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## THE EFFECT OF 2,6-DIMETHYLPYRIDINE N-OXIDE ON COGNITIVE FUNCTIONS AND EMOTIONAL STATE OF RATS FOLLOWING ITS LONG-TERM ORAL ADMINISTRATION

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**Background.** Global anthropogenic environmental pollution, intensification of production, daily physical, emotional and psychogenic stress on humans, as well as the working environment provoke a variety of diseases, fatigue, and cognitive impairment, etc. This may be attributed to a reduced nonspecific resistance of the health system and the development of stressful conditions. Therefore, one of the focal points of preventive toxicology is the development of agents with adaptogenic properties that would prevent the occurrence of harmful effects under the influence of stressors. The aim of the present study was to examine the effect of 2,6-dimethylpyridine N-oxide on cognitive functions and emotional status of rats following its long-term oral administration.

**Materials and Methods.** The plant growth regulator (PGR) Ivin (2,6-dimethylpyridine N-oxide, 99.9%) was chosen for the study. Adaptogen *Eleutherococcus* was used as a reference agent. The study was conducted on Wistar Hannover rats divided into 2 cohorts. Each cohort included the following groups: 1 – intact animals, 2 – control (distilled water), 3 and 4 – Ivin at doses of 13.0 and 0.013 mg/kg (1/100 and 1/100000 LD<sub>50</sub>), respectively, 5 – *Eleutherococcus* at a dose of 50 mg/kg. Exposure period – 28 days, oral route of administration. The state of the central nervous system was assessed by behavioural reactions in the Morris Water Maze and the Elevated Plus Maze.

**Results.** Ivin at doses of 13 and 0.013 mg/kg increased the ability to learn and to form short- and long-term memory in rats, as evidenced by a decrease in the average time of platform location in the Morris Water Maze test. Ivin at a dose of 0.013 mg/kg significantly increased the number of rearings in closed arms of the Elevated Plus Maze, indicating its anxiolytic effect.



The anti-anxiety effect of Ivin needs to be confirmed by additional studies in the Open Field and/or Hole-board tests. The effectiveness of Ivin in terms of the studied parameters was similar or exceeded those of the known adaptogen *Eleutherococcus*.

**Keywords:** 2,6-dimethylpyridine N-oxide, cognitive functions, emotional state, rat, Morris Water Maze, Elevated Plus Maze

## INTRODUCTION

Global anthropogenic environmental pollution, intensification of production, daily physical, emotional and psychogenic stress on humans, as well as the working environment provoke a variety of diseases, fatigue, and cognitive impairment, etc. (Vyunytska, 2018; Vyunytska *et al.*, 2022, Zarudna & Mural, 2015; Kalka, 2015). This may be attributed to a reduced nonspecific resistance of the health system and the development of stressful conditions. Environmental pollutants are among the prevalent factors that have a stressful effect on the organism. Their long-lasting impact on populations of various organisms, including humans, causes a decrease in functional reserves, and a disruption of homeostasis and resistance, which can lead to environmentally caused pathology (Nakonechnaya *et al.*, 2013; Yermishev & Marchak, 2020; Marakushin *et al.*, 2020). Therefore, an important objective of preventive toxicology is to avert harmful effects of chemical, physical and biological environmental factors and, if necessary, to correct the disturbed homeostasis in humans, cultivated plants, and livestock (Magrelo & Magrelo, 2023). One of the areas of such studies may be the search for synthetic substances with adaptogenic properties that would prevent the occurrence of harmful effects in isolation or in combination with a stressor, in particular with pesticides and heavy metals. In this regard, Ivin (2,6-dimethylpyridine N-oxide), which is a widely used plant growth regulator (PGR) in Ukrainian agriculture (Register of Pesticides, 2024), is a promising adaptogen to adverse environmental conditions. The protective role of Ivin against extreme factors, such as temperature, salt stress, and heavy metal ions, has been experimentally proven in plants (Ponomarenko & Iutynska, 2011; Grishko & Demura, 2009; Havrys *et al.*, 2013; Ryabchenko *et al.*, 2006).

Our previous works have shown that Ivin is a low-toxic substance for mammals. Under low-dose exposure to laboratory animals, Ivin provides antioxidant, antihypoxic, hepatoprotective, genoprotective, membrane-stabilising effects, intensifies protein and nucleic acid synthesis, and can play an important role in reducing the toxicity of many xenobiotics (Vasetska *et al.*, 2021; Vasetska *et al.*, 2022, Vasetska *et al.*, 2023a, Vasetska *et al.*, 2023b; Vasetska & Zhminko, 2021). It can be assumed that Ivin can also increase the organism's resistance to various types of stress. In this regard, it is important to study the influence of Ivin on the functional state of the central nervous system of rats using neurobehavioural tests.

The aim of the present study was to examine the effect of 2,6-dimethylpyridine N-oxide on cognitive functions and emotional status of rats following its long-term oral administration.

## MATERIALS AND METHODS

In the present study, the PGR Ivin – 2,6-dimethylpyridine N-oxide (99.9%, liquid, clear from colourless to yellowish, miscible with water), produced by NE ISTC 'Agrobiotech', Kyiv, Ukraine, was used. The adaptogen *Eleutherococcus* extract (manufactured by

PJSC FF 'Viola', Zaporizhzhia, Ukraine (UA/11560/01/01)), was chosen as a reference substance (Todorova, 2021). The experiments were conducted on mature Wistar Hannover male rats (100 animals weighing 230–240 g). The rats were taken from the Specific Pathogen Free (SPF) nursery of the State Enterprise "L. I. Medved's Research Center of Preventive Toxicology, Food and Chemical Safety", the Ministry of Health of Ukraine, and placed in a conventional vivarium. The animals had spent a 7-day acclimatization period under the veterinary supervision, after which they were divided into two cohorts (50 rats per cohort). The first cohort of animals was used for the Morris water maze test (MWM), and the second cohort was used for the Elevated plus maze (EPM). According to the experimental design, both cohorts of animals were divided into groups (10 rats per group): Group 1 – intact animals (untreated male rats), Group 2 – control animals (male rats treated with distilled water as a vehicle), Group 3 – male rats treated with Ivin at a dose of 13.0 mg/kg body weight (1/100 LD<sub>50</sub>, subtoxicity dose), Group 4 – male rats treated with Ivin at a dose of 0.013 mg/kg body weight (1/100000 LD<sub>50</sub>, no effect dose), Group 5 – male rats treated with Eleutherococcus (as a positive control) at a dose of 50 mg of extract/kg body weight (therapeutic dose). Solutions of the test substances were prepared extempore with distilled water as a solvent (vehicle).

The test substances were orally administered to rats for 28 days using a gavage in the morning at the same time. The volume solution administered to the stomach did not exceed 1.0 mL per 100 g of animal weight. Male rats were kept in standard vivarium conditions for studying the functional state of the central nervous system.

Changes in body weight on days 0, 7, 14, 21, and 28 were measured as integral indicators of the studied substances' effect on the body of male rats. Daily clinical examination of animals was carried out to identify possible symptoms of intoxication and neurological disorders (Moser, 2000).

Behavioural reactions were tested according to the Guidelines (Stefanov, 2001) in compliance with the stable environment (temperature, lighting mode and air humidity), free access to feed and water, testing procedure, etc (Hånell & Marklund, 2014).

The cognitive function (learning and memory ability) in the first cohort of males was assessed in the *Morris Water Maze* proposed by R. G. Morris (Vorhees & Williams, 2006). The *Morris Water Maze* test allows to assess the effect of substances on learning ability and the long-term and short-term memory formation dynamics. Animals were placed in the maze and were required to traverse the maze and escape by locating a platform that was hidden beneath the surface of water. The amount of time required to transverse the maze was recorded. If the platform was not found within the set time (120 seconds), the animal was shown its location and removed from the maze, and the trial was recorded as unsuccessful. Testing was conducted after the end of the treatment period (day 28) for five consequent days in the morning at the same time. On each testing day, two trials were performed. The mean time to escape from the Morris maze on the first day and over five days in total was calculated.

The *Elevated Plus Maze* test was used to assess the level of anxiety and exploratory activity (Walf & Frye, 2007; Biedermann *et al.*, 2017). During the test, the animal was placed on the starting platform of a plus-shaped apparatus elevated above the ground with two open and two closed arms. The number of entries to the open and closed arms of the maze, rearing, head dips, the number of defecations/urination, and grooming acts were recorded. The tests were conducted on days 0 (before treatment), 14, and 28 of exposure at the same time each morning. Evaluating behavior in the

Elevated Plus Maze test before treatment ensures the establishment of baseline behavior, standardization of experimental procedures, control for individual differences, and assessment of behavioral stability.

The animal model study was conducted in accordance with the principles of bioethics and the requirements of the Medical and Biological Research Ethics Commission of the "L. I. Medved's Research Center of Preventive Toxicology, Food and Chemical, Ministry of Health, Ukraine" (State Enterprise) (Minutes No. 9/1 dated 09.05.2020).

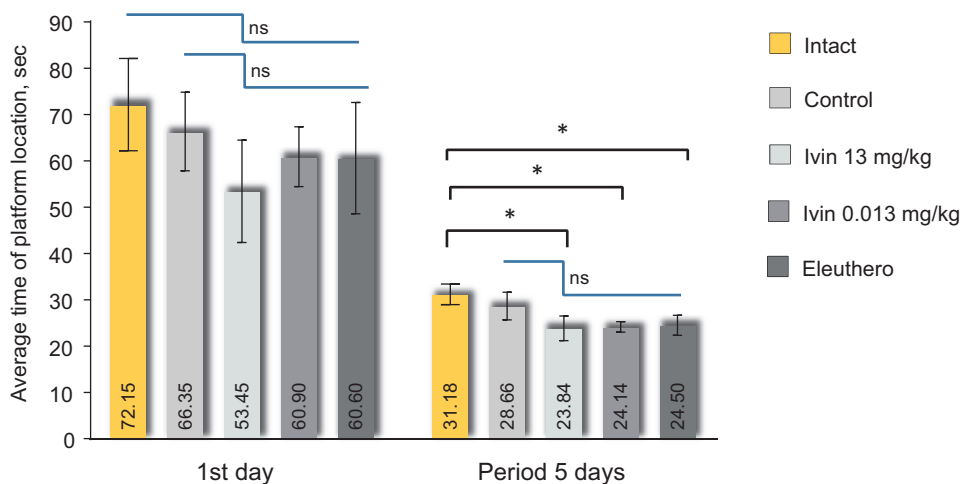
Statistical processing of the obtained data in Morris Water test was carried out using ordinary one-way ANOVA followed by Fisher's LSD test. The scores of Elevated Plus Maze test were analysed using a two-way ANOVA with repeated measures, with the factors being the effects of treatment (five experimental groups) and treatment duration (0, 14, or 28 days) followed by Fisher's LSD test when appropriate. The data were tested for normality of distribution according to Shapiro-Wilk test. The results were presented in the form of mean values and square deviation of the mean ( $M \pm m$ ). Differences were considered significant at  $p \leq 0.05$  for all tests.

## RESULTS AND DISCUSSION

Exposure to Ivin did not result in clinical symptoms of intoxication and mortality in experimental animals. The behaviour of the animals did not differ from the control. There were no significant changes in body weight in experimental, control and intact animals. Special attention in the study of adaptogenic drugs was paid to the cognitive function, since its impairment can lead to disturbances in memory, thinking and other cognitive processes of the brain (Morris, 1984).

The difference in time spent by the animals to locate the platform on the first day of training can be used for estimating the coherence of short-term memory mechanisms and the efficiency of memorisation (Bromley-Brits *et al.*, 2011, D'Hooge & De Deyn, 2001). There were no statistically significant differences found in cognitive abilities in males on the first day of testing, which were orally administered Ivin at doses of 0.013 and 13.0 mg/kg, as well as the reference substance Eleutherococcus, but a positive trend compared to intact animals was observed: the average time of platform location was 60.9, 53.5 and 60.6 seconds, respectively, compared to 72.1 seconds in intact animals.

The changes in time spent on the platform location in the period from 1 to 5 days of testing allowed to estimate the state of long-term memory of animals and their long-term learning ability (Bromley-Brits *et al.*, 2011, D'Hooge *et al.*, 2001). It was found that the administration of the Eleutherococcus enhanced the learning ability of males - the average time of platform location was 24.5 sec, compared to 31.2 sec in intact animals during the trial period (that decreased by 21.5 %,  $p = 0.048$ ). The effect of Ivin on memory and learning was not worse than Eleutherococcus: under the influence of Ivin at doses of 0.013 mg/kg and 13.0 mg/kg, the average time for platform location was 24.1 sec and 23.8 sec respectively, which was significantly less by 22.8% ( $p = 0.038$ ) and 23.7% ( $p = 0.031$ ), respectively compared to intact animals, from the 1st to the 5th day of testing. (see **Figure**). There were no statistically significant differences compared to the control animals, but a positive trend to decrease was retained. Thus, according to the test results, it can be concluded that Ivin in both tested doses increases the ability of animals to learn and form short- and long-term memory at the same level as Eleutherococcus.



The average time spent by the animals to locate the hidden platform in the Morris Water Maze test on the 1-st day and over the period of 5 days of testing ( $M \pm m$ ,  $n = 10$  per group)

**Note:** \* –  $p < 0.05$  difference is statistically significant compared to intact animals, ns – no significant difference compared to the control or intact animals (using one-way ANOVA followed by Fisher's LSD test)

The evaluation of the possible anxiolytic effect of the test substance was performed with the second cohort of rats in the Elevated Plus Maze. The analysis of the behavioural reactions of males in the Elevated Plus Maze enabled to assess their reaction to new environments, the degree of emotional reaction of anxiety and fear, and exploratory activity (Hall, 1936; Walf *et al.*, 2007). The data obtained in the EPM test were analysed by two-way ANOVA to examine the effects of treatment of Ivin and the treatment duration. The two-factor analysis of variance revealed no significance either for the factor of treatment or for the factor of duration of treatment, or for the relationship between these factors for most indicators (number of entries into open/closed arms of the maze, hangings from the platform, defecation, urination, grooming acts) (see **Table**). However, for vertical activity of rats (number of rearings) there was significance for the main effect of treatment factor ( $p = 0.002$ ,  $F(4, 135) = 4.470$ ), no significance for the effect of treatment duration factor ( $p = 0.099$ ,  $F(2, 135) = 2.347$ ) and no significance for the relationship between these factors ( $p = 0.165$ ,  $F(8, 135) = 1.493$ ). The following Fisher's LSD test showed that on the 14th day of observation, the animals exposed to Ivin at a dose of 0.013 mg/kg exhibited a statistically significant increase in rearings into the closed arms (10.50 rearings) of the maze compared to the intact ( $p = 0.0215$ , 6.50 rearings) and control animals ( $p = 0.006$ , 5.7 rearings). On the 28th day, the number of rearings was 12.60, which is significantly higher compared to the intact ( $p = 0.002$ , 7.20 rearings) and control animals ( $p = 0.0005$ , 6.50 rearings), and in comparison with the initial data ( $p = 0.011$ , 8.20 rearings). Under exposure to Ivin at a dose of 13 mg/kg, there were significant changes of rearing number ( $p = 0.044$ ) in comparison with control animals on the 14th observation day only. No significant changes were detected under exposure to Eleutherococcus, but a tendency to increasing the number of rearings in the closed arms of the maze was detected with the most effect compared to the control animals.

Behavioural reactions of males in the Elevated Plus Maze ( $M \pm m$ ,  $n = 10$  per group)

Indicators	Test day	Intact	Control	Ivin – 13 mg/kg	Ivin – 0.013 mg/kg	Eleuthero – 50 mg/kg
Rearing in closed arms	0	6.40±1.41	8.70±0.67	5.90±0.97	8.20±1.00	7.20±0.99
	14	6.50±1.61	5.70±0.92	9.20±1.46 <sup>b</sup>	10.50±1.26 <sup>ab</sup>	8.10±1.37
	28	7.20±1.20	6.50±1.29	8.60±1.45	12.60±1.25 <sup>abc</sup>	9.80±1.01
Rearing in open arms	0	0.10±0.10	0.00±0.00	0.30±0.21	0.00±0.00	0.30±0.21
	14	0.00±0.00	0.20±0.13	0.00±0.00	0.00±0.00	0.20±0.20
	28	0.00±0.00	0.00±0.00	0.00±0.00	0.30±0.15	0.00±0.00
Entries into closed arms	0	1.70±0.30	1.60±0.27	1.40±0.27	1.30±0.21	1.60±0.27
	14	1.80±0.47	1.90±0.41	1.40±0.31	1.70±0.26	1.60±0.34
	28	2.10±0.48	2.50±0.48	2.30±0.60	2.10±0.46	1.80±0.36
Entries into open arms	0	0.50±0.27	0.40±0.22	0.40±0.27	0.30±0.21	0.30±0.15
	14	0.50±0.17	1.00±0.30	0.20±0.13	0.40±0.16	0.40±0.22
	28	1.10±0.38	0.60±0.22	0.80±0.29	0.40±0.22	0.50±0.27
Hangings	0	2.10±0.50	2.20±0.57	1.70±0.72	1.50±0.62	1.60±0.45
	14	2.70±1.05	2.80±0.85	2.10±0.55	1.70±0.50	2.40±0.52
	28	3.20±0.98	2.40±0.75	3.60±0.83	2.10±0.78	1.70±0.58
Defecation	0	1.60±0.43	0.70±0.30	0.90±0.41	1.60±0.56	1.60±0.67
	14	0.20±0.20	0.00±0.00	0.20±0.20	0.20±0.20	0.30±0.30
	28	0.40±0.31	0.00±0.00	0.20±0.20	0.30±0.30	0.50±0.50
Urination	0	0.80±0.20	0.70±0.21	0.40±0.27	0.30±0.21	0.30±0.15
	14	0.30±0.15	0.40±0.16	0.20±0.13	0.40±0.16	0.40±0.22
	28	0.70±0.21	0.60±0.22	0.80±0.29	0.40±0.22	0.50±0.27
Grooming	0	0.00±0.00	0.00±0.00	0.00±0.00	0.30±0.15	0.20±0.13
	14	0.00±0.00	0.00±0.00	0.30±0.15	0.10±0.10	0.20±0.13
	28	0.40±0.16	0.00±0.00	0.30±0.15	0.20±0.13	0.10±0.10

**Note:** <sup>a</sup> –  $p < 0.05$  difference is statistically significant compared to intact animals; <sup>b</sup> –  $p < 0.05$  difference is statistically significant compared to control animals; <sup>c</sup> –  $p < 0.05$  difference is statistically significant compared to initial data (using two-way ANOVA followed by Fisher's LSD test)

In addition to significant changes in exploratory activity, a decrease in the number of defecations was observed in males after Ivin exposure on days 14 and 28 compared to the initial data. Considering the same changes in the group of intact animals and the decrease in the number of defecations as an objective indicator of the degree of intestinal congestion, it is possible to conclude that this is not associated with the influence of Ivin.

It is known that rearings indicate the exploratory activity of animals in a new situation (Hall, 1936). According to H. M. Nanjappaiah and co-authors (2017), the increase of such activity after exposure testifies to its axiolytic activity. However, other researchers (Walf *et al.*, 2007; File & Wardill, 1975; Figueiredo Cerqueira *et al.*, 2023) consider that changes in one component of behaviour do not evidence its antianxiety effect.

Thus, the data obtained in the Elevated Plus Maze indicate an ambiguous result and challenge the anxiolytic properties of Ivin. The anxiolytic effect of Ivin needs to be confirmed by additional studies in the Open Field and/or the Hole-board tests.

By its adaptation properties Ivin is similar to the classical adaptogen Eleutherococcus.

## CONCLUSIONS

1. The 2,6-dimethylpyridine N-oxide (Ivin) at doses of 13 and 0.013 mg/kg enhanced the learning ability and formation of short- and long-term memory in rats, as is indicated by a reduced average time of platform location in the Morris Water Maze test.

2. Ivin at a dose of 0.013 mg/kg significantly increased the number of rearings in the closed arms of the Elevated Plus Maze, which suggested its possible anxiolytic effect. Further tests (the Open Field and/or Hole-board tests) are required to confirm this anxiolytic effect.

3. The revealed changes in the behavioral reactions of rats may be associated with adaptogenic effects of Ivin. The effectiveness of Ivin in terms of the studied parameters was similar or exceeded those of the known adaptogen Eleutherococcus.

## COMPLIANCE WITH ETHICAL STANDARDS

**Conflict of Interest:** there were no commercial or financial relationships that could be interpreted as a potential conflict of interest.

**Human Rights:** this article does not contain any studies with humans.

**Animal studies:** all international, national and institutional guidelines for the care and use of laboratory animals were followed.

## AUTHOR CONTRIBUTIONS

Conceptualization, [V.O.]; methodology, [V.O., R.I.]; validation, [V.O., R.I.]; formal analysis, [V.O., R.I.]; investigation, [V.O., R.I.]; writing – original draft preparation, [V.O.]; writing – review and editing, [V.O., R.I.]; visualization, [V.O., R.I.]; project administration, [V.O.]. All authors have read and agreed to the published version of the manuscript.

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## ВПЛИВ 2,6-ДИМЕТИЛ-N-ОКСИД ПІРИДИНУ НА КОГНІТИВНІ ФУНКЦІЇ ТА ЕМОЦІЙНИЙ СТАН ЩУРІВ ЗА ТРИВАЛОГО ПЕРОРАЛЬНОГО НАДХОДЖЕННЯ В ОРГАНІЗМ

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**Вступ.** Глобальне антропогенне забруднення довкілля, інтенсифікація виробництва, повсякденне фізичне, емоційне та психогенне навантаження на людину, а також специфіка професійного середовища сприяють виникненню низки захворювань, втоми, порушення когнітивних функцій і т. п., що може бути пов'язане зі зниженням неспецифічної опірності організму та розвитком стресових станів. Тому одним із напрямів профілактичної токсикології є розробка препаратів з адаптогенними властивостями, які б запобігали виникненню шкідливих ефектів за впливу стресогенних факторів. Мета дослідження – з'ясувати характер впливу 2,6-диметил-N-оксид піридину на когнітивні функції й емоційний стан щурів за тривалого перорального надходження в організм.

**Матеріали та методи.** У роботі використано 2,6-диметил-N-оксид-піридин, 99.9% (Івін). Препаратом порівняння слугував адаптоген Елеутерокок. Дослідження проведено на 100 самцях щурів Wistar Hannover, розподілених на дві когорти порівну. Кожна когорта включала такі групи: 1 – інтактні тварини, 2 – контроль (дистильована вода), 3 і 4 – Івін у дозах 13,0 і 0,013 мг/кг (1/100 та 1/100000 від ЛД<sub>50</sub>) відповідно, 5 – Елеутерокок у дозі 50 мг/кг. Експозиція – 28 днів, спосіб введення – перорально. Стан центральної нервової системи оцінювали за поведінковими реакціями у "Водному лабіринті Морріса" та "Піднятому хрестоподібному лабіринті".

**Результати і висновки.** Івін у дозах 13 і 0,013 мг/кг підвищує здатність до навчання та формування коротко- і довготривалої пам'яті у щурів, про що свідчить зниження середнього часу знаходження прихованої платформи у тесті "Водний лабіринт Морріса". Івін у дозі 0,013 мг/кг вірогідно підвищував кількість стійок у закритих рукавах хрестоподібного лабіринту, що свідчить про його анксиолітичну дію. Для підтвердження або спростування протитривожного ефекту Івіну доцільно провести додаткові дослідження в тестах "Відкрите поле" та/або "Норковий рефлекс". Ефективність Івіну за дослідженими показниками не поступалась або була вища від ефективності відомого адаптогена Елеутерококу.

**Ключові слова:** N-оксид-2,6-диметил піридину, когнітивні функції, емоційний стан, щури, водний лабіринт Морріса, піднятий хрестоподібний лабіринт

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