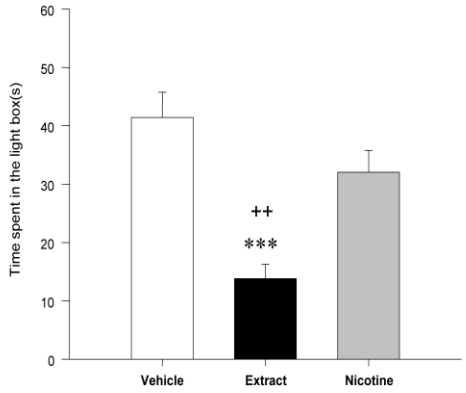

Fig.3 Mean ± S.E.M. The time spent at light during 5-min in light/dark box test for nicotine and extract of alkaloids rats injected intraperitoneally (0.5 mg/kg) 35 min before the test. The number of animals per group is six.

*p< .05, **p < .01, ***p < .001 extract vs vehicule group. +p< .05, ++p < .01, +++p < .001 extract vs nicotine group. (one-way ANOVA followed by tukey test),(n= 6 rats per group)

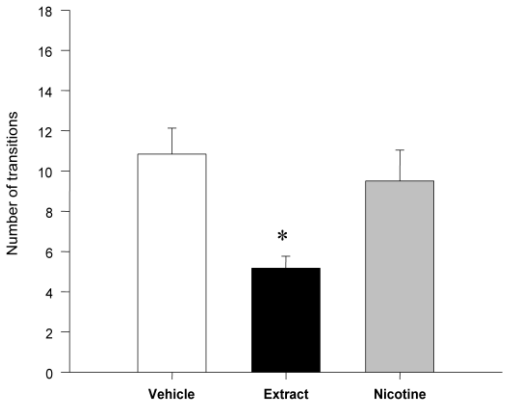

Fig.4 Mean ± S.E.M. The number of transfers between two compartments during 5-min in light/dark box test for nicotine and extract of alkaloids rats injected intraperitoneally (0.5 mg/kg) 35 min before the test. The number of animals per group is six.

*p< .05, **p < .01, ***p < .001 extract vs vehicule group. +p< .05, ++p < .01, +++p < .001 extract vs nicotine group. (Kruskal-Wallis One Way Analysis of Variance on Ranks followed by tukey test),(n= 6 rats per group)

C. *Effect of nicotine and the extract in the Marble burying test*

Fig 5 illustrates the significant differences in marble burying between the three testing groups [F(2,17)= 12.2, P = <0.001]. Rats treated with the extract (0.5 mg/kg) buried the most marbles while vehicle- and nicotine (0.5 mg/kg)-treated rats buried the least. The behavioral responses were statistically significant between vehicle vs extract and extract vs nicotine but no effect was found between vehicle and nicotine (Tukey's test).

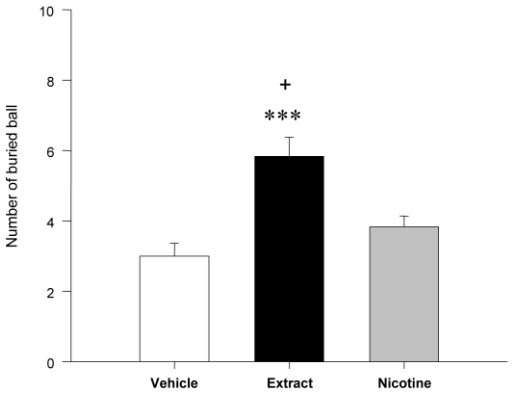

Fig.5 Mean ± S.E.M. The number of transfers burying marble during 30-min in marble buried test for nicotine and extract of alkaloids rats injected intraperitoneally (0.5 mg/kg) 35 min before the test. The number of animals per group is six.

*p< .05, **p < .01, ***p < .001 extract vs vehicule group. +p< .05, ++p < .01, +++p < .001 extract vs nicotine group. (one-way ANOVA followed by tukey test),(n= 6 rats per group)

IV. DISCUSSION

In the present study we have studied the effect of nicotine alone or combined with minor alkaloids extracted from the tobacco plant on motor activity. The results show that the extract differs from pure nicotine in the various

behavioral tests. The results add further arguments to the growing evidence that minor alkaloids play a role in the psychoactive properties of tobacco plant.

The main important result is to report that the extract differed from nicotine in behavioral evaluation. The extract corresponds to pure alkaloids and the concentration of nicotine in the extract has been shown to be 95-97%. Thus, the concentration of nicotine injected from the extract or from nicotine powder is somehow similar. According to previous data, the difference could be related to the presence of the other alkaloids contained in the extract.

In agreement with previous data [26], we show that nicotine enhances horizontal locomotor hyperactivity. Surprisingly, the extract reduced locomotor activity. Although the hyperlocomotor activity can be paralleled by an enhancement of mesoaccumbal DA transmission, our data suggest that these opposite results are related to other factors.

Nicotine has been reported to affect anxiety in a complex manner and this has been considered as an important component of the addiction to nicotine [20]. Here we found that nicotine (0.5 mg/kg i.p.) did not modify the number of transition between the boxes, the time spent in light box or the number of marbles burying. These results suggest that nicotine has no effect on anxiety in our tests. This result contrasts with data reporting that nicotine induces anxiogenic [2, 20, 27, 33] or anxiolytic effects [3, 20]. In other studies in adult male animals, however, nicotine has been reported to have no effect on anxiety-related behaviors [1]. There are a number of possible reasons for these conflicting findings, including different methods of assessing anxiety and their environment, different nicotine doses and dosing regimens, and different strains of animals [13, 33]. The important finding is that the extract of tobacco produces distinct effects compared to pure nicotine. It would correspond to anxiogenic effets in the light/dark box test and anxiolytic effects in the marble burying test. of note, the decrease in locomotor activity observed with the tobacco extract may have limited the transitions of the animals to cross light and dark boxes, dampening the idea that the reported effects in the light/dark box are related to enhanced anxiety. In any case, although we don't have clear explanation for these opposite directions regarding anxiety, the effects observed with the tobacco extract are clear-cut compared to nicotine. Indeed, it has been reported that the addition of the minor alkaloids to nicotine favors enhances locomotor activity and sensitization, increases the rewarding efficacy of nicotine across several doses and strengthens the motivation of rats to obtain nicotine [4, 22]. The effect of nicotine stimulating mesolimbic/mesocortical DA transmission is thought to underline its rewarding, reinforcing and locomotor stimulant effects [21].

According to the different responses observed in behavioral tests, it is possible that the alkaloids other than nicotine either directly bind this peculiar receptor, or indirectly favor its interaction with nicotine. In general, the half-life of the tobacco alkaloids nornicotine and cotinine (7.2-8.5h and 9,8-13,6 h) is longer compared to nicotine (0.7-1.4h) in plasma and brain [24, 14, 5]. Thus, although their affinity and concentrations are lower compared to nicotine [6], they may have effects on their own [30, 32] and one cannot exclude that their accumulation impacts cholinergic transmission. It has been reported that nicotine and minor alkaloids inhibit the activity of serononergic neurons in the dorsal raphe nucleus and, in line with our results, the inhibition was much more pronounced with a tobacco extract [32].

The finding that the tobacco extracts distinctly affect behavior compared to nicotine is in line with a growing number of biochemical, behavioral and electrophysiological studies [4, 22, 23, 32]. Further studies are required to better elucidate the precise nature of the mechanisms of the alkaloids of tobacco plant in brain, and their combination with nicotine. In this direction, Studies are continued in our laboratory.

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