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LUMINESCENCE ANALYSIS OF BLOOD COMPONENTS IN STUDIES OF THE NEUROPROTECTIVE PROPERTIES OF THE DRUG "SEMAX" IN CEREBRAL ISCHEMIA

I. M. Vlasova,* D. E. Buravtcov, and A. M. Saletsky

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Luminescence-spectral analysis is used to study the neuroprotective effect of the drug Semax on the level of oxidative stress in cerebral tissues during cerebral ischemia through estimation of the free-radical damage to low density lipoproteins in animal blood serum. It is found that Semax decreases the oxidative stress in cerebral tissues during ischemia by reducing the production of free-radical compounds owing to activation of an inflammation line of protection for the organism.

Keywords: *luminescence, fluorescent probe, blood serum, cerebral ischemia, low density lipoproteins, Semax*

Introduction. In previous studies of optical spectroscopic techniques, we have observed differences in the low density lipoproteins (LDLP) in the blood serum of animals with experimentally induced cerebral ischemia compared to a control group of healthy animals: following cerebral ischemia the size of the serum LDLP increases while their density decreases. These changes, involving free-radical oxidative damage to unsaturated fatty acids in the outer amphipathic phospholipid layer of the LDLP in the serum of animals following cerebral ischemia, have also been observed by correlation spectroscopy of laser scattered light [1] and by Rayleigh scattering spectroscopy [2], as well as by fluorescence analysis [3].

We have explained the detected changes in the LDLP owing to cerebral ischemia in terms of a free-radical theory for oxidative stress. Elevated production of active forms of oxygen (AFO) occurs during ischemia for two reasons. The first is the breakdown of oxidative phosphorylation in cerebral tissues, which leads to the formation of the superoxide radical $O_2^{-\bullet}$. The second is the activation of an inflammation line of defense of the organism during necrosis of brain tissue, i.e., activation of macrophages and neutrophils which produce a lot of free-radical compounds ($O_2^{-\bullet}$, H_2O_2, $NO^{\bullet}$) owing to their fermentative systems (NADPH-oxidases, xanthioxidases, NO-synthases, lipoxygenases). The buildup of free-radical products in the brain tissue during ischemia leads eventually to their entry into the blood and to damage to components of the blood, in particular, to modification of blood lipoproteins (especially the LDLP) [1–3].

In recent medical analytical research there has been increased interest in studies of the protective mechanisms in the brain which protect it from damage during cerebral ischemia. Considerable attention has been devoted to the study of the protective action of ischemic preconditioning. Ischemic preconditioning is a series of short "brief ischemia-reperfusion" cycles, i.e., a sequence of "blood flow pinch- blood flow recovery" cycles, in an organ that precede a prolonged global ischemia of that organ. In fact, ischemic preconditioning is aging of the organ by small periods of ischemia before it undergoes prolonged global ischemia. A protective effect from ischemic surgical preconditioning in terms of free-radical lipo-peroxide damage to the LDLP in animal blood which delay cerebral ischemia has been observed by spectroscopic methods [4,5].

Studies of medical drugs with a neuroprotective action currently play a major role in research on the mechanisms for protection of the brain from damage during ischemia. One such drug is the synthetic polypeptide Semax (methionin–glutamine–histidine–phenylalanine–proline–glycine–proline, Met-Glu-His-Phe-Pro-Gly-Pro), whose pentide sequence has been patented by the Institute of Molecular Genetics of the Russian Academy of Sciences. Semax, a syn-

*To whom correspondence should be addressed.

M. V. Lomonosov Moscow State University, Leninskie Gory, 119992 Moscow, Russia; e-mail: vlasovairina1979@mail.ru. Translated from Zhurnal Prikladnoi Spektroskopii, Vol. 76, No. 1, pp. 131–137, January–February, 2009. Original article submitted March 25, 2008.

thetic polypeptide, is an analog of fragment 4–7 of adrenocorticotropic hormone (ACTH) without the hormonal activity. Semax belongs to the group of neuropeptides with an adaptive and nootropic effect, and enhances the resistance of an organism during hypoxia and cerebral ischemia. Because of its neuroprotective properties, during a cerebral thrombosis Semax greatly limits the damage to the brain if it is used immediately after the stroke.

This paper is a study of the mechanism of the neuroprotective action of the drug Semax at the level of oxidative stress in brain tissues during ischemic strokes (cerebral ischemia) evaluated in terms of free-radical damage to LDLP in animal blood serum by means of luminescence-spectral analysis.

Materials and Methods. These studies were carried out on the blood serum of Wistar rats (n = 28; young females of mass 200 ± 30 g). The animals were divided into two different groups: Semax was given intraperitoneally (100 μl per 100 g of mass) to the first group (n = 14) and a physiological solution of the same volume was given to the second (control) group (n = 14). Experimental cerebral ischemia was induced in both groups. The animals were first doped with an intraperitoneal injection of chloral hydrate (350 mg/ml). Total global cerebral ischemia was achieved by cutting off the flow of blood through both interior carotid arteries for 2 h. Semax was first administered to the first group and physiological solution to the second 15 min after the start of the cerebral ischemia procedure. The second administration of Semax and of physiological solution to the first and second groups, respectively, took place after reperfusion of the interior carotid arteries (i.e., after termination of the cerebral ischemia procedure), 15 min after reperfusion (i.e., 2 h 15 min after the start of the cerebral ischemia procedure).

Blood was drawn from the animals' jugular veins. Several samples were taken from both groups of animals in order to evaluate the pharmacological effect of the Semax: sample 1 prior to the cerebral ischemia operation, and samples 2–7, 1, 2, 3, 5, 7, and 10 days, respectively, after the operation. The blood serum used in the subsequent spectroscopic studies was obtained in the standard way from the blood samples [1].

The fluorescence studies were conducted using a Perkin-Elmer LS 55 spectrofluorometer. Samples of serum from both groups of animals were placed without dilution into cuvettes at room temperature to study the effect of Semax on the level of oxidative stress in the brain tissue during ischemia in terms of the oxidative damage to the serum LDLP.

The fluorescence spectra were measured over the range 400–600 nm with irradiation of the serum by light with λ_{exc} = 350 nm and then processed using the Perkin-Elmer FL Winlab program. Fluorescence spectra of the blood serum samples from both groups of animals, obtained on different days, were measured in order to study the kinetics of the action of Semax, and the average spectra for both groups were obtained.

The effect of Semax at the level of oxidative stress of the organism in terms of the damage to the serum LDLP was also studied using a fluorescence molecular probe, rhodamine 6G, which was added to serum samples from both groups at a concentration of 60 μM. The fluorescence of rhodamine 6G in the serum samples was recorded over wavelengths of 540–660 nm with an excitation wavelength of λ_{exc} = 530 nm. The resulting fluorescence spectra were processed by the Perkin-Elmer FL Winlab program. Fluorescence spectra of rhodamine 6G in the serum of both groups of animals obtained on different days were measured in order to study the kinetics of the action of Semax.

Results and Discussion. During these studies, fluorescence spectra (λ_{exc} = 350 nm) of serum samples obtained on different days from both groups of rats (those which received the Semax and the control group which received physiological solution) were obtained. The excitation wavelength for the fluorescence of the serum, λ_{exc} = 350 nm, was chosen [3] because excitation by light with λ_{exc} > 300 nm produces emission owing to the nonprotein components of the blood serum (phospholipid components of the lipoproteins), which are of interest for this work, while excitation by light with λ_{exc} < 300 nm would produce emission from the protein portion of the serum.

Figure 1 shows the fluorescence spectra of serum samples from rats subjected to experimental cerebral ischemia that were given either Semax or the physiological solution. Fluorescence spectra of the serum samples were taken both before the experimental cerebral ischemia procedure and after it on different days in order to evaluate the pharmacological properties of the drug Semax. It is clear that, after the experimental cerebral ischemia procedure, an increased intensity can be observed in the peaks of the fluorescence spectra of the blood serum from both groups of animals compared to the peak intensities prior to cerebral ischemia. The observed effect can be explained in terms of a free-radical theory of oxidative stress. During cerebral ischemia, active forms of oxygen are produced in the brain tissue which then enter the blood flow and damage the phospholipid components of the LDLP, oxidizing them and forming lipoperoxide compounds in place of unsaturated fatty acids in them. The increased intensity of the serum fluo-

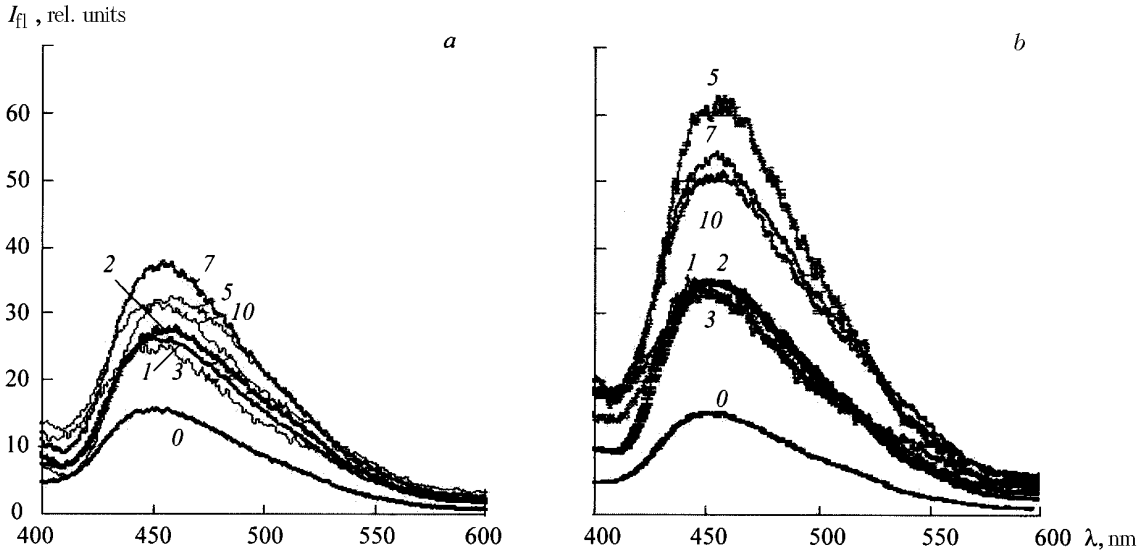

Fig. 1. Fluorescence spectra (λ_{exc} = 350 nm) of the blood serum of rats which received Semax (a) and physiological solution (b) on different days; the numbers on the spectra indicate the day following a model cerebral ischemia; 0 corresponds to a measurement taken on the day of the ischemia procedure.

rescence following cerebral ischemia is caused by the presence of unpaired electrons in the free-radical compounds of the serum from the animals after ischemia and, therefore, by a higher probability of excitation of the molecules of these compounds during irradiation. An examination of the kinetics of the action of Semax over the days following the cerebral ischemia shows that there are differences between the two groups of animals (those which received the Semax and the physiological solution) in the peak intensity of the serum fluorescence spectra.

Figure 2 illustrates the variation in the peak intensity of the serum fluorescence spectra (λ_{exc} = 350 nm) for the animals in the two groups over the days following the model experimental cerebral ischemia. It can be seen that over the first three days following the model experimental cerebral ischemia, the differences in the intensity of the peak in the serum fluorescence spectra between the two groups of animals (those which received the Semax and the physiological solution) are negligible. This indicates roughly equal levels of oxidative free-radical damage to the components of the blood serum. Substantial differences between the two groups show up after the third day following cerebral ischemia. Although an increase is observed in the peak intensity of the serum fluorescence spectra for both groups, the increase for the control group, the animals given the physiological solution, is considerably greater (by a factor of 1.9 compared to the third day following the operation) than for the group of animals who were given Semax (a factor of 1.5 compared to the third day following the operation). Then, regarding the differences between the two groups of animals, it should be noted that in the group which received Semax there is a more monotonic daily variation in the intensity of the peak in the serum fluorescence spectrum, while in the control group, which received the physiological solution, there is a sharp burst of intensity in the peak of the serum fluorescence spectra (on the fifth day following the ischemia simulation).

This effect is most likely explained by the effect of the Semax on the inflammation protection of the organism, which is activated upon necrosis of the brain tissue following the ischemia procedure. The enhanced production of active forms of oxygen and of peroxide oxidation products of lipids in the cells of the brain tissues is a universal response of cells in the initial stage of inflammation owing to necrosis of tissue following ischemia. At an inflammation site formed during tissue necrosis the products of peroxide oxidation of lipids modulate the metabolic activity of phagocytic cells. Thus, the primary products of lipid peroxide oxidation formed owing to disturbances of oxidative phosphorylation have a significant effect on the activation and development of the inflammation reaction. The development of oxidative stress during inflammation both induces the arrival of phagocytic cells into the inflammation site and modulates the receptor properties of these cells, thereby increasing their activity. As the inflammatory reaction de-

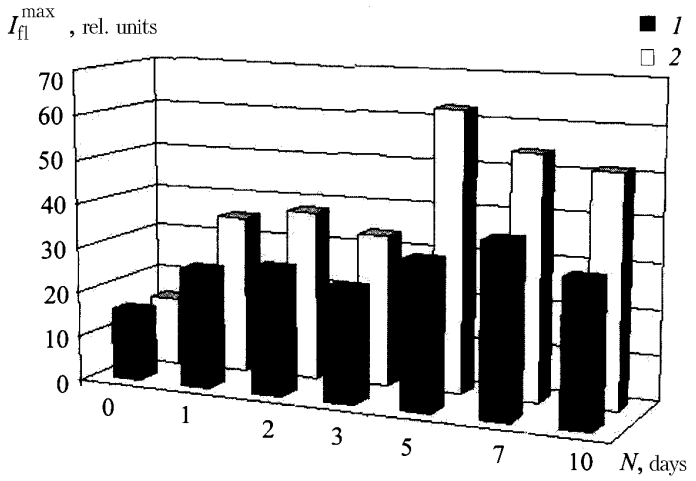

Fig. 2. Intensity of the fluorescence spectrum peak (λ_{exc} = 350 nm) of the blood serum of animals who received Semax (1) or physiological solution (2) on different days after a model experimental cerebral ischemia procedure. Column 0 corresponds to measurements taken before the ischemia procedure.

velops, the toxic fermentates and active forms of oxygen released from the phagocytic cells damage the connective tissue and microvesicles. The elevated level of production of active forms of oxygen by the phagocytic cells causes damage to the proteins, lipids, and nucleic acids in the brain tissues. Oxidation of the lipids leads to the appearance of chemoattractants which facilitate migration of phagocytic cells into the inflammation site. In this way a vicious circle or state of "chronic oxidative stress" is initiated, the maintenance of which requires the arrival of phagocytic cells and the presence of oxidative substrates–unsaturated fatty acids [6].

The inflammatory line of defence of the organism does not set in at once after cerebral ischemia, but only after two or three days; here macrophages and neutrophils are activated, which produce a multitude of free-radical compounds owing to their fermentative systems which damage components of the blood when they arrive in the blood stream. In the group of animals who received the physiological solution, because of the inflammatory line of defence there is significant oxidative free-radical damage of the blood components three days after the experimental cerebral ischemia was induced. In the group of animals who received Semax, such strong oxidative damage to the blood components was not observed three days after induction of the experimental cerebral ischemia. This indicates that Semax influences the level of operation of the inflammatory line of defence during cerebral ischemia: the Semax halts the chronic oxidative stress.

The neuroprotective action of Semax at the level of oxidative stress in brain cells in terms of free-radical damage to the components of the blood during cerebral ischemia was also studied using a fluorescence probe of rhodamine 6G, which is widely used in studies of serum LDLP [3, 5]. Rhodamine 6G has no chemical effect on the phospholipid components of serum LDLP, but, since it has a high fluorescence quantum yield, does serve to emphasize the physical and chemical changes in the serum before and after an ischemia procedure.

Measurements were made of the fluorescence of rhodamine 6G (λ_{exc} = 530 nm) added to samples of blood serum obtained on different days from the rats in both groups, i.e., those who received Semax and the control group who received the physiological solution. Figure 3 illustrates variation in the peak intensity of the fluorescence spectrum (λ_{exc} = 530 nm) of rhodamine 6G added to the serum from the two groups of animals (receiving Semax and control) in the days following the cerebral ischemia procedure. Clearly, after the cerebral ischemia procedure there is an increase in the peak intensity of the fluorescence spectrum of rhodamine 6G added to the serum compared to before the procedure for both groups of animals. This increase in the fluorescence intensity of rhodamine 6G happens because the rhodamine 6G enters the system under study as a fluorescence probe that accepts energy from excited lipid free-radical compounds in the composition of the phospholipids of the LDLP, which show up as a consequence of the ischemic oxidative damage to the phospholipid layer of the LDLP.

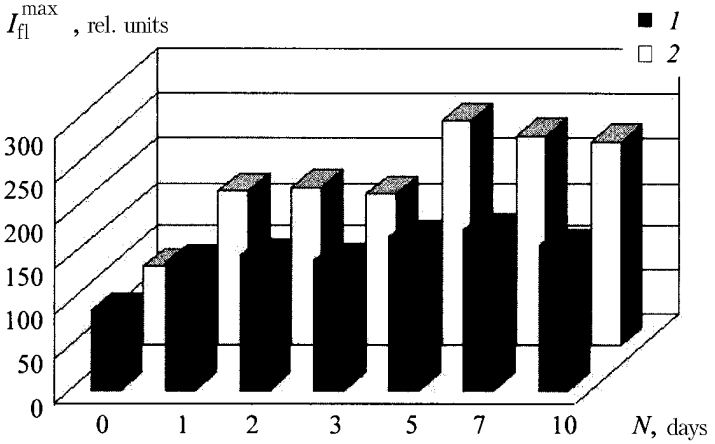

Fig. 3. Intensity of the peak of the fluorescence spectrum (λ_{exc} = 530 nm) of rhodamine 6G (60 μM) added to the blood serum of animals who received Semax (1) or physiological solution (2) on different days after a model experimental cerebral ischemia procedure. Column 0 corresponds to measurements taken before the ischemia procedure.

In an examination of the kinetics of the action of Semax in the days following the cerebral ischemia procedure, it was found that there are differences in the peak intensity of the fluorescence spectra of the rhodamine 6G added to the blood serum for the two groups of animals (those receiving Semax and physiological solution).

It can be seen that, as in the previously examined case of the fluorescence of the blood serum, during the first three days after the simulated cerebral ischemia the difference in the intensities of the peaks of the fluorescence spectra of rhodamine 6G in serum between the two groups of animals (those receiving Semax and physiological solution) was negligible; this indicates that there is a similar level of oxidative free-radical damage to the components of the serum in both groups. However, beginning with the third day after the simulated cerebral ischemia there is a large increase (by a factor of 1.5 relative to the third day after the ischemia) in the fluorescence of rhodamine 6G added to the serum of the animals who received the physiological solution compared to the group of animals who received Semax (by a factor of 1.2 relative to the third day after the ischemia). Thus, the rhodamine 6G fluorescence probe confirms that, when Semax is given to the animals immediately during a cerebral ischemia, there is relatively less damage to the brain tissue in terms of free-radical damage. A therapeutically significant neuroprotective effect of Semax begins to show up on the third day after a cerebral ischemia.

Conclusion. As indicated above, during cerebral ischemia active forms of oxygen are produced more rapidly for two reasons. First, oxidative phosphorylation breaks down in the cerebral tissues. Second, the inflammatory mechanism for protection of the organism sets in, i.e., macrophages and neutrophils are activated and these produce a multitude of free-radical compounds owing to their fermentative systems. Because of the disruption of oxidative phosphorylation in the cells of the cerebral tissues, active forms of oxygen are produced immediately following or within the first day after a cerebral ischemia. The inflammatory line of protection of the organism after necrosis of the cerebral tissue is actuated later — two or three days after the ischemia procedure. These experiments for evaluating the free-radical damage to the components of the serum of animals employing luminescence analysis show that the drug Semax reduces the oxidative stress in cerebral tissue during ischemia by reducing the contribution of the second of these reasons and halting the chronic oxidative stress.

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