Current Topics in Biophysics 2002, 26(1), 129-136

GENERATION OF NITRIC OXIDE AS A PREDICTIVE PARAMETER IN MEDICINE

ANNA BRATASZ[1], BOŻENA POLOK[1], IWONA KUTER[1], IGOR GOŚCIŃSKI[2], WOJCIECH TRACZEWSKI[2], MARIUSZ KRUPA[2], MAREK MOSKAŁA[2], PPRZEMYSŁAW GAŁKA[2], RYSZARD KONIOR[3], STANISŁAW ŁUKIEWICZ[1]

[1]Jagiellonian Universsity, Instituite of Molecular Biology, ul. Gronostajowa 7, 30-387 Kraków;
[2]Collegium Medicum, Department of Neurotraumatology;
[3]Cracow City Hospital, Department of Neuroinfections;

The paper discusses the question whether the intensity of nitric oxide production may serve in human individuals as a marker of prognostic value. This problem is examined partly on the basis of current literature (see introduction and discussion) and partly in the light of our own clinical studies. The latter were limited to neurological patients who delivered samples of the cerebrospinal fluid. This material was examined for the content of nitric oxide using electron spin resonance (EPR) techniques. In adult patients with meningitis the level of nitric oxide was found to be markedly higher as compared with other groups of brain diseases, such as brain traumas and brain tumors. An excessively intense generation of nitric oxide by the brain, expressed in its high concentration in the cerebrospinal fluid, was frequently associated with an increased number of fatal outcomes. In children suffering from meningitis, EPR analysis made it possible to distinguish between viral and bacterial background of disease in a quick and easy way. Altogether the data indicate that nitric oxide level may become in future a useful prognostic marker at least in the case of neurological diseases.

INTRODUCTION

Ranges of nitric oxide production

First of all one has to realize that the amount of nitric oxide generated in the organism may vary in wide limits (Henry, Guisami & Duzastel, 1997). There are two extremes in this regard: nitric oxide underproduction, leading to excessively low levels of this substance in the body, and nitric oxide overproduction which brings about its accumulation in animal system in very high concentrations. Inbetween one can distinguish a range of nitric oxide generation which might be considered normal.

Normal production of nitric oxide

Its level depends on the type of body fluid or tissue. In the case of a cerebrospinal fluid of normal healthy volunteers nitric oxide concentration amounts to between 4 - 10 μmol/l. The same holds true for neurologically healthy patients suffering from headache, hypertension or vertigo as well as for HIV patients at the early stage of disease.

Underproduction of nitric oxide

The level of nitric oxide is slightly lower in Langerhans islets (5 – 8 μmol/l) and in peripheral blood (3 – 4 μmol/l). The concentration of nitric oxide may drop much below 4 μmol/l and approach zero in terminal HIV patients. In endothelial cells isolated from human umbillical veins which represent a classical model for studying the process of in vitro aging of cells, the level of nitric oxide is about one thousand times lower, i.e. reaches the value around 4 nanomol/l. In general, underproduction of nitric oxide is a phenomenon much less frequent than overproduction of this substance.

Overproduction of nitric oxide

One can distinguish 4 types of situations which may lead to an excessive generation of nitric oxide. These are namely: infections, transplant rejection, tumor defense and autoimmune diseases.

As regards the first of them, overproduction of nitric oxide may be brought about by either bacterial, parasitic or viral infection. If the process has a bacterial background, the concentration of this substance may oscillate between 30 μmol/l and 70 μmol/l in septic shock which kills at least 200 thousand people per year in the U.S.A. alone and about 100 thousand in the European Community. Another huge medical problem is a parasitic cerebral malaria which may take 200 - 300 millions of victims over the world and is associated with a level of about 80 μmol/l of nitric oxide in patients revealing an effective defence of the immune system which may promise a favourable

able outcome. Neurological complications observed during this disease are going hand in hand with a significant drop of nitric oxide concentration (to about 40 μmol/l) resulting from a weaker immunity. It may increase, however, substantially as a result of a quinine treatment which strengthens patient's defense system.

Similar relations are found in viral diseases such as chorioretinitis in AIDS patients and Cytomegalo virus infections. In all these cases an increase in the production of nitric oxide during various kinds of sicknesses brought about by bacteria, parasites or viruses, might be ascribed to a massive activation of macrophages and other cells of the immune system which is an expressions of a strong defense reaction of the organism. In this particular case a high level of nitric oxide seems to be associated with a good prognosis. As will be seen later, this is, however, not always the case. In brain disorders one can observe an opposite correlation. Here the elevated amounts of nitric oxide should rather be interpreted as a warning anouncing a possible death of patient.

Transplant rejections is the next group of processes which may be associated with an overproduction of nitric oxide. From the very beginning of the era of heart transplantations, physicians tried to counteract the process of rejection in various ways. One of them was to block nitric oxide synthesis. In fact, some of the inhibitors of nitric oxide synthase II proved to be quite effective in this respect in animal systems.

Inhibition of nitric oxide generation is, however, not always followed by affecting the process of heart rejection. As an example N^{ω}-monomethyl-L-arginine may be given. This is an inhibitor of nitric oxide synthase I which effectively blocks the activity of this enzyme without, however, counteracting transplant rejection. It is not the case with aminoguanidyne which inhibits nitric oxide synthase II, but is able both to prolong graft survival and reduce the rejection grade.

In the case of liver allografts nitric oxide has a good chance to become a predictive parameter. This is due to 3 reasons. First, because its level markedly increases during the acute phase of rejection. Secondly, because it correlates well with the severity of rejection, and finally because it decreases during anti-rejection treatment. Under these circumstances the process of acute rejection becomes detectable by monitoring the level of nitric oxide, whatever would be the method of its quantitative determination.

The third group of factors which may be responsible for overproduction of nitric oxide are associated with the process of tumor defense. Very interesting studies along this line were performed on human carcinoma in nude mice. They have shown that nitric oxide can either promote or inhibit tumor growth depending on its local concentration. If it is low, nitric oxide behaves as a promoter. If it is however high, nitric oxide starts to act as an anti-tumor agent.

As regards human cancers in patients, it is essential to realize that nitric oxide is always produced in the tumor itself, but not in its healthy surroundings. Nitric oxide complexes with hemoglobin have been found to accumulate in the necrotic centre of tumors. Nitric oxide is also frequent in the areas of chronic inflammation of digestive and urogenital systems. The next point is that in breast cancer nitric oxide generation correlates with the differentiation and grade of tumors.

Finally, the neoplasms of the central nervous system are rich in constitutive nitric oxide synthases and an inducible nitric oxide synthase. So, high amounts of nitric oxide can be found in the tumors of neuronal origin, as will be seen later.

The last group of factors which are able to cause overproduction of nitric oxide are autoimmune diseases. One can illustrate this by three examples. As one of them psoriasis, a skin disease of unknown origin may serve. As the next example the whole group of rheumatoid diseases, and in particular arthritis can be quoted. Central nervous system diseases, and especially multiple sclerosis and schizophrenia, might be chosen as the last illustration. Unfortunately, in neither of these three cases of autoimmune diseases, the real reason and mechanism explaining excessive generation of nitric oxide is known.

From this short review it is evident that there is quite a number of disturbances in nitric oxide production in the pathological brain. Just to name them briefly, these are cerebral toxoplasmosis and malaria, schizophrenia and multiple sclerosis, brain infarcts associated with artery occlusion, central nervous system neoplasms, structural injuries of motoneurons or blocks of synaptic transmission. As we said, nobody knows why excessive generation of nitric oxide occurs in all these cases.

In spite of this poor understanding of basic facts, people are trying to prevent and treat such pathological conditions, although it is understandable that great success cannot be expected under these circumstances. The present attempts are based on three pretty obvious approaches, namely on: (1) inhibitors of nitric oxide synthases, (2) complexes with hemoglobin and (3) nicotinamide treatment. All of them have in essence one thing in common: they counteract neurotoxicity.

Types of nitric oxide synthases in the central nervous system

Nitric oxide is biosynthesised in the cells of many tissues (Bentivolgio, Bertini, Mariotti & Peng, 1998) via conversion of L-arginine to L-citruline by a family of nitric oxide synthases (Nathan & Xie, 1994). Once produced, nitric oxide can interact with a number of molecular targets which determine the profile of NO as a major biological mediator, modulator and effector. Three isoenzymes of nitric oxide synthases are responsible for nitric oxide biosynthesis in various tissues (Nathan & Xie, 1994). Neuronal constitutive nitric oxide synthase (NOS1) is expressed in neurones and is responsible for the physiological production of nitric oxide in neural tissues. Nitric oxide synthase NOS3 is mainly confined to endothelium and produces nitric oxide which contributes to the regulation of vascular tone. Nitric oxide produced by these two constitutive NOSes participates in physiological processes. However, inducible NOS (NOS2) is normally not expressed in the majority of cells, but may be induced by the action of various proinflamatory cytokines.

NOS1 and NOS3 are Ca^{+2} and calmodulin dependent isoforms, whereas Ca^{+2} does not regulate NOS2 activity. Activation of guanylate cyclase (GMP) regulated by nitric oxide can increase the release of cyclic guanosine monophosphate (cGMP) and can facilitate neuronal transmission (Garthwait, Charles & Chess-Williams, 1988), promote vascular relaxation (Furchgott & Zawadzki, 1980), inhibit platelet and leukocyte aggregation and adhesion (Radomski & Palmer, 1987) and regulate brain fluid balance (Faraci & Heistad, 1992). Reduction of basal nitric oxide release may predispose to hypertension, thrombosis, vasospasm, and arteriosclerosis (Kugiyama, Sugiyama, Matsumura, Ohta, Doi & Yasue, 1996). On the other hand a high level of nitric oxide which occurs during excessive NOS2 expression is generally toxic (Nathan & Xie, 1994; Dawson, 1995). It is associated with an unspecified immunological response, with the pathological symptoms of several diseases and with the destruction of tissue, taking place in the case of graft rejection, septic shock (Moncada, Palmer & Higgs, 1991) and some neurological diseases (Nicotera, Bonfoco & Brune, 1995; Molina, Jimenez-Jimenez, Orti-Pareja & Navarro, 1998).

In the central nervous system astrocytes, microglia and macrophages (Bentivolgio *et al.*, 1998) are capable of the expression of NOS2 and produce nitric oxide. Oligodendrocytes are able to express NOS2 in rat but there are no data pertaining patients.

Mechanisms of nitric oxide action

Nitric oxide is cytotoxic, causing cell death by necrosis or apoptosis. This molecule induces apoptosis in many types of cells including thymocytes, T cells, myeloid cells and neurons. Nitric oxide induces mitochondrial permeability which causes generation of the reactive oxygen species (Beckman, Beckman, Chen, Marshall & Freeman, 1990). Nitric oxide and peroxynitite are also damaging to cellular functions due to lipid peroxidation, consumpion of intracellular anti-oxidants (Barker, Bolanos, Land, Clark & Heales, 1996), DNA damage (Inoue & Kawanishi, 1995) and inhibit several very important enzymes such as aconitase, glyceraldehyde-3-phosphate dehydrogenase, and ribonucleotide reductase.

The question has sometimes been formulated as to the correlation between the intensity of nitric oxide generation and deviation from the normal functions of the organism (Lincoln, Hayle & Burnstock, 1997). The facts did not confirm the existence of such a connection. The only what could be observed so far is a number of examples indicating that nitric oxide deficiency or excess may sometimes be associated with certain pathological conditions. The previous part of introduction contains some data which illustrate this sort of connection.

An especially attractive group of phenomena, confirming the importance of nitric oxide balance, are the functions played by this substance in the activity of brain which is the main subject of the present paper. Two of these functions are determined by the action of nitric oxide on neurones. They express themselves as neurotoxicity or neuroprotection. The other two are due to the phenomena brought about by nitric oxide in the synapses. They are known as synaptic plasticity and modulatory activity.

Moderate concentrations of nitric oxide are engaged under normal conditions in cell communication and especially in neuronal transmission. Excessive amounts of nitric oxide produced in the brain area, however, as a rule deleterious. That is why monitoring of nitric oxide level in various brain diseases appears to be justified. For this reason such measurements were undertaken in our laboratory on adult patients with various brain disfunctions and on children with meningitis. Significant variations in the level of nitric oxide could be observed between individual patients. An attempt was undertaken in this paper at correlating the concentration of nitric oxide in the cerebrospinal fluid of neurological patients, with the viral and bacterial background of infection or with the

incidence of fatal outcomes. One could also observe that a decrease in the elevated amounts of nitric oxide was found to be associated with the improvement in patients condition. Therefore, it may happen that nitric oxide will become in future a useful parameter of prognostic value, at least in the case of certain types of diseases.

MATERIAL

Cerebrospinal fluid

Cerebrospinal fluid (CSF) was obtained by lumbar puncture during routine diagnostic examination. Two groups of patients were chosen:

(1) adult neurological patients treated at the Department of Neurotraumatology, Collegium Medicum, Jagiellonian University.
(2) children with meningitis from Cracow City Hospital, Department of Neuroinfections.

The studies were performed on 102 samples from 45 adult patients (including 37 men and 8 women) aged 42.3 ± 1.42 years (between 16 and 69 years). CSF was sometimes examined more than once in the same patient at various stages of disease. A variety of conditions were treated such as subdural, epidural and intracerebral haematomas, cerebral and brain stem contusions, duraplastics, scull bones fractures, brain tumors or infections.

The second group consisted of children with bacterial and viral meningitis.

The examination was performed on 45 children aged between 1 month and 14.5 years.

The level of NO was determined before starting the treatment.

Analytical reagents

Sodium dithionite and human lyophilised haemoglobin used for the determination of NO were produced by Sigma-Aldrich Co.

METHODS

Handling of CSF samples

The samples of CSF, obtained by lumbar puncture, were stored at +4 °C for about one hour before preparation. Haemoglobin (Hb) in the amount of 17 mg mixed with 9 mg of sodium dithionite ($Na_2S_2O_4$) were solved in 0.1 ml PBS (phosphate buffer saline) and added to 0.5 ml of cerebrospinal fluid. After 7 minutes the mixture was frozen to liquid nitrogen temperature and stored at about –73 °C until the moment of EPR measurement. Haemoglobin served as a spin trap. Sodium dithionite was added to reduce ferrous iron and nitrite to nitric oxide. Samples were prepared under argon in view of a very high contamination of air with nitric oxide.

Controls were prepared in the same way (17 mg Hb + 9 mg $Na_2S_2O_4$ + 0,6 (0.1 + 0.5) ml PBS).

EPR determination

The measurements were performed at 77 K with 300 E Bruker X-band EPR spectrometer. The following instrumental parameters were as a rule used: 20 mW microwave power, 5 Gs modulation amplitude, 100 kHz modulation frequency, 10 ms time constant, 500 Gs scan range, 9.48 GHz microwave frequency, 21 sec scan time, 10 scans and receiver gain 4E5. EPR triplet signal amplitude was measured as the height of a second line of hyperfine structure of Hb-NO EPR signal. DPPH (1,1-diphenyl-2-picrylhydrazyl) was used as a standard (g = 2.003).

Data are expressed as mean and SEM (standard error of mean).

RESULTS

In the present study the level of nitric oxide was determined in cerebrospinal fluid of patients with many kinds of neurological diseases.

Adult patients were divided into three groups. Those suffering from: (1) craniocerebral trauma (58 samples from 32 patients); (2) brain tumors (37 samples from 9 patients); (3) meningitis (7 samples from 4 patients). Meningitis was sometimes associated with other diseases. Neurological condition was estimated by Glasgow Coma Score (GCS) and outcome of treatment by Glasgow Outcome Scale (GOS).

The second group of patients were children aged 1 month to 14.5 years, with bacterial and viral meningitis (Tab. 2).

The level of nitric oxide in cerebrospinal fluid was measured using spectroscopy of electron paramagnetic resonance (EPR) and spin trapping.

Type of the brain disease and NO level

Many types of pathological conditions were included into the present study (Tab. 1). As one can see from Fig. 1 the level of NO in the CSF of patients with meningitis (2856 ± 1218 [a.u.]; mean ± SEM) is distinctly higher than in two other groups of brain diseases and is significant statistically. In the case of tumors and brain traumas this level is comparable and amounts to 661 ± 179 [a.u.] for brain tumors or 874 ± 174 [a.u.] for brain lesions.

Tab. 1. Clinical diagnosis and number of samples and patients

Clinical diagnosis	No. of samples	No. of patients
TRAUMA: haematomas: subdural, epidural, intracerebral; cerebral contusions; brain stem damages; dura-plastics; brain abscesses; scull bones fractures	58	32
BRAIN NEOPLASMS: astrocytoma; glioma malignum oligodendroglioma; blastoma	37	9
MENINGITIS	7	4
Total number	102	45

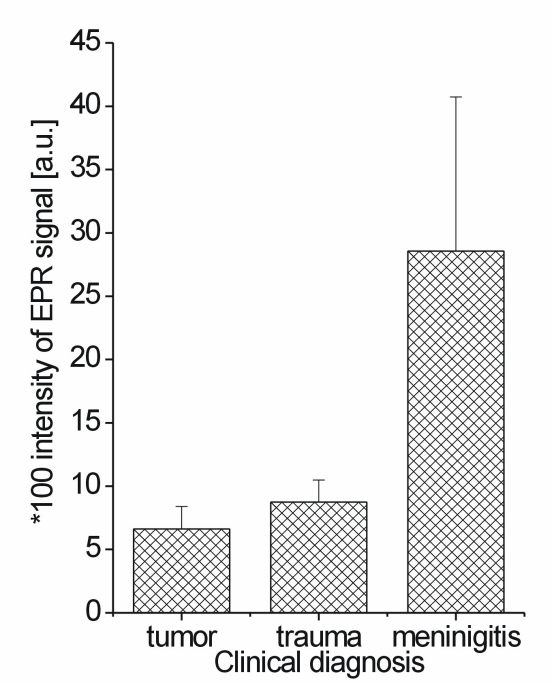

Fig.1. EPR signal amplitudes due to NO In various types of brain diseases

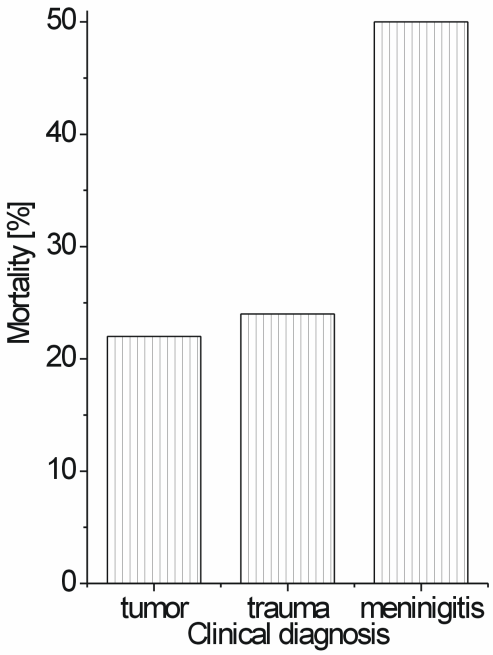

Fig. 2. Mortality in various types of brain diseases

Type of brain disease and percentage of surviving patients

As one can see from Fig. 1 and 2, the concentration of NO in CSF and mortality seem to be different in various types of brain diseases. It is clearly the highest in the case of meningitis in adult patients and comparable for patients with tumors or brain traumas.

Amount of NO in CSF and incidence of fatal outcomes

A correlation can be observed between the content of NO in CSF of patients from all 3 groups of brain diseases and their mortality. Highly elevated NO levels in CFS seem to be associated with a decreasing chance of survival (Fig. 3). The highest increase in NO level (over 3000 [a.u.] of EPR signal) may additionally find its expression in the values of GCS parameter and outcome of treatment (GOS; Fig. 4).

Level of NO during meningitis in children

The examined cases of meningitis were caused by different types of bacteria and viruses (tab. 2). All analytical procedures were performed before starting the treatment of children.

As one can see from Figure 5, the content of NO in CSF of children with bacterial meningitis (1669 ± 184) is considerably higher as compared with the amount of NO in the same disease of viral origin (14 ± 14). As a result, EPR technique makes it possible to differentiate between bacterial and viral meningitis in a very reliable way.

It normally takes about one hour to perform an EPR examination of the CSF, whereas traditional bacteriological methods require a period of at least 24 h to analyse the same material. Time is critical in the treatment of bacterial meningitis where immediate application of appropriate antibiotics may decide about life or death of patients. One has to realise that in viral meningitis antibiotics are harmful for bacterial flora and undesirable as a therapeutic modality.

The present results indicate that EPR methodology makes it possible to determine the amount of NO in CSF much sooner. They also show a correlation between the level of NO and a type of meningitis so that EPR spectroscopy might represent a useful adjuvant strategy for differentiating viral and bacterial meningitis.

DISCUSSION

Nitric oxide concentration which depends on the intensity of its local generation makes usually possible to predict the future outcome of a patient. In the present paper the discussion has been limited to our own neurological observations. The survey of literature which can be found in the introduction supplies in addition a variety of opinions on the predictive value of nitric oxide level.

The first question to be asked here is, whether a very high amount of nitric oxide in the organisms is always a bad sign, as we tend to believe in the light of our own studies?

Literature seems to indicate that this is not a rule. As one could notice reading introduction, overproduction of nitric oxide due to infections may sometimes induce a massive generation of nitric oxide by the macrophages which is a symptom of an energetic defence response of the immune system and is associated with the expectation of a positive outcome. In the case of sepsis, however, attempts at saving the patient involved sometimes the application of appropriate NOS inhibitors in order to reduce the intensity of nitric oxide generation (Hussein, Beerahee, Grover, Jordan, Jeffs, Donaldson, Zaccardelli, Colice, Guntupalli, Watson & Vincent, 1999).

The process of transplant rejection in animal systems can be effectively monitored by measuring the concentration of nitric oxide in peripheral blood. This approach does not seem, however, to have the acceptance in the case of human cardiac transplantations. Physiciance are rather conserva-

Tab. 2. Clinical diagnosis and number of samples.

Clinical diagnosis	No. of samples
BACTERIAL: Neiseria meningitalis, Haemophilus influenzae, E. coli, Enterobacter, Diplococcus pneumoniae	45
VIRAL: Paramyxovirus paratides, Coxsachie virus, Varicellae-zoster, ECHO group viruses	10

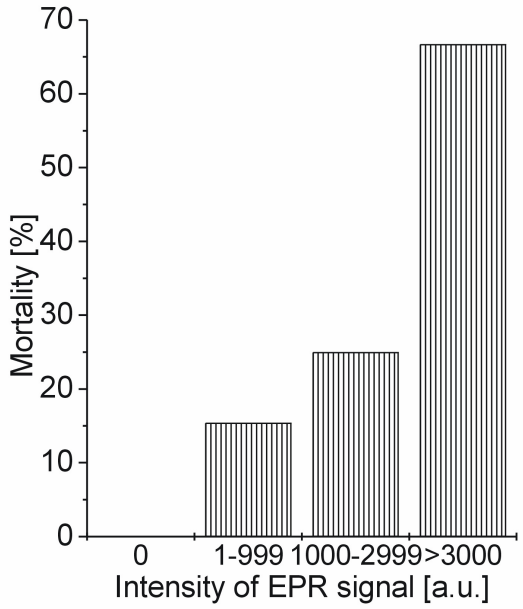

Fig. 3. Nitric oxide concentration in cerebral spinal fluid and probability of fatal outcomes.

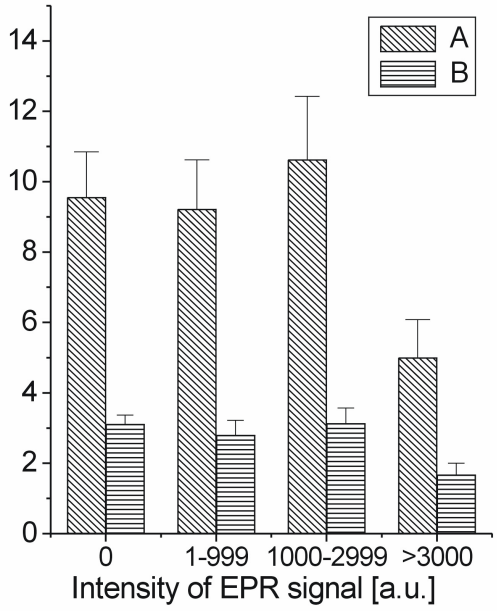

Fig. 4. NO level in CSF of patients with brain diseases and its correlation with GCS (A) and GOS (B). Mean and SEM.

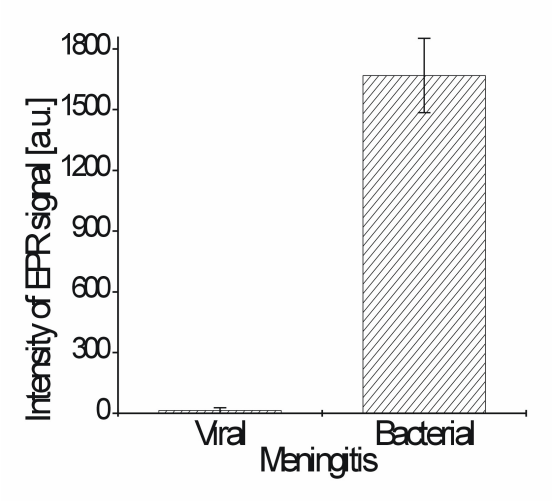

Fig. 5. Possibility of differentiating between viral and bacterial meningitis in children based on the amount of NO in the CSF.

tive and trust only calssical histopathological examination of myocardial biopsies. They choose this approach in spite of the fact that it is invasive, more risky and stressful for the patient.

In oncology, he determination of nitric oxide in body fluids does not have so far any clinical applications, except for the tumors of the central nervous system. Here the examination of cerebrospinal fluid for the content of nitric oxide has been introduced by our group and has a chance of becoming a useful prognostic marker. The amount of nitric oxide in the ascitic fluid which accumulats in the body cavity of certain terminal cancer patients, is recently under investigation in our laboratory.

In the case of patients with arthritis one can estimate detectable concentrations of nitric oxide in the synovial fluid using EPR spectroscopy. The prognostic value of this parameter will be evaluated in the near future (Zając, 2001).

In the light of quantitative EPR studies on samples of the examined cerebrospinal fluid one can formulate a couple of questions.

One of them is the problem whether the kind of clinical diagnosis is associated with the amount of nitric oxide found in the sample. The answer is clear cut for patients with meningitis who distinctly differ from the other two groups of diseases. This would indicate that brain infections release a much stronger response of inducible NOS-es than brain lesions and brain traumas.

The next question is whether a different amount of nitric oxide in three examined groups of pathologies is followed by a different number of patients who do not survive. It turned out that meningitis is at the first place from the point of view of high mortality.

If all three groups of diseases are taken together, one can find out that the level of nitric oxide in the cerebrospinal fluid is distinctly connected with the number of deceasing patients, those revealing its highest amount being must likely to die.

Very impressive are the results obtained in the case of children suffering from meningitis. The EPR data indicate that the level of nitric oxide is drastically different depending on viral or bacterial origin of the disease. It is worth emphasizing that the examination is much easier and faster upon application of EPR technique. A value of a future prognostic parameter seems to be likely and justified in this case as well.

CONCLUSIONS

It is essential to remember from the review of literature that high levels of nitric oxide are not always deleterious, they are sometimes beneficial for the organism.

Clinical observations based on EPR data presented in this paper indicate that: (1) type of brain disease seems to determine an average content of nitric oxide in the CSF of patients, (2) very elevated amount of nitric oxide in the CSF is associated with a bad prognosis including a high probability of a fatal outcome, (3) EPR measurement of nitric oxide concentration in CSF of children with meningitis makes it possible to differentiate in an easy and quick way between viral and bacterial type of this disease.

All these facts seem to justify an expectation that the level of nitric oxide in the CSF of neurological patients has a good chance to become in future a useful indicator of a prognostic value.

REFERENCES

Barker J. E., Bolanos J. P., Land J. M., Clark J. B. & Heales S. J. (1996). Glutathione protests astrocytes from peroxynitrite-mediated mitochondrial damage: implications for neuronal/astrocyte trafficking and neurogeneration. *Developmental Neuroscience.*, **18**, 391-396.

Beckman J. S., Beckman T. W., Chen J., Marshall P. A. & Freeman B. A. (1990). Apparent hydroxyl radical production by peroxynitrite: implications for endothelial injury from nitric oxide and superoxide. *Proceedings of the National Academy of Sciences of the United States of America.*, **87**, 1620-1624.

Bentivolgio M., Bertini G., Mariotti R. & Peng Z.-C. (1998). Normal and pathological expression of nitric oxide synthase in the central nervous system. [In:] Moncada S., Nistico G., Bagetta G. & Higgs E.A.

(Eds.), *Nitric Oxide and the Cell. Proliferation, Differentiation and Death*. London: Portland Press Ltd.

Dawson V. L. (1995). Nitric oxide: role in neurotoxicity. *Clinical & Experimental Pharmacology & Physiology*, **22**, 305-308.

Faraci F. M. & Heistad D. D. (1992). Does basal production of nitric oxide contribute to regulation of brain-fluid balance? *American Journal of Physiology*., **262**, H340-H344.

Furchgott R. F. & Zawadzki J. V. (1980). The obligatory role of endothelial cells in the relaxation of arterial smooth muscle by acetylcholine. *Nature*., **288**, 373-376.

Garthwait J., Charles S. L. & Chess-Williams R. (1988). Endothelium-derived relaxing factor releaseon activation of NMDA receptors suggests role as intercellular messenger in the brain. *Nature*., **336**, 385-388.

Henry Y. A., Guisami A. & Duzastel B. (1997). *Nitric Oxide Research from Chemistry to Biology: EPR Spectroscopy of Nitrosylated Compounds*. Chapman and Hall and R.G. Landes Bioscience: Austin, Texas,.

Hussein Z., Beerahee M., Grover R., Jordan B., Jeffs R., Donaldson J., Zaccardelli D., Colice G., Guntupalli K., Watson D. & Vincent J. -L. (1999). Pharmacokinetics of nitric oxide synthase inhibitor L-N^G-methylarginine hydrochloride in patients with septic shock. *Clinical Pharmacology Therapeutics.*, **65**, 1-9.

Inoue S. & Kawanishi S. (1995). Oxidative DNA damage induced by simultaneous generation of nitric oxide and superoxide. *FEBS Letters*. **371**(1), 86-8.

Kugiyama K., Sugiyama S., Matsumura T., Ohta Y., Doi H. & Yasue H. (1996). Suppression of atherosclerotic changes in cholesterol-fed rabbits treated with an oral inhibitor of neutral endopeptidase 24.11 (EC 3.4.24.11). *Arteriosclerosis, Thrombosis & Vascular Biology*., **16**, 1080-1087.

Lincoln J., Hayle C. H. V. & Burnstock G. (1997). *Nitric oxide in health and disease.* Cambridge University Press: Cambridge.

Molina J. A., Jimenez-Jimenez F. J., Orti-Pareja M. & Navarro J. A. (1998). The role of nitric oxide in neurodegeneration. potential for pharmacological intervention. *Drugs Agig.*, **12**, 251-259.

Moncada S., Palmer R.M. & Higgs E.A. (1991). Nitric oxide: physiology, pathophysiology, and pharmacology. *Pharmacological Reviews*., **43**, 109-142.

Nathan C. & Xie Q. (1994). Regulation of biosynthaesis of nitric oxide. *The Journal of Biological Chemistry.*, **269**, 13725-13728.

Nicotera P., Bonfoco E. & Brune B. (1995). Mechanisms for nitric oxide-induced cell death: involvement of apoptosis. *Adv Neuroimmunol*., **5**, 411-420.

Radomski W.M. & Palmer R.M. (1987). The anti-aggregating properties of vascular endothelium: interaction between prostacyklin and nitric oxide. *British Journal of Pharmacology*., **92**, 639-646.

Zając A. (2001). *Pomiary tlenku azotu i ceruloplazminy przy użyciu spektroskopii EPR w chorobach autoimmunizacyjnych.* Unpubslished MSc. Thesis, Jagiellonian University, Kraków.