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INCREASED LYMPHOCYTE CASPASE-3 ACTIVITY IN PATIENTS WITH SCHIZOPHRENIA

POVEĆANA AKTIVNOST KASPAZE-3 U LIMFOCITIMA OBOLELIH OD SHIZOFRENIJE

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Summary: A growing body of evidence indicates that cortical brain cells of schizophrenic patients are vulnerable to apoptosis. As apoptosis is an important mechanism in organism modeling during development, active since the early phase of intrauterine life, it could be involved in the pathogenesis of schizophrenia. To test this hypothesis, caspase-3 activity was determined in peripheral blood mononuclear cells from 30 patients with schizophrenia and from 30 age and gender matched healthy subjects by a colorimetric commercially available kit. Consistent with increased susceptibility to apoptosis, caspase-3 activity in lymphocytes of patients with schizophrenia was significantly increased (0.111±0.055 μmol/mg protein, $p<0.05$) in comparison with those in the matched control group (0.086±0.030 μmol/mg protein). The highest activity was obtained in the group showing almost equally positive and negative symptoms (0.159±0.096 μmol/mg protein) and it was significantly higher ($p<0.05$) compared to the group with a relative predomination of positive symptoms (0.100±0.029 μmol/mg protein). Caspase-3 activity in patients receiving typical antipsychotic drugs (0.124± 0.071 μmol/mg protein) was not significantly different from that in patients treated with atypical antipsychotics (0.104±0.039 μmol/mg protein). To our knowledge to date, this has been the first demonstration that there is a significant increase in caspase-3 activity, determined in native cells, in patients with schizophrenia, indicating a dysregulated apoptotic mechanism in this disease.

Keywords: apoptosis, caspase-3 activity, lymphocytes, schizophrenia

Kratak sadržaj: Sve je više dokaza da su kortikalne ćelije mozga obolelih od shizofrenije osetljive na apoptozu. Kako je apoptoza aktivna od rane faze intrauterinog života, važan mehanizam u modelovanju organizma tokom razvoja, mogla bi biti uključena u patogenezu shizofrenije. U cilju testiranja ove hipoteze određivana je aktivnost kaspaze-3, kolorimetrijskom metodom, u mononuklearnim ćelijama periferne krvi kod 30 obolelih od shizofrenije i 30 zdravih osoba slične starosti i pola. Shodno povećanoj osetljivosti na apoptozu, aktivnost kaspaze-3 u limfocitima obolelih od shizofrenije je značajno veća (0,111±0,055 μmol/mg proteina, $p<0,05$) u poređenju sa vrednostima kontrolne grupe (0,086±0,030 μmol/mg proteina). Najviša aktivnost je dobijena u grupi bolesnika sa skoro podjednako izraženom pozitivnom i negativnom simptomatologijom (0,159±0,096 mmol/mg proteina) i bila je značajno viša ($p<0,05$) od vrednosti grupe sa relativnom predominacijom pozitivnih simptoma (0,100±0,029 μmol/mg proteina). Nije nađena značajna razlika u aktivnosti kaspaze-3 između bolesnika tretiranih tipičnim (0,124±0,071 μmol/mg proteina) odnosno atipičnim (0,104±0,039 μmol/mg proteina) antipsihoticima. Prema našim saznanjima ovo su prvi rezultati koji pokazuju da je aktivnost kaspaze-3 značajno povećana u nativnim ćelijama obolelih od shizofrenije što ukazuje na disregulaciju apoptotičnog mehanizma u ovoj bolesti.

Ključne reči: apoptoza, aktivnost kaspaze-3, limfociti, shizofrenija

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Introduction

Schizophrenia is a major chronic debilitating psychiatric disorder associated with social, occupational, behavioral, and cognitive impairment. Besides a strong genetic component (1, 2), the mechanisms playing a role in the pathophysiology of this disease are not

known yet. Many individuals manifest the disorder in the absence of genetic loading. A number of human genes, expressed in the brain, are located in chromosomal loci associated with schizophrenia and are potential candidate genes (3, 4). However, none of these gene polymorphisms approached an acceptable genome-wide significance (5).

According to the neurodevelopmental hypothesis, schizophrenia seems to be caused by a combination of factors including genetic vulnerability and environmental factors that occur during individual development (6, 7). However, neurodevelopment does not include important characteristics of schizophrenia such as: protracted period of symptomatic dormancy between the putative insult and the emergence of symptoms, the progressive clinical deterioration that affects at least a subgroup of patients, and progressive neurostructural changes in certain cortical and ventricular brain structures. In order to identify a pathophysiological mechanism that could explain the progressive elements of schizophrenia and its relationship with neurodevelopment, oxidant stress, glutamate excitotoxicity, neurochemical sensitisation and a dysregulation of apoptosis were considered.

Indications of apoptotic mechanism dysregulation include: reduced neuronal densities in the prefrontal cortex (8), reduced incidence rates of cancer (9), negative disease association between schizophrenia and rheumatoid arthritis (10), and absence of evidence of gliosis in the brain tissue of schizophrenics (11). Although much less DNA fragmentation in individuals with schizophrenia than in healthy controls was found (12) the results of Coulson (13) based on the studies of apoptotic nuclei, caspase-3 activity, and protein levels of Bcl-2, Bax and $p53^{P392Ser}$ in dermal fibroblast cell lines suggested significant abnormalities in the regulation of apoptosis in schizophrenia that did not occur in non-schizophrenic psychiatric disorders, and healthy subjects. The exact role of apoptosis in schizophrenia remains uncertain, but the potential involvement of non-lethal localized apoptosis appears plausible (14).

Considering the evidence, the aim of this study was to assess the activity of caspase-3 in peripheral blood mononuclear cells of patients with schizophrenia.

Patients and Methods

Patients were recruited at the Clinic of Psychiatry and the Clinic for Mental Health Protection of the Clinical Centre Niš. The subjects were assessed with the Diagnostic Interview for Psychosis by two independent psychiatrists, and diagnosed according to DSM-IV and ICD-10. The patients with coincidental immune, inflammatory and liver diseases, as well as epilepsy, neurological disorders and mental retardation were excluded from the study. For the disease evaluation and clinical management of the patients the Positive and Negative Syndrome Scales (PANSS) were used. This scale includes three subscales: the positive scale has 7 items, negative also 7 items and the general psychopathology scale includes 16 items. Each patient is tested according to PANSS and each item is scored by two independent psychiatrists. Patients are selected on the basis of total score. The values of total score higher than 3 indicated predominance of the positive syndrome, those lower than -8 indicated predominance of the negative syndrome, and the score values between -8 and 3 indicated a mixed type, i.e. almost equal expression of positive and negative syndromes.

The study included 30 schizophrenic patients (19 males, 11 females; mean age 31.1±8.1) and 30 healthy controls (19 males, 11 females; mean age 29.1±6.9), without any somatic or mental disorder, recruited at the Department for Blood Transfusion, who were matched according to age, gender, marital status, education, living conditions and settings, and habits.

All the subjects provided written informed consent, and the study was approved by the Clinical Centre Niš Ethics Committee.

Venous blood was collected in vacutainer tubes containing potassium EDTA as anticoagulant. Peripheral blood mononuclear cells were isolated immediately from peripheral blood using lymphocyte separation medium (PAA, Pashing, Austria). In brief, the blood was mixed with equal amounts of physiological saline and layered over lymphocyte separation medium. Samples were centrifuged on 400 x g for 30 minutes at room temperature. Separated lymphocytes were collected**,** centrifuged at 200 x g and washed two times with physiological saline. Lymphocyte suspension was resuspended in 50 μL of chilled cell lysing buffer and frozen until caspase-3 activity determination. Before caspase-3 activity analysis resuspended frozen cells were thawed and centrifuged for one minute in a microcentrifuge (10000 x g). Then, the cell supernatant was transferred to a fresh tube, and protein concentration was determined in each sample on an autoanalyzer AU-400 (Olympus, Japan). Further, the assay was performed according to the instructions of the manufacturer. Samples were assayed for caspase-3 activity using a colorimetric commercially available kit (Bio Source Europe S.A, Novellas, Belgium). The caspase-3 colorimetric protease assay is based on the caspase ability to recognize the amino acid tetrapeptide sequence Asp-Glue-Val-Asp (DEVD). The kit includes optimized buffers and substrate, DEVD-pNa, composed of the chromophore, p-nitroanilide (pNa), and a synthetic tetrapeptide, DEVD. After the cleavage of the substrate by caspase-3 during 2 hour incubation at 37 °C, the absorbance of pNa is quantified using a

microplate reader at 405 nm. Caspase-3 activity was calculated using the molar extinction coefficient for pNa, and expressed per mg protein.

Data analysis was performed using the SigmaStat computer program. The difference between groups was tested by the Mann-Whitney Rank Sum Test (ANOVA). P values less than 0.05 are presented as significant. Correlations between caspase-3 activity and demographic and clinical characteristics of patients were assessed using Pearson's coefficients.

Results

The demographic characteristics of patients with that schizophrenia and healthy controls are presented in *Table I*. Both groups consisted of the same number of males and females. The age of patients at the first onset of the disease was between 18 and 34 years, and the duration of psychiatric disease was between 6 months and 15 years.

PANSS scores in patients with schizophrenia included PANSS positive score, PANSS negative score, PANSS total score as well as PANSS general psychopathology score for each individual patient involved in this study were presented (*Table II*). It was observed that in 15 patients positive symptoms showed relative predominance, negative ones in 9 patients, and in 6 patients both types of symptoms were almost equally expressed.

Patients with schizophrenia had significantly higher lymphocyte caspase-3 activity (0.111±0.055 μmol/mg protein; $p<0.05$) in comparison with the control group (0.086±0.030 μmol/mg protein) (*Figure 1*). When the patients were divided into groups according to the PANSS scores, the highest activity was observed in the group showing almost equally positive and negative symptoms (0.159± 0.096 μmol/mg protein) and this enzyme activity was significantly higher compared to the group with the relative predominance of positive symptoms (0.100± 0.029 mmol/mg protein). Caspase-3 activity in patients receiving typical antipsychotic drugs (TAD) (0.124± 0.071 mmol/mg protein) was not significantly different from that of patients treated with atypical antipsychotics (AAD) (0.104±0.039 mmol/mg protein) (*Figure 2*). There was not any significant correlation between lymphocyte caspase-3 activity and heredity (r=0.123), the first onset of the disease (r=–0.211) and the duration of psychiatric disease (r=–0.206). Also, the values of enzyme activity in male and female patients were not statistically different.

Discussion

As far as we know, this is the first study undertaken to evaluate caspase-3 activity *in vivo*, in lymphocytes of patients with schizophrenia. Caspase-3 is the main executor caspase capable of degrading a number of cell substrates during activation. This terminal protease can be activated by both apoptotic pathways. In the extrinsic pathway, it is activated by caspase-8, the caspase that is recruited to an activated Fas complex, i.e. through FasL ligation to the Fas receptor that sets in motion a series of events resulting in cell death. In the intrinsic, mitochondrial pathway, which is most frequently implicated in central nervous system apoptosis (15), caspase-3 is activated by the cleavage of caspase-9 (16). The inhibition of caspase-3 activity blocks DNA fragmentation in response to Fas (17, 18), and a variety of other apoptotic stimuli, placing this caspase at a central control point. Mice deficient in two effectors, caspase-3 and -7, died immediately after birth with defects in cardiac development (19). The engagement and activity of apoptotic pathways may favor either cell death or differentiation. During neuronal

Table I Demographic and clinical characteristics of patients with schizophrenia.

		Schizophrenia	Control
Male/female (N)		19/11	19/11
Age (years)		31.1±8.1	29.1±6.9
Heredity (+/–)		12/18	–
Patient age at the disease onset	<20 20–24 25–29 30–34	10 4 9 7	– – – –
Duration of psychiatric disease (years)	<1 1–3 3–5 >5	5 5 8 12	– – – –
PANSS total score		91±14	–
PANSS positive score		24±7	–
PANSS negative score		23±10	–
PANSS general psychopathology		48±9	–

Table II Individual PANSS scores in patients with schizophrenia.

Patient	PANSS positive score	PANSS negative score	PANSS total score	PANSS general psychopathology
p1	13	29	–16	50
p2	18	27	–9	49
p3	26	22	+4	47
p4	17	23	–6	46
p5	23	39	–16	75
p6	25	11	+14	41
p7	35	18	+17	48
p8	23	40	–17	68
p9	20	16	+4	43
p10	31	43	–12	51
p11	34	38	–4	51
p12	34	34	0	55
p13	21	30	–9	42
p14	36	25	+11	49
p15	19	40	–21	57
p16	25	7	+18	49
p17	11	12	–1	34
p18	25	13	+12	43
p19	15	24	–9	56
p20	20	12	+8	40
p21	24	15	+9	50
p22	31	14	+17	54
p23	39	18	+21	54
p24	27	16	+11	44
p25	19	14	+5	31
p26	28	24	+4	33
p27	18	23	–5	42
p28	31	30	+1	52
p29	22	18	+4	39
p30	19	30	–11	47

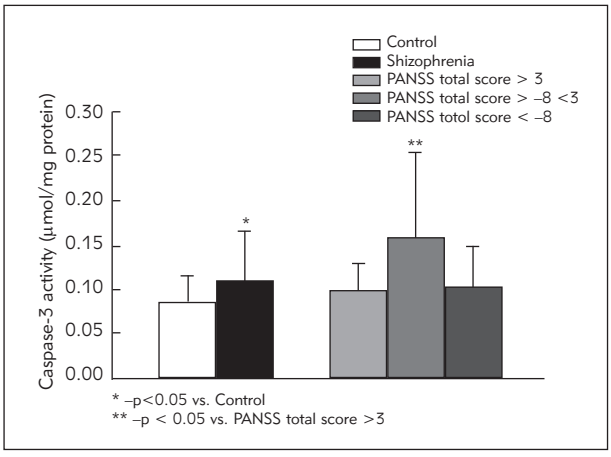

Figure 1 PBMC caspase-3 activity in patients with schizophrenia.

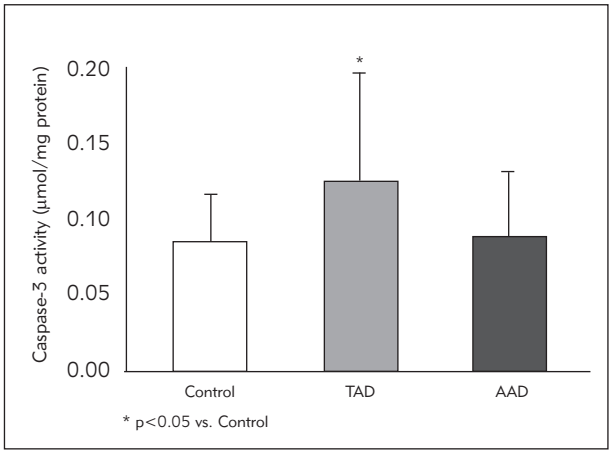

Figure 2 PBMC caspase-3 activity in patients with schizophrenia treated with tipical or atipical antipsychotics.

stem cell differentiation into neurons and glia a large percentage of these cells undergoes apoptosis. However, caspase-3 activity is elevated in nonapoptotic differentiating cell populations (20). This finding suggests that the targeted inhibition of this protease in neuronal cells may have unintended consequences. Caspase-3 processing, p53 phosphorylation, and p53 transcriptional activation increased at 3 days of differentiation, with no evidence of apoptosis (21) suggesting that apoptosis-associated factors such as caspases and p53 temporally modulate cell signaling and the differentiation of neuronal stem cells. Also, high caspase-3 activity may be involved in the maintenance of synaptic plasticity (22).

As caspase-3 is the end point of both apoptotic pathways we have chosen this enzyme activity to evaluate apoptotic ability *in vivo*, in patients with schi-

zophrenia, and we have shown that caspase-3 activity is significantly increased in the lymphocytes of those patients. Another *in vitro* study based on cultured dermal fibroblast cells showed anomalous apoptotic mechanisms in schizophrenic patients (23) suggesting that altered apoptosis may be observable in all somatic cell types in schizophrenia. Contrary to this finding, Jarskog et al. (24) did not find any significant difference in the level of caspase-3 in a postmortem tissue study of temporal cortex in patients with schizophrenia compared with healthy controls.

In two case-control studies and in one family study the gene of the key glutathione-synthesizing enzyme, glutamate cysteine ligase modifier (GCLM) subunit, is shown to be strongly associated with schizophrenia. GCLM gene expression was decreased in patients' fibroblasts (25). Since oxidative stress is a strong inductor of caspase-3 (26) leading to cellular damage via apoptosis (27) a dysregulation of GSH metabolism may be one of the vulnerability factors contributing to the development of the disease. S-nitrosoglutathione is shown to protect brain dopamine neurons from oxidative stress (28). S-nitrosylation of caspase-3 itself is an important regulator of caspase function (29) in physiological conditions, but the highly significant increase in nitric oxide concentration in schizophrenic patients (30) may act in two different ways: either by increasing caspase-3 S-nitrosylation thereby lowering its activity and cell apoptosis (31), or by initiating apoptosis and elevating the enzyme activity which we showed in this study.

A probable explanation for the decreased cortical volume observed in patients with schizophrenia is reduced neuropil and neuronal size, rather than a loss of neurons. A developmental concept of synaptic apoptosis suggests that the activation of this process may be localized in terminal neurites without neuronal death (14). Synaptic apoptosis represents a potential mechanism of synaptic remodeling and elimination of synapses, in physiological as well as pathological conditions, which may have a direct influence on synaptic plasticity (32). It is thought that in Alzheimer's disease this form of synaptic loss has a direct effect on early cognitive depletion that ends in neuronal death (14). A few studies showed that synaptosome apoptosis might be activated by various pro-apoptotic stimuli (33). The focal application of glutamate to distal dendrites *in vitro*, can cause a localized increase in caspase-3 activity in synaptic terminals without its propagation to the neuronal soma (14). Similarly, β-amiloid induces the localized degeneration of distal hypocampal neurites with apoptotic morphology without the induction of cell death in compartmentalized cell cultures (34).

Previous studies have suggested that the high level of caspase-3 activity in brain tissue postmortem, which frequently accompanies neurodegenerative disorders, is not due to schizophrenia in which it is unchanged or lowered in the temporal cortex of chronic patients (24). However, this does not exclude apoptosis in the early stages of the disease. Jarskog et al. (14) hypothesized that apoptotic activity might contribute to the progressive neurostructural changes in the prodromal stage and the first episodes of psychosis, and cortical neuropil is the main substrate which is damaged by this process. It is supposed that caspase-3 and/or other effector caspases could be sensitive to an activation i.e. to a time limited increase in pro-apoptotic stimuli (oxidative stress, glutamate excitotoxicity) at the beginning of the schizophrenic process. As a consequence, clinical and neurostructural deteriorations could be expressed followed by pro-apoptotic stress depletion and caspase-3 activity normalisation.

On the basis of all known evidence our results may be interpreted in several ways: 1) Elevated caspase-3 activity may be a consequence of increased pro-apoptotic stress. Alternatively, high Bax/Bcl-2 ratio may induce the release of cytochrome c from mitochondria followed by caspase activation. 2) There is a possibility that this finding is a result of antipsychotic drugs acting (35). It is shown that typical antipsychotic haloperidol may induce neuronal apoptosis (36). Others (37) noted that a one-month application of haloperidol, quetapine and closapine increases caspase-3 activity by 40–50%. Qing et al. (38) found that atypical antipsychotics prevent apoptosis through the modulation of superoxide dismutase 1 expression. 3. In favor of our results is the finding of Miyaoka et al. (39) who showed that the treatment of schizophrenics with minocycline, a caspase inhibitor, led to a significant improvement of the clinical status of patients with schizophrenia, which points to the important role of caspase-3 in the pathogenesis of schizophrenia.

In conclusion, although limited data is available about apoptotic markers and potential inducers of apoptosis *in vivo* obtained in small patient groups with schizophrenia, all of them, including our own results, indicate a dysregulated apoptotic process in schizophrenia.

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Conflict of interest statement

The authors stated that there are no conflicts of interest regarding the publication of this article.

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