ANNALES
UNIVERSITATIS MARIAE CURIE-SKŁODOWSKA
LUBLIN – POLONIA
VOL. XXII, N 3, 20 SECTIO DDD 2009

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Influence of acute administration of diazepam and novel anxiolytic Selank on rabbits' spontaneous behaviour

Wpływ jednorazowego podania diazepamu i nowego anksjolitycznego preparatu Selank na spontaniczne zachowanie się królików

Anxiety is an unpleasant emotional state experienced by people. If pathological or excessive, it is undesirable, it may be a cause of disturbance in human life. Therefore in recent years anxiolytics have become increasingly popular in current use. Diazepam and other benzodiazepines are the most widely prescribed psychotropic drugs that have a variety of effects, including sedative, anticonvulsant, anxiolytic and muscle relaxant activity [4]. They are relatively safe for a short-term treatment, although their sides effects are known [2]. Psychological dependence and physical withdrawal symptoms after abrupt cessation of moderate to high doses have been observed after a long-term use of benzodiazepines [22]. Additionally chronic administration of diazepam has been reported to produce tolerance to its effects [16].

Selank is a synthetic analog of a natural tetrapeptide tuftsin, an integral part of the Fc heavy chain of immunoglobulin G with the sequence Thr-Lys-Pro-Arg. Tuftsin posseses several biological functions connected with the immune system [13]. Some studies have shown that addition of amino acid sequences to regulatory peptides leads to the production of biologically active analogs. Selank contains tuftsin sequence joined with tripeptide Pro-Gly-Pro [1]. In comparison to its precursor the heptapeptide stimulates functions of the immune system weaker [3] but exerts stronger central effects [12].

It is possible to employ the tuftsin analog in the future as a new potential anxioselective drug without behavioral side effects [11]. In the present studies we compared anxiolytic activity of a frequently used compound diazepam and the new tuftsin analog Selank after acute administration. We were interested in testing the hypothesis that Selank has no behavioral side effects. To do this, we examined distinctive types of spontaneous behaviour, typical of rabbits, next the behaviour after the agents' administration. Finally, we compared the results, thus defining the activity and side effects of the used substances.

MATERIAL AND METHODS

Animals. Twenty male Chinchilla rabbits weighing approximately 3250 g were used. Animals were divided into 2 equal groups. They were kept under standard conditions at room temperature of 20±2°C with proper air circulation and natural light-dark cycle. Food and water were available

at all times. Rabbits were brought to the experimental room, placed in the experimental cage and acclimatized to the surrounding conditions 1 h before starting any experiment. In order to avoid possible influences of light-dark cycle on animals' behaviour, the experiments were conducted between 10.00 a.m. and 1.00 p.m.

Drugs. Diazepam was obtained from Polfa, Poznań in injection ampules with the concentration of 5 mg/ml and injected intravenously (*vena marginalis*) at a dose of 1 mg/kg, 35–40 min before the experimental session. Selank was synthesized in the Institute of Molecular Genetics of the Russian Academy of Medical Sciences in Moscow. The synthesis, standardization and analyses of monoacid content were conducted with the use of HPLC and TLC (plates with Silica Gel Silufol, Cavalier of the Czech Republic) in the Laboratory of Regulatory Peptides of the Institute of Molecular Genetics of the Russian Academy of Medical Sciences in Moscow (V.N. Nezabivat'ko) and with HPLC in the Medical University of Lublin, Poland (S. Kawka). Selank was administered with an automatic measuring pipette at a dose of 200 µg/kg, diluted in distilled water up to 200 µl into each nose opening, 15 min before initiation of the experiment.

Procedure. Six forms of rabbits' behaviour: tension, orientation-searching reactions, comfort, grooming, water and food intake were distinguished with the purpose of the estimation of the substances' influence on different animals' reactions. The tension phase was manifested by the tension posture, immobility of the animals, acceleration of breathing and increase in the tension of skeletal muscles. The orientation-searching reactions meant changes in motor activity including exploratory behaviour. The comfort phase was the relaxation of the animals, sleepiness and decrease in the muscle tension. Grooming was the nursing activity. Each daily session of behaviour observation lasted 2 h. Duration of each phase was measured in seconds with a stopwatch.

The rabbits were divided into 2 equal groups. On the first day of the experiment, in both groups spontaneous behaviour was tested. On the second day, 100 ml of distilled water was administered into each nostril of the rabbit with the automatic measuring pipette in group 1. Group 2 received injections of diazepam. On the third day, group 1 received Selank. The procedure of experiments was worked out in the Institute of Pharmacology of the Russian Academy of Medical Sciences in Moscow. The experiments were conducted in accordance with the ethical standards for the humane treatment of animals and Polish legislation concerning animal experimentation.

Statistics. The statistical significance of dissolvent and substance influence on the duration of the distinguished phases was calculated by the Student's test. $P<0.05$ was taken as significant.

RESULTS

The reaction of distilled water was excluded before testing Selank influence. Substances were tested on 2 separated groups of animals. As a result, rabbits' behaviour in control groups exhibited some differences. The rabbits treated with diazepam and Selank showed reduction of the tension phase duration from 15s to 0s and from 110s to 9s, respectively. These changes were not statistically significant, probably due to low initial values, but diazepam completely eliminated the tension phase and its shortening after Selank administration was more than 90% (Fig. 1).

There was a significant effect of diazepam on the orientation-searching reactions. The substance shortened this phase from 1950s to 580s ($p<0.05$). The orientation-searching behaviour was not influenced by tuftsin analog (slight reduction from 840s to 770s) (Fig. 2).

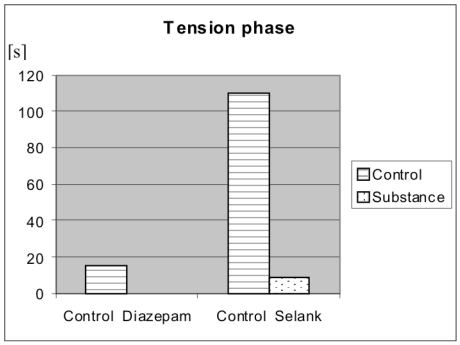

Fig. 1. Tension phase

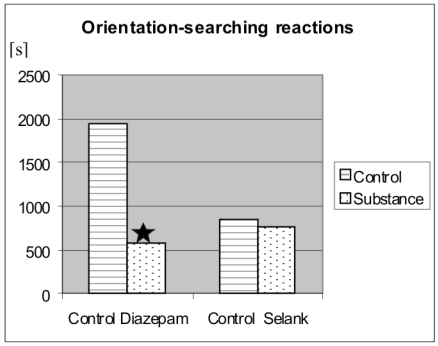

Fig. 2. Orientation-searching reactions

The results demonstrated the differences between diazepam and Selank effects on the comfort phase. Diazepam prolonged the phase from 4500s to 6300s significantly ($p<0.05$), tuftsin analog reduced it minimally from 5100s to 4900s (Fig. 3).

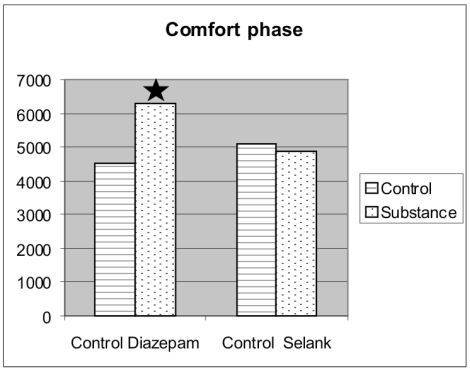

Fig. 3. Comfort phase

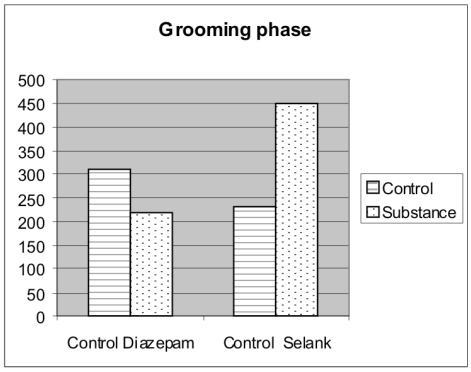

Fig. 4. Grooming

Intra-nasal administration of Selank and its dissolvent caused caring reactions. The duration of grooming after administering distilled water increased from 230s to 450s. Changes observed after tuftsin analog administration were of the same value. Therefore, the results were not taken under consideration. Diazepam did not alter grooming (slight reduction from 310s to 220s) (Fig. 4).

There was a significant decrease in food intake from 360s to 50s following diazepam injection ($p<0.05$). In comparison with benzodiazepine agonist, Selank did not influence eating significantly (the rise from 780s to 850s) (Fig. 5).

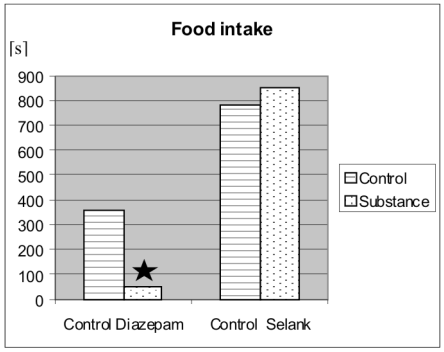

Fig. 5. Food intake

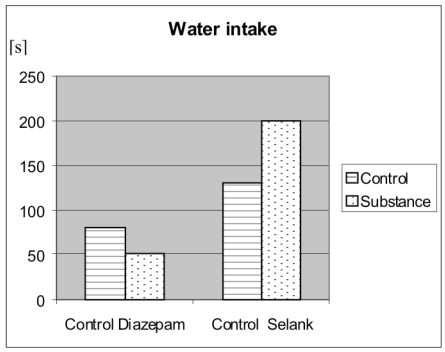

Fig. 6. Water intake

Diazepam and Selank did not alter drinking habits. Changes from 80s to 50s and from 130s to 200s, respectively, were not significant (Fig. 6).

DISCUSSION

In our experiments diazepam and Selank did not exhibit anxiolytic activity. Both substances caused the reduction of behaviours typical of the tension phase (tension, fear, anxiety) but these changes were not statistically significant. Diazepam has been reported to be a standard anxiolytic drug [2, 4, 22]. It binds to central (CBR) and peripheral (PBR) benzodiazepine receptors. Its therapeutic effects are mediated through the CBR, which is the recognition site on the ionotropic GABA receptor that gates chloride ion channels [8, 9]. Diazepam was active in all experimental models of anxiety-like behaviour, including the elevated plus maze and the open field. In the first test diazepam showed dose-dependent increases in both the number of entries into and the time spent in the open arms in comparison to the same parameters of the control group [5, 15]. The open field examines anxiety-related behaviour characterized by the normal aversion of the animal to an open, bright, area. It allows to observe and estimate a wide variety of behaviours including rearing, grooming and defecation. Diazepam tended to suppress anxiogenic activities [5]. It exerted a dose-dependent anxiolytic effect in rats in tests for anxiety but the most effective doses of diazepam depended on the rat strains and stocks [2]. According to Kalueff and Tuohimaa [10], the dose of 1mg/kg has been reported to produce an anxiolytic, non-sedating influence.

Selank shows anxiolytic properties with the nootropic component but unlike benzodiazepines it has no behavioral side effects. In additionm, searching and exploratory reactions are increased by Selank. Probably an amino acid, lysine determines both behavioral effects, whereas the presence of threonine is responsible for the anxiolytic activity [1]. Selank affects the learning process in animals with initially decreased learning ability after a single dose and progressively enhances this function after repeated doses administration [12]. The studies suggest that the tuftsin analog affects tissue levels of most known neurotransmitters in the CNS including norepinephrine (NE), dopamine (DA), serotonin (5HT) and its metabolite 5-hydroxyindoleaceticacid (5-HIAA). In acute treatment, changes in the monoamines concentration occur 10 min after Selank administration and achieve the maximum within 30 min to 2 h. Dopamine levels are found to increase in hypothalamus and cerebral cortex. A decrease in the content of NA and 5HT, a rise in 5-HIAA concentration are shown in

hypothalamus and brain stem. Due to the fact that DA is a precursor of NA, Semenova et al. suggest that Selank influences dopamine beta-hydroxylase activity. Beta-hydroxylase catalyses reaction of dopamine metamorphosis into NA. Additionally, the importance of the monoamine systems in the mediation of certain behaviours in stress and in anxiety states leads to the suggestion that antianxiety properties of Selank are related to changes in the concentration of these monoamines [17]. It also enhances leucocytes chemotaxis, motility and leucopoiesis [3].

Selank was tested in the open field model. Rapid movements of rodents in the periphery zone, the numbers of excursions to the central, dangerous one and vertical rearing were investigated. Animals with the "passive" type of the emotional stress response showed both an increase in peripheral and central movement activity in the open field in comparison to the control group. Changes in the "active" type of the emotional stress response group were not statistically significant. In the Persholt forced swimming test the tuftsin analog exerted anxiolytic activity causing a decrease in both the immobility time and the number of the episodes of immobility. The duration of active swimming and the time to the first episode of immobility were increased after Selank administration [11]. Seredenin et al. [19] noticed that the magnitudes of the anti-anxiety effects of Selank were dose-dependent, without the production of sedation. Semenova et al. [18] and Neznamov et al. [14] emphasised the anxiolytic properties of the heptapeptide. In patients, a single dose of Selank reduced anxiety, emotional and muscular tension, restlessness, fatigue. It improved activity and ability to work [14].

According to a report by Semenova et al. [18], single administration of Selank exerted a stimulatory effect on orientation and exploratory behaviour of animals in the open field although this effects depended on the season. The present study demonstrated that Selank did not influence the orientation-searching reactions under spontaneous, normal conditions. It increased motor activity in stress [11, 17].

In general, Selank has been considered to have both anti-anxiety properties and an activating influence on animals' behaviour [11]. Additionally, it affected learning and memory processes in rats with normal and functionally decreased learning ability [12]. As compared with the heptapeptide, diazepam reduced the orientation-searching reactions markedly at the dose of 1mg/kg in our experiments. Bert et al. [2] observed that the locomotor activity was suppressed in Harlan-Wistar rats following the administration of diazepam at the doses from 2mg/kg. On the other hand, in the Harlan-Fischer rats the benzodiazepine induced hyperlocomotion at a dose of 0.5mg/kg and sedation at a higher dose of 3mg/kg. Other authors reported that doses from 0.3 to 5.0mg/kg did not alter the locomotor activity, whereas 10.0 mg/kg induced hyperactivity [15].

In the present experiments diazepam induced sedation, it increased the duration of the comfort phase significantly as compared to the tuftsin analog, which had no influence on this phase. Consistent with these findings, it has been reported that benzodiazepines are sedative compounds [20, 22]. By contrast, tuftsin analog did not produce sedation in animals [19]. Neznamov et al. [14] observed a significant decrease in somnolence in humans after Selank administration.

An accepted view is that the grooming investigation is very important in many anxiety studies and anxiety leads to increased grooming duration and frequency. Kalueff and Tuohimaa [10] showed no clear correlation between anxiety, anxiolytic and anxiogenic drugs and grooming scores. In their experiments diazepam did not alter either the duration of grooming or the number of grooming bouts at any dose administered. We found that diazepam did not influence grooming. Selank effect was not analysed as it was explained above.

Previous studies showed that benzodiazepines enhanced food intake [6, 15, 20] and the central benzodiazepine receptors mediated this effect [9]. The brain stem, especially the parabrachial nucleus, was implicated in enhancement of feeding by diazepam. It was suggested that the facilitation of eating was produced due to direct effects on appetite and palatability [20]. It was also reported that in

rats chronic diazepam treatment induced a smaller body weight increase than in controls [9]. In our experiments diazepam suppressed eating. The observations were consistent with the findings of Rex et al. [15]. The authors showed that in the food consumption test in the home cages and in the food consumption in the open field diazepam did not increase the feeding in rats. Our results demonstrated that Selank did not alter food intake. Czabak-Garbacz et al. [7] investigated the influence of a long-term treatment with tuftsin analog on rats' body weight. In their study similar results were observed both in rats injected with the heptapeptide and those receiving distilled water. In both groups the mean body weight increased more slowly than in the control one. Therefore, the changes could not be produced by Selank.

We found that diazepam and Selank did not influence water intake. Stout and Weiss [21] reported that animals given diazepam drank more times than animals injected with physiological saline. But the compound did not induce the amount of water consumed and did not reduce latency to begin drinking. Those experiments demonstrated that the enhancement of water intake by benzodiazepine was associated rather with the sedative effects of the substance than the influence of the drug on drinking behaviour.

CONCLUSIONS

Selank seems to be a selective anxiolytic compound without any influence on rabbits' spontaneous behaviour, including motor activity, eating, drinking. Additionally, as compared to many popular anxiolytic drugs it does not produce sedation.

Acknowledgement. The work was supported by grants from Medical University of Lublin (PW 434/00).

REFERENCES

1. Ashmarin I. P., Samonina G. E., Lyapina L. A. et al.: Natural and hybrid ("chimeric") stable regulatory glyproline peptides. Pathophysiology, 11, 179, 2005.
2. Bert B., Fink H., Sohr R., Rex A.: Different effects of diazepam in Fischer rats and two stocks of Wistar rats in tests of anxiety. Pharmacol. Biochem. Behav., 70, 411, 2001.
3. Bulatova N. R., Romanova E. A., Krinskaya A. V. et al.: Poststressor correction of the functional macrophage activity by tuftsin and its derivatives. (Russian) Biull. Eksp. Biol. Med., 108, 64, 1989.
4. Burke F. T., Miller L. G., Moerschbaecher J. M.: Acute effects of benzodiazepines on operant behavior and *in vivo* receptor binding in mice. Pharmacol. Biochem. Behav., 1994, 48(1), 69-76.
5. Clement Y., Chapouthier G.: Biological bases of anxiety. Neurosci. Biobehav. Rev., 22(5), 623, 1998.
6. Cooper S. J.: Palatability-dependent appetite and benzodiazepines: new directions from the pharmacology of GABA A receptor subtypes. Appetite, 44(2), 133, 2005.
7. Czabak-Garbacz R., Cygan B., Wolański Ł., Kozlovsky I. I.: Influence of long-term treatment with tuftsin analogue TP-7 on the anxiety-phobic states and body weight. Pharmacol. Rep., 58, 562, 2006.
8. Houston A. J., Wong J. C. L., Ebenezer I. S.: Effects of subcutaneous administration of the gamma-aminobutyric acid A receptor agonist muscimol on water intake in water-deprived rats. Physiol. Behav., 77, 445, 2002.

9. Jing X., Wala E. P., Sloan J. W.: The effect of PK 11195, a specific antagonist of the peripheral benzodiazepine receptors, on body weight in rats chronically exposed to diazepam. Pharmacol. Res., 42(3), 227, 2000.
10. Kalueff A. V., Tuohimaa P.: Mouse grooming microstructure is a reliable anxiety marker bidirectionally sensitive to GABAergic drugs. Eur. J. Pharmacol., 508, 147, 2005.
11. Kozlovskaya M. M., Kozlovski I. I., Val'dman E. A., Seredenin S. B.: Selank and short peptides of the tuftsin family in the regulation of adaptive behaviour in stress. Neurosci. Behav. Physiol., 33, 853, 2003.
12. Kozlovski I. I., Danchev N. D.: The optimizing action of the synthetic peptide Selank on a conditioned active avoidance reflex in rats. Neurosci. Behav. Physiol., 33, 639, 2003.
13. Lang O., Mezö G., Hudecz F., Köhidai L.: Effect of tuftsin and oligotuftsins on chemotaxis and chemotactic selection in Tetrahymena pyriformis. Cell. Biol. Int., 30, 603, 2006.
14. Neznamov G. G., Telashova E. S., Bochkarev V. K., Koschelev V. V.: Novel anxiolytic Selank: Results of the phase II clinical trials. Eur. Neuropsychopharmacol., 15, suppl 2, 159, 2005.
15. Rex A., Stephens D. N., Fink H.: "Anxiolytic" action of diazepam and abecranil in a modified open field test. Pharmacol. Biochem. Behav., 53(4), 1005, 1996.
16. Scott S. J, Smith P. F., Darlington C. L.: Quantification of the depressive effects of diazepam on the guinea pig righting reflex. Pharmacol. Biochem. Behav., 47(3), 739, 1994.
17. Semenova G. P., Gurevich E. V., Kozolvskaya M. M., Gromova E. A.: The role of the brain monoaminergic systems in the effects of tuftsin and its analogue on animal emotional behaviour. (Russian) Fiziol. Zh. SSSR. Im I. M. Sechenova, 75, 759, 1989.
18. Semenova T. P., Kozlovskaya M. M., Zuikov A. V. et al.: Seasonal effects of Selank on the behaviour of hibernating animals. (Russian) Biull. Eksp. Biol. Med., 140, 658, 2005.
19. Seredenin S. B., Blednov Y. A., Kozlovski I. I.: Studies of the anti-anxiety action of an analog of the endogenous peptide tuftsin in inbred mice with different phenotypes for emotional stress reactions. Zh. Vyssh. Nerv. Deyat., 48(1), 153, 1998.
20. Söderpalm A. H. V., Berridge K. C.: Food intake after diazepam, morphine or muscimol: microinjections in the nucleus accumbens shell. Pharmacol. Biochem. Behav., 66(2), 429, 2000.
21. Stout J. C., Weiss J. M.: An animal model for measuring behavioral responses to anxiogenic and anxiolytic manipulations. Pharmacol. Biochem. Behav., 47(3), 459, 1994.
22. Tallmann J. F., Paul S. M., Skolnick P. P., Gallager D. W.: Receptors for the age of anxiety: pharmacology of the benzodiazepines. Science, 207, 274, 1980.

SUMMARY

The present study compared effects of the CBR agonist diazepam and a synthetic analog of tuftsin Selank on six forms of rabbits' spontaneous behaviour. The experiments were performed in 2 groups of animals. In the first group diazepam was injected intravenously at a dose of 1 mg/kg, in the other Selank was administered intranasally at a dose of 200 μg/kg. Each daily session lasted 2h. Diazepam reduced orientation-searching reactions and food uptake and it prolonged the comfort phase. Tuftsin analog did not influence the examined reactions.

STRESZCZENIE

Celem badania było porównanie wpływu pochodnej benzodwuazepiny – diazepamu i syntetycznego analogu tuftsyny – Selank na sześć rodzajów spontanicznego zachowania się królików. Eksperymenty były wykonywane w dwu grupach zwierząt. W pierwszej grupie podawano dożylnie diazepam w dawce 1 mg/kg, w drugiej grupie podawano donosowo Selank w dawce 200 µg/kg. Każde doświadczenie trwało 2h. Diazepam redukował reakcje orientacyjno-poznawcze i pobieranie pokarmu, wydłużał fazę komfortu. Analog tuftsyny nie zmieniał czasu trwania badanych faz.