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NEUROPROTECTIVE PROPERTIES OF N-PHENYLACETYL-L-PROLYLGLYCINE ETHYL ESTER NASAL GEL IN AN EXPERIMENTAL MODEL OF MULTIPLE SCLEROSIS EQUIVALENT

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Ключевые слова: *назальный гель, специфическая активность, церебропротектор, этиловый эфир n-фенилацетил-l-пролилглицина*

Abstract. Neuroprotective properties of n-phenylacetyl-l-prolylglycine ethyl ester nasal gel in an experimental model of multiple sclerosis equivalent. Burlaka B.S., Belenichev I.F., Nefedov O.O., Aliyeva O.G., Bukhtiyarova N.V. *The purpose of this research is to study the specific activity of our developed nasal dosage form with n-phenylacetyl-l-prolylglycine ethyl ester. The experiments were performed on 260 white outbred rats weighing 190-220 g. Experimental allergic encephalomyelitis (EAE) was induced by a single subcutaneous inoculation of an encephalitogenic mixture (EHM) in Complete Freund's Adjuvant (CFA) based on 100 mg of homologous spinal cord homogenate; 0.2 ml of CFA (the content of killed mycobacteria 5 mg/ml) and 0.2 ml of physiological saline per animal. There were five groups of animals in the study: 1) intact; 2) control - untreated with EAE, received saline solution; 3) animals with EAE that received basic treatment - methylprednisolone (MP), 3.4 mg/kg, intraperitoneally slowly in saline no more than 1/10 of the CBV rat; 4) animals with EAE treated with MP + Noopept, at a dose of 10 mg/kg; 5) animals with EAE treated with MP + Citicoline (Ceraxon, Ferrer Internacional S.A., Spain) D003U1 series, 500 mg/kg, intragastrically. The integrative functions of rats' brain with EAE were studied using the "Open Field" method with an arena of own production with dimensions 80x80x35 cm. The study of memory was carried out using the radial labyrinth LE760 (AgnTho's, Sweden). Capture and image recording was carried out using a color video camera SSC-DC378P (Sony, Japan). Video file analysis was performed using Smartv 3.0 software (Harvard Apparatus, USA). As a result of the studies on experimental model of multiple sclerosis with a nasal gel containing ethyl ester of n-phenylacetyl-l-prolylglycine revealed the presence of normotimic activity, antidepressant and anxiolytic effects, an increase in the total activity of the central nervous system. The results obtained indicate a high neuroprotective and nootropic activity of the Noopept intranasal gel. By the degree of influence on reducing the number of errors in working memory, the Noopept gel significantly exceeds monotherapy with methylprednisolone and combination therapy with methylprednisolone and citicoline. A further study of the effect of the developed nasal gel on the morphofunctional indices of sensorimotor cortical neurons under experimental multiple sclerosis, as well as on the content of HSP70 in the animal brain, is promising.*

Реферат. Нейропротективні властивості назального гелю з етиловим ефіром n-фенілацетил-l-пролілгліцину на експериментальній моделі еквіваленту розсіяного склерозу. Бурлака Б.С., Белєнічев І.Ф., Нефьодов О.О., Алієва О.Г., Бухтіярова Н.В. *Мета цього дослідження – вивчення специфічної активності розробленої нами назальної лікарської форми з етиловим ефіром n-фенілацетил-l-пролілгліцину. Досліди виконані на 260 білих безпородних щурах масою 190-220 г. Експериментальний алергічний енцефаломієліт (ЕАЕ) індукували шляхом одноразової підшкірної інокуляції енцефалітогенної суміші (ЕГС) в повному ад'юванті Фрейнда (ПАФ) з розрахунку 100 мг гомогенату гомологічного спинного мозку; 0,2 мл ПАФ (вміст убитих мікобактерій 5 мг/мл) і 0,2 мл фізіологічного розчину на тварину. У дослідженні було п'ять груп тварин: 1) інтактні; 2) контрольні – неліковані з ЕАЕ, отримували фізіологічний розчин; 3) тварини з ЕАЕ, які одержували базове лікування – метилпреднізолон (МП), 3,4 мг/кг, внутрішньоочеревинно, повільно у фізіологічному розчині, об'ємом не більше 1/10 ОЦК щура; 4) тварини з ЕАЕ, які одержували МП + ноопепт, у дозі 10 мг/кг; 5) тварини з ЕАЕ, які одержували МП + Цитиколін (Цераксон, «Ferrer Internacional S.A.», Іспанія) серія D003U1, 500 мг/кг, внутрішньошлунково. Інтегративні функції головного мозку щурів з ЕАЕ вивчали за допомогою методики «Відкрите поле» з використанням арени власного виробництва розмірами 80x80x35см. Дослідження пам'яті проводили за допомогою радіального лабіринту LE760 (AgnTho's, Sweden). Захоплення і запис зображення проводився за допомогою кольорової відеокамери SSC-DC378P (Sony, Japan). Аналіз відеофайлу проводився за допомогою програмного забезпечення Smartv 3.0 (Harvard Apparatus, USA). У результаті проведених досліджень на експериментальній моделі розсіяного склерозу, назального гелю з етиловим ефіром n-фенілацетил-l-пролілгліцина виявлено наявність нормотимічної активності, антидепресивної й анксіолітичної дії, збільшення загальної активності ЦНС. Отримані результати свідчать про високу нейропротективну й ноотропну активності інтраназального гелю «Ноопепт». За ступенем впливу на зниження кількості помилок робочої пам'яті гель «Ноопепт» достовірно перевершує монотерапію метилпреднізолоном і терапію комбінації метилпреднізолону з цитиколіном. Перспективними є подальше вивчення впливу розробленого назального гелю на морфофункціональні показники нейронів сенсомоторної кори в умовах експериментального розсіяного склерозу, а також на утримання HSP70 у головному мозку тварин.*

The development of drugs or new dosage forms for the treatment of cerebrovascular diseases, is an urgent problem of modern pharmacy and medicine. As active ingredients in pharmacotherapeutic preparations, a wide range of compounds of various therapeutic groups for the treatment of acute and chronic manifestations of cerebrovascular pathologies are used. The use of peptides as active pharmaceutical ingredients in dosage forms is promising [12, 14].

At the department of Drug Technology, Pharmacology and Medical Formulations of Zaporizhzhya State Medical University within the framework of the research topic "Development of the composition, technology and biopharmaceutical studies of pharmacotherapeutic systems for transmucosal delivery of drugs", as a result of complex physical, chemical, microbiological and biopharmaceutical studies, a new, gel, nasal form with an active pharmaceutical ingredient – n-phenylacetyl-l-

prolylglycine ethyl ester (noopept), at a concentration of 1% is developed [3, 10].

A common chronic autoimmune disease of the nervous system is multiple sclerosis (MS). This pathology is characterized by damage to the myelin sheath of nerve fibers with their further decay. As a result of this in patients there is a decrease in intelligence, memory impairment, impaired behavior, emotional functions, deep and superficial sensitivity which significantly negatively affects the quality of life of patients.

To increase the effectiveness of MS therapy, it is advisable to use universal therapeutic approaches which will include neuroprotection aimed at regulating the balance of immune and neurotrophic factors, remyelination processes. Given the current level of knowledge about neuroprotection in MS, the optimal neuroprotective therapy of this disease from the standpoint of practical neurology is carried out by using primary and secondary neuroprotection agents against the background of the administering of remyelinating drugs. A generally recognized model of multiple sclerosis in laboratory animals is experimental allergic encephalomyelitis (EAE), which has multiple sclerosis-like clinical manifestations and pathogenetic mechanisms [2, 4, 6, 7, 8].

The purpose of this research was to study the specific neuroprotective activity of a nasal dosage form with n-phenylacetyl-l-prolylglycine ethyl ester.

MATERIALS AND METHODS OF RESEARCH

The experiments were performed on 260 white outbred rats weighing 190-220 g, obtained from the nursery of the Institute of Pharmacology and Toxicology of the Academy of Medical Sciences of Ukraine. The duration of the quarantine (acclimatization period) for all animals was 14 days. During quarantine each animal was examined daily (behavior and general condition), animals were observed in cells (morbidity and mortality) twice a day. Before the start of the study animals meeting the criteria for inclusion in the experiment were divided into groups using the randomization method. Inappropriate animals were excluded from the quarantine study. Cages with animals were placed in separate rooms. Light mode: 12 hours – light, 12 hours – darkness. The air temperature was maintained within 19-25° C, relative humidity – 50-70%. Temperature and humidity were recorded daily. The ventilation mode was established, providing about 15 room volumes per hour. Experimental animals were kept on the same rations under ordinary conditions in vivarium. Animals were housed in standard cages – 5 rats per cage. Diet – feed grain, bread, root crops (beets, carrots) [9].

Experimental allergic encephalomyelitis (EAE) was induced by a single subcutaneous inoculation of an encephalitogenic mixture (EHM) in Complete Freund's Adjuvant (CFA) based on 100 mg of homologous spinal cord homogenate; 0.2 ml of CFA (the content of killed mycobacteria 5 mg/ml) and 0.2 ml of physiological saline per animal.

There were five groups of animals in the study:

1) intact (10 rats);

2) control – untreated with EAE, received saline solution (20 rats);

3) animals with EAE that received basic treatment – methylprednisolone (MP), 3.4 mg/kg, intraperitoneally slowly in saline no more than 1/10 of the CBV rat (20 rats);

4) animals with EAE treated with MP + Noopept at a dose of 10 mg / kg (20 rats);

5) animals with EAE treated with MP + Citicoline (Ceraxon, Ferrer Internacional S.A., Spain) D003U1 series, 500 mg / kg, intragastrically (20 rats).

The drugs were administered 2 days after EAE induction: methyl prednisone for 7 days, and noopept and citicoline for 14 days (latent phase + clinical phase until the peak of the disease). Control and intact rats during the entire course of treatment received intraperitoneal and intragastric saline in similar volumes. All studies were performed on the 17th day of the experiment.

The integrative functions of the brain of rats with EAE were studied using the “Open Field” method using an arena of own production with dimensions 80x80x35cm. The animal was placed in the middle of one of the sides with its muzzle facing the wall, after which it was allowed to freely move around the arena for 8 minutes. We estimated the total distance traveled (cm), the total motor activity (cm 2/s), the structure of activity (high, low activity, inactivity,%), the number of fading and occurrences in the center, the distance traveled near the wall (cm) and in the central regions of arena (cm,%), vertical search activity (the number of racks on the hind legs at the wall and in the center), the number of short and long grooming events, the number of acts of defecation and urination [1, 5, 11].

The study of memory was carried out using the radial labyrinth LE760 (AgnTho's, Sweden). The study was conducted in complete silence. The reference memory (the general long-term idea of the structure of the labyrinth and the location of food that was formed in the animal during the training) and the number of reference memory errors (the first visit to a previously closed beam in which the animal never found food) were evaluated, as well as working memory (short-term the animal’s idea of the location of food in a particular experiment) and

the number of errors in working memory (repeated visits to the beam in which the animal previously found or did not find food). In addition, the distance covered and the overall physical activity were evaluated. During the experiments, the influence of external and internal visual, olfactory, and auditory stimuli was excluded. Assessment of animal behavior was carried out by a laboratory assistant who was not aware of the animal's belonging to a specific experimental group. Image capture and recording was carried out using a color video camera SSC-DC378P (Sony, Japan). Video file analysis was performed using Smartv 3.0 software (Harvard Apparatus, USA) [11, 13].

The results of the study were processed using the statistical package of the licensed program STATISTICA® for Windows 6.0 (StatSoft Inc., No. AXXR712D833214FAN5), as well as SPSS 16.0 and Microsoft Excel 2003. Separate statistical procedures and algorithms are implemented in the form of specially written macros in the corresponding programs. For all types of analysis, differences at $p<0.05$ were considered statistically significant.

RESULTS AND DISCUSSION

Many patients with MS experience insomnia or intermittent superficial sleep, but its disturbance is not a direct consequence of the disease. They are usually caused by secondary factors: stress, spasticity, limited physical activity, or depression. Therefore, an assessment of the ability of antidepressants to enhance drug sleep in rats with EAE was made. The effectiveness of the Noopept intranasal gel and citicoline, in this series of experimental studies were evaluated using two indicators: the time of falling asleep (latent period of sleep) and its duration in rats with EAE. Drug sleep was induced by the administration of thiopental sodium (30 mg/kg) (Table 1).

Table 1

The characteristic of drug sleep, in rats with EAE, on the background of the combined administration of methylprednisolone with noopept and citicoline (M±m, n=10)

Experimental groups	Dose mg/kg	Time to fall asleep (sec.)	Duration of sleep (min.)
EAE (control)	**-**	**62.7±12.7**	**26.5±3.7**
EAE + MP	**3.4**	**54.8±10.0**	**36.7±3.4**
EAE + MP + Noopept	**3.4+10**	**27.1±5.2*[2]**	**63.1±5.1*[2]**
EAE + MP + Citicoline	**3.4+25**	**29.1±5.4**	**55.6±5.8*[2]**

Note: * - $p \leq 0.05$ in relation to control indicators, 1 - $p \leq 0.05$ in relation to citicoline, 2 - $p \leq 0.05$ in relation to methylprednisolone.

It was established that during noopept and citicoline usage, unidirectional dynamics of changes in the latent period and duration of sleep was observed. So, against the background of the administration of citicoline sleep in rats with EAE was prolonged by 109.8% ($p \leq 0.05$), compared with the control group. When using the Noopept intranasal gel, this indicator changed even more – falling asleep time decreased by 56.7% ($p \leq 0.05$), sleep duration increased by 138.1% ($p \leq 0.05$) compared with EAE group.

When assessing specific parameters of the open field technique, it was found that the induction of EAE adversely affected the behavioral characteristics of animals. EAE induction led to a significant decrease in the distance traveled by animals on the 16th day of the experiment by 1.4 times (Table 2).

The free distance in absolute units decreased 1.87 times, but the free distance increased 5.8 times as a percentage of total motor activity. Also, in animals of the control group, an increase of 2.58 times the number of fading was found, and the immobility of animals increased by 1.87 times. All these facts indicate the formation of anxiety in animals with EAE, as well as inhibition of search activity in them. EAE induction did not affect the number of free racks of animals, but led to an increase in racks near the wall. The number of acts of short grooming was also reduced 3 times against the background of the unchanged amount of long grooming. This fact also indicates increased anxiety, excitability, irritability of animals, a decrease in feelings of comfort. In animals of the control group, a decrease in high activity was noted, which indicated a low emotionality and a decrease in the search and research activity of animals. In rats of the control group, a 1.4-times increase in low activity was observed, which indicates the extinction of research activity to assimilate a new situation.

Table 2

The effect of drugs on the tentative research activity of rats with EAE

Index	Intact	Control (EAE)	EAE+MP	EAE+MP+ citicoline	EAE+MP+ noopept
The number of entries in the center, units	1	2	2	1	1
High activity,%, P±m	7.83±1.44	5.23±1.00	5.87±1.8	7.11±1.1	7.77±1.2*[2]
Low activity,%, P±m	61.71±7.08	87.3±4.0	85.1±3.1	87.2±4.0	75.2±2.6
Inactivity,%, P±m	30.47±6.59	52.3±4.3	53.0±4.4	41.0±3.4	34.0±2.4*
Inactivity, units, M±m	284±35	533±21	495±21	410±23*[2]	385±15*[2]
Distance traveled, cm, M±m	4161.8±290.7	2902.0±311.1	3014.1±346.5	3422.1±216.2	3887.2±218.2*[12]
Free distance, cm, M±m	59.3±26.3	32.7±11.2	23.4±8.2	33.6±9.2	38.4±12.5
Fading, unit, M±m	284±35	733±21	784±23*	534±18*	417±22*[12]
Free distance,%, P±m	1.43±0.61	8.30±1.2	8.7±1.6	9.2±2.6	7.2±1.3
The distance to the wall, cm, M±m	4102.44±289.5	3211.2±511.8	3771.3±231.2	3877.2±244.2	3755.1±234.6
Stand at the wall, units, M±m	4±1	6±1	5±1	4±1	4±1
Free stand, units, M±m	4	2±1	1	2	4*[12]
Grooming short, units, M±m	3±1	1	1	1	3*[12]
Long grooming, units, M±m	1	1	1	1	1
Defecation, units, M±m	3	2	2±1	2	2
Urination, units, M±m	1±1	1	1	1	1

Note: * - p≤0.05 in relation to control indicators, 1 - p≤0.05 in relation to citicoline, 2 - p≤0.05 in relation to methylprednisolone.

The course introduction of rats with EAE – methylprednisolone did not affect the indicative research activity and emotional behavior in the test "open field". At the same time, methylprednisolone slightly increased the number of fading, which indicates an increase in anxiety. The course introduction of rats with EAE against the background of methylprednisolone, citicoline, led to some improvement in the tentative research activity and behavior of animals. So, in this group of animals, a significant decrease in inactivity and the number of fading was noted, indicating a decrease in anxiety, irritability of animals and restoration of emotional behavior. The course introduction of rats with EAE on the background of methylprednisolone, intranasally noopept, led to a more complete restoration of the lost research functions and components of the emotional behavior of animals. Thus, in the group of rats treated with noopept, a significant increase in locomotor activity (an increase in the total distance traveled by 1.3 times), an increase in research and search activity (an increase in high activity by 1.5 times and a decrease in immobility by 1.4 times) were observed. It is worth noting that an increase in high activity can also be regarded as a decrease in the "efficiency" of research and search activity, since the rat makes an excessive number of "extra" movements and requires more time to master a new situation. Positive, in the action of noopept, in EAE conditions, was a decrease in anxiety (a 1.7-times decrease in the number of fading) and an increase in the emotionality of animals (a 3-times increase in grooming and a 2-times increase in struts on the wall). Moreover, by the effect on the indicators of anxiety (fading) and emotional activity (reducing inactivity and grooming), the noopept gel significantly exceeds Citicoline. The more pronounced effect of the noopept gel compared to Citicoline is explained by the fact that when administered intranasally, peptides, probably via the extraneuronal pathway, pass through the olfactory epithelium both in the brain region associated with memory and learning processes, as well as in the brain structure responsible for emotional status (forebrain and limbic regions). As a result, Noopept, with intranasal administration, has anxiolytic and nootropic effects.

When assessing specific learning indicators, in the radial labyrinth, it was found that the animals had cognitive dysfunction 27 days after the induction of EAE. The overall activity of the animals of the control group decreased (Table 3).

When reproducing the results of training animals, it was found that on the 27th day after the induction of EAE, the number of working memory errors increased by 5.75 times and the number of reference memory errors by 5 times, which indicated an impairment of memory functions in animals with EAE. Our data are in line with the concept of the formation of cognitive deficits in MS. Administration of methylprednisolone did not affect the cognitive performance of animals with EAE. The course administration of citicoline with methylprednisolone did not affect the indicator of the total activity of animals during training in the labyrinth, but significantly reduced by 2 times the number of reference memory errors and by 25% the number of working memory errors. The introduction of noopept with methylprednisolone led to a significant increase in the total activity of animals with EAE during training by 25.3%, as well as to a 2.5-times decrease in the number of reference memory errors and by 45% in the number of working memory errors.

Table 3

Effect on rat learning and memory in EAE conditions (M±m)

Groups of animals	Total activity, cm^2/s	Number of reference memory errors	Number of working memory errors
Intact	**24380.9±1242.4**	**2**	**4±1**
Control (EAE)	**17865.5±1143.1**	**10±1**	**23±1**
EAE + MP	**18121.5±1022.1**	**10±1**	**20±1**
EAE + MP + Noopept	**22387.4±1077.4***	**4±1*[2]**	**11±1*[12]**
EAE + MP + Citicoline	**19675.2±1121.4**	**5±1*[2]**	**15±1*[2]**

Note: * - $p \leq 0.05$ in relation to control indicators, 1 - $p \leq 0.05$ in relation to citicoline, 2 - $p \leq 0.05$ in relation to methylprednisolone.

To assess the effectiveness of the neuroprotective effect of the studied drugs, the following was calculated: 1) the duration of the latent period of EAE; 2) the total number of rats with moderate and severe course of the experimental equivalent of multiple sclerosis (as a percentage of the number in the group); 3) the average clinical index at the peak of EAE; 4) the average cumulative index of the "disease"; 5) the average duration of the "disease".

The severity of neurological impairment was scored according to the clinical index. The clinical index (Clin) was determined on a scale: muscle weakness of one limb – ½ point, paresis – 1 point, paralysis – 1 ½ point. When several limbs were involved in the process, the points were summarized. Absence of impairments was taken as 0 point, fatal outcome – 6 points. Animals with a clinical index of ½-2 ½ points were assigned to the group with a mild form of the experimental equivalent of multiple sclerosis; 3-6 points corresponded to severe EAE. For an integrative assessment of the severity of EAE for each animal, a cumulative index (Cumul) was calculated – the sum of individual clinical indices for the period of the "illness". The results of the studies indicate that the development of neurological disorders of varying severity was recorded during the induction of EAE in animals of the control group; a lethal outcome of the disease was observed in one of 10 rodents (10%). In the area of inoculation, inflammation was noted that persisted for more than 20 days (Table 4).

After EAE induction, in rats of the control group, the first neurological disorders were recorded on days 9-11. The peak of the clinical manifestations of allergic encephalomyelitis in most animals developed on days 12-14 and lasted an average of 4 days; the duration of EAE was 15.9+1.1 days with an average cumulative index of 27 points. Moreover, at the peak of the clinical manifestations of EAE, the number of animals with a clinical index of ½-2 ½ points was 41.7% of rats, which corresponded to a mild degree of "disease", and severe EAE was observed in 58.3% of rodents (clinical index 3-6 points).

It was found that the administration of methylprednisolone eliminated lethal cases, completely prevented the development of neurological disorders in 10% of animals, and also reduced the number of rodents with severe EAE to 30%. In this case, neurological symptoms of EAE proceeded for a shorter period (duration of EAE decreased by 1.9 times, $p<0.05$) and in mild or moderate severe form (the clinical index

at the peak of the disease decreased by 1.3 times ($p<0.05$), cumulative – almost 2.6 times ($p<0.05$).

The course administration of citicoline, along with the administration of methylprednisolone, eliminated fatal cases, completely prevented the development of neurological disorders in 40% of animals, and also reduced the number of rodents with severe EAE to 10%. At the same time, neurological symptoms of EAE proceeded for a shorter period (duration of EAE was shortened by 2.5 times, $p<0.05$) and in mild or moderate severe form (the clinical index at the peak of the disease decreased 2.28 times ($p<0.05$), cumulative 4.43 times ($p<0.05$).

The course administration of noopept, accompanied by the administration of methylprednisolone also completely eliminated fatal cases, completely prevented the development of neurological disorders in 40% of animals, and also reduced the number of rodents with severe EAE to 10%. At the same time, neurological symptoms of EAE proceeded for a shorter period (the duration of EAE was shortened by 2.78 times, $p<0.05$) and in mild or moderate severe form (the clinical index at the peak of the disease decreased by 3 times ($p<0.05$), cumulative 4.88 times ($p<0.05$).

Table 4

The effect of noopept and citicoline on the course of EAE at the background of basic therapy with methylprednisolone (M±m)

Indicators	Groups of animals			
	control, EAE (n=10)	methylprednisolone (MP) (n=10)	noopept +MP (n=10)	citicoline + MP (n=10)
% sick animals (total / hard)	90/70	80/30	60/10*[2]	60/10*[2]
Average Clin Index at the peak of EAE, points	2,4±0,5	1,85±0,5	0,8±0,4*[2]	1,05±0,15*[2]
Average Cumul index, points	28,8± 1,5	10,4±0,9*	5,9±0,7*[2]	6,5±0,5*[2]
Duration of EAE, days (student test)	15,9 ± 1,1	8,4 ± 0,9*	5,7 ± 0,4*[2]	6,2 ± 0,5*[2]

Note. * - $p\leq0.05$ in relation to control indicators, 1 - $p\leq0.05$ in relation to citicoline, 2 - $p\leq0.05$ in relation to methylprednisolone.

CONCLUSIONS

1. In the studied nasal form, on the model of experimental multiple sclerosis, the presence of normotimic activity, antidepressant and anxiolytic effects, an increase in the total activity of the central nervous system was revealed.
2. According to the degree of influence on reducing the number of working memory errors, in experimental multiple sclerosis, the Noopept gel significantly exceeds monotherapy with methylprednisolone and therapy with a combination of methylprednisolone and citicoline. The results obtained indicate a high neuroprotective and nootropic activity of the Noopept intranasal gel.
3. A further study of the effect of the developed nasal gel on the morphofunctional indices of sensorimotor cortical neurons under experimental multiple sclerosis, as well as on the content of HSP70 in the animal brain is prospective.

Conflict of interests. The authors declare no conflict of interest.

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