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Neuroprotective Effects of Glycine for Therapy of Acute Ischaemic Stroke

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Key Words
Acute cerebral ischaemia · Neuroprotection · Clinical trials · Glycine

Abstract
The aim of this randomized, double-blind, placebo-controlled trial was to assess the safety and the efficacy of the pharmaceutic drug glycine in 200 patients with acute (<6 h) ischaemic stroke in the carotid artery territory. Fifty patients received placebo, 49 glycine 0.5 g/day, 51 glycine 1.0 g/day and 50 glycine 2.0 g/day for 5 days in each group. The efficacy of glycine was assessed by clinical analysis, by an enzyme-linked immunosorbent assay of levels of blood serum autoantibodies to NMDA-binding proteines, by detection of excitatory (glutamate, aspartate) and inhibitory (glycine, GABA) amino acid concentrations and lipid peroxidation products (TBARS) in CSF. The trial confirmed the safety profile of the glycine treatment. Slight sedation was observed in 9 patients (4.5%) as a side-effect. Other marked side-effects or adverse events were absent. The glycine treatment at the dose of 1.0–2.0 g/day was accompanied by a tendency to a decreased 30-day mortality (5.9% in 1.0 g/day glycine and 10% in 2.0 g/day glycine groups vs. 14% in the placebo and 14.3% in 0.5 g/day glycine groups), to an improved clinical outcome on the Orgogozo Stroke Scale ($p < 0.01$) and the Scandinavian Stroke Scale ($p < 0.01$) and to a favourable functional outcome on the Barthel index ($p < 0.01$ in 1.0 g/day glycine vs. placebo group in patients with no or mild disability). An early normalization of autoantibody titres to NMDA-binding proteins in serum was found ($p < 0.01$ vs. placebo), a reduction of glutamate and aspartate levels ($p < 0.05$ vs. placebo), an increase in GABA concentrations ($p < 0.01$ vs. placebo in severe stroke patients) and also a reduction of TBARS levels ($p < 0.05$ vs. placebo) in CSF by day 3. Thus, the trial suggests that sublingual application of 1.0–2.0 g/day glycine started within 6 h after the onset of acute ischaemic stroke in the carotid artery territory is safe and can exert favourable clinical effects. These results will be verified in further trials with a larger number of patients.

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Introduction

The inhibitory amino acid glycine deserves attention as an important participant of the complex biochemical cascade in focal cerebral ischaemia [1–6]. On the one hand, glycine as an N-methyl-*D*-aspartate (NMDA) glutamate receptor co-agonist facilitates NMDA receptor functions in submicromolecular concentrations by occupation of the strychnine-insensitive glycine site [7–19]. On the other hand, the facilitatory action of glycine appears at concentrations of below 0.1 μ*M* and the binding site of the NMDA receptor is saturated between 10 and 100 μ*M* [20]. Addition of a larger concentration of glycine (100 μ*M*, 1 m*M*) after oxygen deprivation is not responsible for the long-term modulation of NMDA receptor activity in the rat hippocampus [21], and increased extracellular concentrations of glycine, such as those observed in ischaemia (10–100 m*M*), do not potentiate NMDA-evoked depolarization in vivo and, thereby, excitotoxicity [22, 23]. At the same time, glycine has an inhibitory effect by interaction with glycine receptors and γ-aminobutyric acid (GABA) receptors [24–26]. High doses of glycine and of its agonists have anticonvulsant activity [27–33] and potentiate the anticonvulsant effect of clinically established anti-epileptic drugs [34]. It could be difficult to explain the revealed effects if activation of excitatory glutamate systems is supposed to be the main mechanism of glycine action.

Besides, glycine has general metabolic effects and plays an important role as a conjugator of low-molecular-weight toxic substances generated during the ischaemic processes [35–37].

The pharmaceutic drug glycine was developed in the Laboratory of Metabolic Regulators of the Medical Scientific and Production Complex 'Biotics' (Moscow, Russia). It comprises the inhibitory amino acid glycine and a pharmaceutic carrier – 0.5–2.0 mass% of methyl cellulose (for sublingual or in-the-cheeck application). Experiments with glycine demonstrated good penetration of the drug through the receptor zones [38]. Administration of 20 mg/kg of glycine in rats with frontal lobe focal ischaemia resulted in a significant elevation of glycine concentration and an increase in the velocity of the GABA cycle in different brain structures including also the area of the ischaemic focus [39]. Besides, a significant reduction of the concentrations of products of oxidative stress in the brain infarction zone and a significant normalization of latency periods of conditioned (behaviour) reflexes were shown in rats under glycine treatment [40, 41].

Safety trials with glycine in normal subjects (including rats and healthy volunteers) were performed in 1992 and demonstrated the safety profile of the glycine treatment and absence of marked side-effects except a slight sedative state [42]. The drug glycine in doses of 300–600 mg/day was shown to possess antistress, stress-protective and nootropic effects [42].

Beneficial mediator and metabolic properties of the inhibitory amino acid glycine obtained in experiments and clinics as well as the safety profile and favourable effects of the pharmaceutic drug glycine demonstrated in animal models with focal cerebral ischaemia provide good evidence of possible neuroprotective properties of the pharmaceutic drug glycine in patients with acute ischaemic stroke.

The purpose of the present study was to investigate the safety and the efficacy of the pharmaceutic drug glycine in patients with acute ischaemic stroke and the ability of this drug to ameliorate the outcome of those patients.

Methods

The trial was performed when the approval of the Ethics Committe of the study centre was obtained, and the patients or their legal representatives gave written or witnessed informed consent to participate in the trial.

Patients

Patients with acute ischaemic stroke in the carotid artery territory were eligible for inclusion in the trial if they (1) were admitted to the Intensive Stroke Unit at the Department of Neurology of the Russian Medical University within the first 6 h after the onset of stroke, (2) were within the age range of 45–75 years, (3) were conscious or mildly obtunded [baseline Orgogozo Stroke Scale (OSS) score more than 15]. Patients who had experienced a previous stroke with residual neurological impairment, suffered from any other disorder interfering with neurological or functional assessment or who had a life-threatening concurrent illness were excluded from participation in the trial. Other exclusion criteria were congestive heart failure, acute myocardial infarction (within the previous 6 weeks), ECG findings of ventricular arrhythmia, second- or third-degree atrioventricular block.

The trial initially included 212 patients (out of 775 screened patients before randomization) who were eligible for inclusion (fig. 1). Twelve patients with either intracerebral haemorrhage or ischaemic stroke in the vertebrobasilar territory were excluded from the primary efficacy analysis after randomization (the study protocol allowed a CT scan to be performed within 24 h after the start of trial medication). The target population consited of 200 patients with ischaemic stroke in the carotid artery territory (male/female = 110/90; mean age 63.7 ± 10.1 years); 115 patients suffered from left hemispheric stroke, 85 from right hemispheric stroke. There were no significant differences between the treatment groups in the target population with regard to demographic and baseline characteristics (table 1).

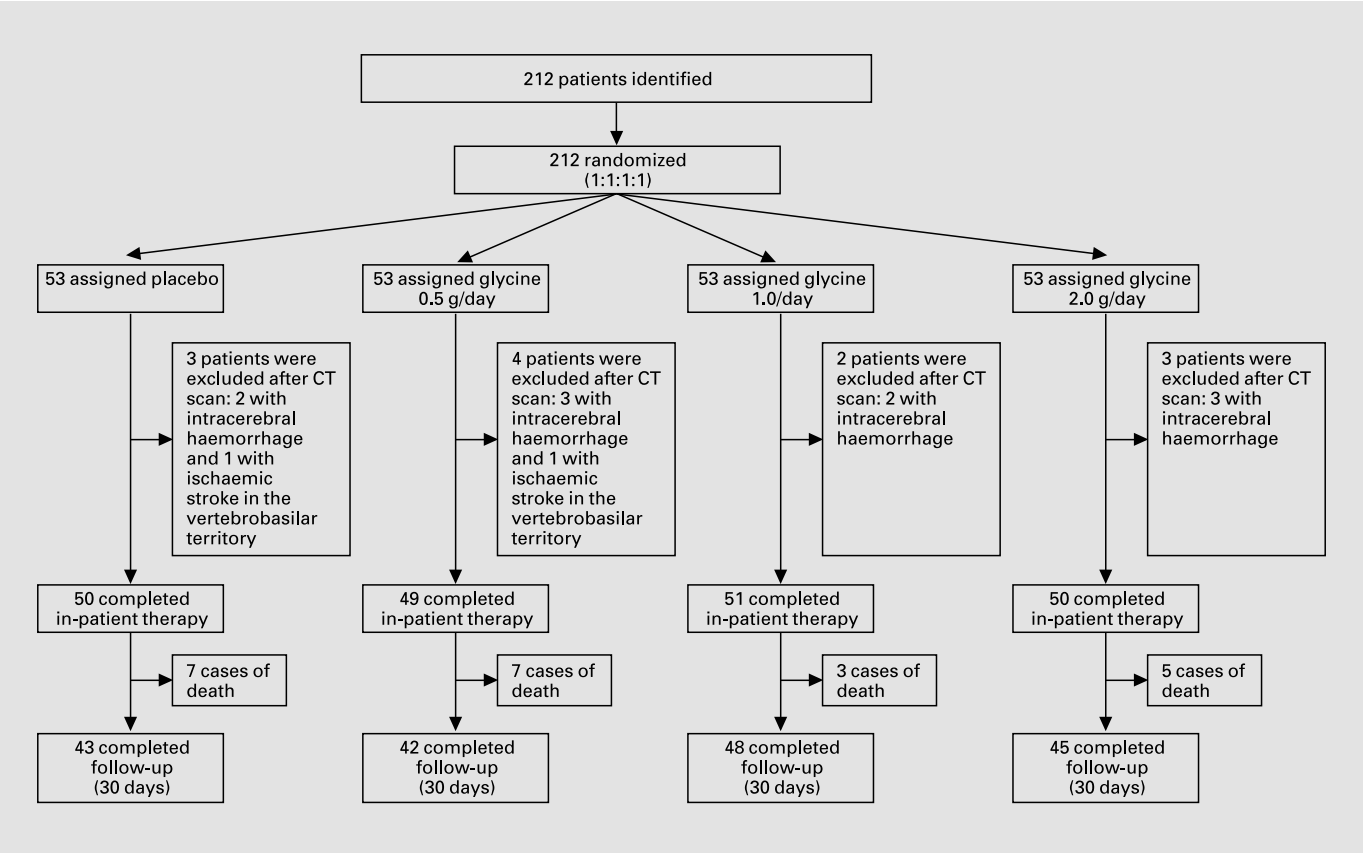

Fig. 1. Trial profile.

Table 1. Demographic and baseline characteristics of the target population (200 patients with ischaemic stroke in the territory of the carotid artery)

Characteristic	Placebo	Glycine			p
		0.5 g/day	1.0 g/day	2.0 g/day	
	(n = 50)	(n = 49)	(n = 51)	(n = 50)	
Sex (M/F)	28/22	25/24	27/24	30/20	NS
Median age, years	65.1 ± 9.5	64.5 ± 10.5	67.3 ± 8.7	66.7 ± 9.7	NS
Left/right hemisphere localization	32/18	28/21	28/23	27/23	NS
Mean inclusion time ± SEM, h	5.5 ± 0.2	5.3 ± 0.2	5.4 ± 0.2	5.2 ± 0.3	NS
Mean OSS score at start ± SEM	41.24 ± 2.6	41.06 ± 2.4	38.88 ± 1.9	40.9 ± 2.1	NS
Patients with baseline OSS score ≤ 40 (severe stroke)	19 (38%)	20 (40.8%)	21 (41.2%)	20 (40%)	NS
Patients with baseline OSS score 41–64 (moderate stroke)	23 (46%)	22 (45%)	23 (45.1%)	24 (48%)	NS
Patients with baseline OSS score ≥ 65 (mild stroke)	8 (16%)	7 (14.2%)	7 (13.7%)	6 (12.0%)	NS
Mean SSS score at start ± SEM	27.0 ± 1.4	27.1 ± 1.3	26.48 ± 1.3	26.91 ± 1.2	NS

Treatment Regimen

Within 6 h after the onset of stroke, individual random and blind assignment was performed on patients to receive sublingual treatment with placebo or one of three doses of glycine: 0.5, 1.0 or 2.0 g/day for 5 days. Tablets of placebo and glycine were similar in appearance and taste. The personnel at the trial site, outcome assessors, the personnel of the Safety Monitoring Committee involved in conducting or monitoring the trial and also data analysis were blinded to the trial drug codes. Concomitant treatment with calcium channel blockers, piracetam (Nootropil), drugs with neurotrophic and neuromodulatory properties (such as gangliosides, low-molecular-weight peptides) and other experimental stroke drugs was prohibited throughout the trial. Routine therapy included haemodilution and Aspirin (in all patients) and glycerol (in all cases of severe strokes). The same background therapy allowed us to compare stroke outcomes in the placebo and glycine groups.

Assessments

Baseline Assessments. A medical history, general physical and neurological examinations, ECG, haematological and biochemical tests and investigations of cerebrospinal fluid (CSF) were included. A CT scan of the brain was performed within the first 24 h after the start of trial medication. Neurological status was assessed by the Scandinavian Stroke Scale (SSS) [43] and the OSS [44], and the functional status was assessed by the Barthel index [45, 46]. The SSS and OSS are both graded by 10 neurological items; for the SSS and OSS, the maximum score (absence of neurological deficit) is 66 and 100, respectively, and the minimum score is 0. Based on OSS scores, patients were divided into several groups corresponding to severity of ischaemic stroke; a score ≥ 65 corresponded to mild stroke, one from 64 to 41 to moderate stroke, one from 40 to 26 to severe stroke and one ≤ 25 to extremely severe stroke. The initial division had been kept up throughout the trial; the SSS score assessment and other measures were considered within the initial OSS score groups. The Barthel index evaluates 10 activities of daily living with a maximal value of 100 and a minimal value of 0; a score from 0 to 45 corresponds to severe disability, one from 50 to 70 to moderate disability, one from 75 to 95 to mild disability and a score of 100 equals no disability [46].

Blood pressure, heart rate and ECG were repeated within 6–12 h after the initiation of therapy and on days 3, 5, 7, 14, 21 and 30. Neurological assessments were made at the start on admission within the first 6 h, at the end of the glycine treatment (on day 6) and at the end of the trial (on day 30). The Barthel index was estimated on day 30.

The following methods of laboratory monitoring were selected taking into consideration beneficial mediator and metabolic properties of the pharmaceutic drug glycine observed in experiments and clinics. Laboratory investigations of blood [the detection of autoantibodies (autoAB) to glutamate NMDA receptors] were carried out on admission to hospital and 6, 9, 12 and 24 h after the onset of stroke (monitoring within the first 24 h), and also on days 3 and 5 (within the early acute period). Investigations of CSF [determination of glutamate, aspartate, glycine and GABA levels and concentration of thiobarbituric-acid-reactive substances (TBARS)] were carried out within the first 6 h before the glycine treatment and were repeated on day 3. No complications related to lumbar puncture were observed. Mortality and adverse events were followed over the entire trial period of 30 days. Safety was followed by the Safety Monitoring Committee, which reviewed all reports of death and adverse events.

Protocol-specified study end points were the safety, neurological outcome according to the OSS and SSS, functional outcome according to the Barthel index and mortality on day 30.

Blood Serum Test for the Detection of AutoAB to Glutamate NMDA Receptors. We used an enzyme-linked immunosorbent assay (an ELISA technique) to test levels of blood serum autoAB to glutamate NMDA-binding proteins (NMDA-BP) on admission to hospital, 6, 9, 12 and 24 h after the onset of stroke, and on days 3 and 5. This diagnostic test was developed in the Institute of Human Brain of the Russian Academy of Sciences [47] for immunoferent analysis of autoAB to phencyclidine-binding membrane protein, isolated from NMDA receptors of human brain tissue. The test uses a 96-well plate with connected NMDA-BP, isolated from post-mortem human brain cortex and polyclonal antibodies to NMDA-BP. Serum was diluted to 1:50, and polyclonal antibody as a standard (0.01–400 ng/ml) was applied to the immunosorbent. The plate was incubated for 30 min at 37°C and then washed by a 0.05 *M* phosphate buffer, pH 7.4, containing 0.05% of Tween-20. Rabbit antibodies to human immunoglobulin labelled with horseradish peroxidase were added (0.1 ml), and the plate was incubated for 35 min at 37°C. After incubation the wells were washed with the buffer and distilled water. The reaction was stained by orthophenylenediamine in 0.05 *M* citrate buffers, pH 4.3, and a brown colour of reaction was monitored at 490 nm on a microplate rider (Dynatech MR 4000, UK). The curve of dependence of optical density from the concentration of polyclonal antibodies to NMDA-BP was derived and used for calculation of autoAB concentration in blood serum (ng/ml). In previous studies we detected the level of NMDA-BP autoAB in the blood serum of healthy persons (n = 200, age 35–75 years) which was 0.3–1.5 ng/ml [47].

Determination of Glutamate, Aspartate, Glycine and GABA Levels in CSF. Amino acid analysis was performed in CSF by high-performance liquid chromatography with electrochemical detection. CSF samples were obtained within the first 6 h after the stroke onset, before the first dose of glycine, and on day 3. CSF was diluted in 1:20 of 0.1 *N* $HClO_4$; it was centrifuged for 10 min at 10,000 *g* precipitate proteins. The supernatant (50 μl), *l*-homoserine (0.02 mg/nl) and ophtalaldehyde-sulphite reagent (25 μl) were incubated for 20 min at 20°C in 0.1 *M* sodium tetraborate buffer, pH 9.5. Then the sample (20 μl) was injected on a reverse-phase column (3 × 150 mm, C-18) and diluted by 0.01 *M* phosphate buffer, pH 5.6, containing 0.05 m*M* EDTA, 5% methanol (v/v). The mixture of *l*-homoserine (0.02 mg/ml) in 0.1 *M* sodium tetraborate buffer (50 μl), pH 9.5, was used as internal standard. The flow rate was 1 ml/min with electrochemical detection of glutamate and glycine by use of carbon electrode LC-4B (Bioanalytical Systems Inc., USA). All reagents used were from Sigma (St. Louis, Mo., USA) and Fluka (Buchs, Switzerland). The concentration of amino acid neurotransmitters (μmol/l) in CSF was calculated on a calibrated curve: $S_{\text{amino acid}}/S_o$, where $S_{\text{amino acid}}$ is the square of the amino acid peak and S_o is the square of the internal standard peak on the chromatogram. In previous studies we determined amino acid concentrations in CSF before peridural anaesthesia in 20 surgical patients without neurological disorders. Normal CSF concentrations of amino acids (aspartate 1.22 ± 0.21, glutamate 4.48 ± 0.74, glycine 15.15 ± 1.4, GABA 0.97 ± 0.34 μmol/l) were used as controls in the present study.

Determination of Lipid Peroxidation Processes (Products) in CSF Samples. Determination of lipid peroxidation processes in CSF samples was performed by measuring TBARS according to Ohkawa et al. [48] within the first 6 h before the glycine treatment and on day 3. Briefly, 10% (w/v) homogenate was mixed with sodium dodecyl sul-

Table 2. Mortality and causes of death according to treatment group

Characteristic	Placebo	Glycine		
	(n = 50)	0.5 g/day (n = 49)	1.0 g/day (n = 51)	2.0 g/day (n = 50)
Mortality				
All patients	7/50 (14.0%)	7/49 (14.3%)	3/51 (5.9%)*	5/50 (10.0%)
Patients with baseline OSS score ≥40 (mild and moderate stroke)	2/31 (6.5%)	2/29 (6.9%)	0/30 (0%)	1/30 (3.3%)
Patients with baseline OSS score 25–40 (severe stroke)	2/11 (18.2%)	2/12 (16.7%)	1/11 (9.1%)	2/13 (15.4%)
Patients with baseline OSS score ≤25 (extremely severe stroke)	3/8 (37.5%)	3/8 (37.5%)	2/10 (20.0%)	2/7 (28.6%)
Causes of death				
Cerebral oedema	5	3	1	1
PA thrombo-embolism	1	2	1	2
Cardiac failure	1	1	1	2
Secondary stroke	–	1	–	–

* $p < 0.05$, compared with the placebo group. PA = Pulmonary artery.

phate, acetate buffer (pH 3.5) and aqueous solution of thiobarbituric acid. After heating at 95°C for 60 min, the red pigment produced was extracted with n-butanol-pyridine mixture and measured by absorbance at 532 nm. All results were expressed as nanomoles per millilitre. In previous studies we determined the concentration of lipid peroxidation products in CSF before peridural anaesthesia in 20 surgical patients without neurological disorders, which was 2.42 ± 0.7 nmol/l, and this was used as control measure in the present study.

Statistical Analysis

Demographic and baseline disease characteristics were compared with the use of the Cochran-Mantel-Haenszel test for general association for nominal categorical variables (e.g. sex) and a one-way ANOVA for continuous variables (e.g. age). One-sample t tests were performed with the use of descriptive statistics for each heart rate and blood pressure to evaluate changes versus baseline values.

The primary efficacy analysis was performed on a target population basis as prespecified in the protocol, i.e. on all 200 patients with carotid artery territory ischaemic stroke who were correctly included in the trial according to the inclusion and exclusion criteria and who completed the follow-up within 30 days.

Descriptive statistics and frequency distributions were generated for the study and point data. The Wilcoxon matched-pairs signed-rank test was used to analyse the statistical significance for the changes in measured parameters, and the Mann-Whitney U test was used for pairwise and group comparisons. The dynamics of biochemical parameters was assessed using ANOVA. Mortality rates and disability levels on the Barthel index among the treatment groups were compared with the use of Fisher's exact test.

Clinical assessment of the glycine groups showed that there was no significant difference between the 1.0 and 2.0 g/day glycine groups. A post hoc subgroup analysis regarding biochemical parameters was performed on the 0.5 and 1.0–2.0 g/day glycine groups.

All statistical tests were interpreted at the 5% two-tailed significance level.

Results

The trial confirmed the safety profile of the glycine treatment. Slight sedation was observed in 9 patients (4.5%) as a side-effect. Other marked side-effects or adverse events were absent. The glycine treatment had no statistically significant effects on ECG and haemorheological parameters.

Compared to placebo, there was a lower mortality in the 2.0 g/day glycine group and a significant decrease in mortality in the 1.0 g/day group ($p < 0.05$; table 2).

A quantitative time-related analysis of the dynamics of neurological deficit (as measured by the mean shift from baseline on the OSS and the SSS) performed by intention-to-treat and on-treatment methods revealed more rapid improvement of neurological deficits up to days 6 and 30 in patients of the 1.0 and 2.0 g/day glycine groups versus placebo (tables 3, 4), which was more pronounced in the 1.0 g/day group. In the 0.5 g/day glycine group, a tendency towards acceleration of improvement was noted only in patients with mild to moderate stroke (OSS score >40) up to day 30 (table 4).

Table 3. Change in neurological outcome as measured by the mean shift from baseline on the OSS and SSS: intention-to-treat analysis

Stroke scale		Placebo (n = 53)	Glycine		
			0.5 g/day (n = 53)	1.0 g/day (n = 53)	2.0 g/day (n = 53)
At day 6					
OSS	Patients with mild to moderate stroke (OSS >40)	+14.05 ± 1.2 (n = 32)	+14.43 ± 1.5 (n = 30)	+22.05 ± 1.3** (n = 31)	+16.93 ± 1.3 (n = 31)
	Patients with severe stroke (OSS ≤40)	+5.5 ± 1.0 (n = 21)	+5.89 ± 0.9 (n = 23)	+20.17 ± 1.6** (n = 22)	+16.11 ± 1.6** (n = 22)
SSS	Patients with mild to moderate stroke (OSS >40)	+5.11 ± 0.8 (n = 32)	+5.24 ± 0.7 (n = 30)	+7.25 ± 0.6* (n = 31)	+6.85 ± 0.7 (n = 31)
	Patients with severe stroke (OSS ≤40)	+3.57 ± 0.9 (n = 21)	+3.22 ± 0.9 (n = 23)	+12.26 ± 1.2** (n = 22)	+10.10 ± 1.1** (n = 22)
At day 30					
OSS	Patients with mild to moderate stroke (OSS >40)	+18.64 ± 2.0 (n = 32)	+24.54 ± 2.8* (n = 30)	+33.52 ± 1.9** (n = 31)	+27.20 ± 2.2** (n = 31)
	Patients with severe stroke (OSS ≤40)	+13.85 ± 3.1 (n = 21)	+16.23 ± 3.9 (n = 23)	+34.04 ± 2.6** (n = 22)	+31.95 ± 2.7** (n = 22)
SSS	Patients with mild to moderate stroke (OSS >40)	+9.52 ± 1.0 (n = 32)	+15.80 ± 1.2** (n = 30)	+21.09 ± 0.7** (n = 31)	+19.94 ± 1.2** (n = 31)
	Patients with severe stroke (OSS ≤40)	+10.27 ± 2.4 (n = 21)	+11.12 ± 2.6 (n = 23)	+23.19 ± 1.7** (n = 22)	+22.25 ± 1.9** (n = 22)

Patients who died during the 30-day study period were assigned the worst score of each of the neurological scales after their death. Positive values indicate improvement. * $p < 0.05$, ** $p < 0.01$, compared with placebo.

Table 4. Change in neurological outcome as measured by the mean shift from baseline on the OSS and SSS: on-treatment analysis

Stroke scale		Placebo (n = 50)	Glycine		
			0.5 g/day (n = 49)	1.0 g/day (n = 51)	2.0 g/day (n = 50)
At day 6					
OSS	Patients with mild to moderate stroke (OSS >40)	+14.28 ± 1.1 (n = 31)	+14.58 ± 1.4 (n = 29)	+22.28 ± 1.3** (n = 30)	+17.09 ± 1.2 (n = 30)
	Patients with severe stroke (OSS ≤40)	+5.77 ± 0.9 (n = 19)	+6.32 ± 0.9 (n = 20)	+20.42 ± 1.5** (n = 21)	+16.72 ± 1.5** (n = 20)
SSS	Patients with mild to moderate stroke (OSS >40)	+5.15 ± 0.7 (n = 31)	+5.28 ± 0.6 (n = 29)	+7.31 ± 0.5* (n = 30)	+6.91 ± 0.7 (n = 30)
	Patients with severe stroke (OSS ≤40)	+3.74 ± 0.8 (n = 19)	+3.40 ± 0.7 (n = 20)	+12.61 ± 1.1** (n = 21)	+10.87 ± 1.0** (n = 20)
At day 30					
OSS	Patients with mild to moderate stroke (OSS >40)	+18.92 ± 1.9 (n = 31)	+24.77 ± 2.8* (n = 29)	+33.97 ± 1.7** (n = 30)	+27.61 ± 2.1** (n = 30)
	Patients with severe stroke (OSS ≤40)	+14.78 ± 2.9 (n = 19)	+17.47 ± 3.7 (n = 20)	+34.71 ± 2.5** (n = 21)	+33.35 ± 2.6** (n = 20)
SSS	Patients with mild to moderate stroke (OSS >40)	+9.70 ± 0.9 (n = 31)	+16.00 ± 1.1** (n = 29)	+21.29 ± 0.6** (n = 30)	+20.10 ± 1.1** (n = 30)
	Patients with severe stroke (OSS ≤40)	+10.83 ± 2.3 (n = 19)	+12.04 ± 2.5 (n = 20)	+23.82 ± 1.6** (n = 21)	+23.48 ± 1.7** (n = 20)

Patients who died during the 30-day study period were assigned the worst score of each of the neurological scales after their death. Positive values indicate improvement. * $p < 0.05$, ** $p < 0.01$, compared with placebo.

Table 5. Functional outcome at day 30: intention-to-treat analysis

Functional outcome	Placebo	Glycine		
		0.5 g/day	1.0 g/day	2.0 g/day
All patients				
Death	8/53 (15.1%)	8/53 (15.1%)	3/53 (5.7%)	6/53 (11.3%)
Severe disability	10/53 (18.9%)	8/53 (15.1%)	1/53 (1.8%)[b, c]	3/53 (5.7%)[a, c]
Moderate disability	21/53 (39.6%)	20/53 (37.7%)	8/53 (15.1%)[a, c]	11/53 (20.7%)
Mild or no disability	14/53 (26.4%)	17/53 (32.1%)	41/53 (77.4%)[b, c]	33/53 (62.3%)
Patients with mild to moderate stroke (OSS >40)				
Death	2/32 (6.3%)	2/30 (6.7%)	0/31 (0%)	1/31 (3.2%)
Severe disability	1/32 (3.1%)	1/30 (3.3%)	0/31 (0%)	0/31 (0%)
Moderate disability	17/32 (53.1%)	14/30 (46.7%)	3/31 (9.7%)[b]	5/31 (16.1%)[a]
Mild or no disability	12/32 (37.5%)	13/30 (43.3%)	28/31 (90.3%)[a]	25/31 (80.7%)
Patients with severe stroke (OSS ≤40)				
Death	6/21 (28.6%)	6/23 (26.1%)	3/22 (13.7%)	5/22 (22.7%)
Severe disability	9/21 (42.9%)	7/23 (30.4%)	1/22 (4.5%)[b]	3/22 (13.7%)
Moderate disability	4/21 (19.0%)	6/23 (26.1%)	5/22 (22.7%)	6/22 (27.3%)
Mild or no disability	2/21 (9.5%)	4/23 (17.4%)	13/22 (59.1%)[a]	8/22 (36.3%)

Severe disability = Barthel score 0–45; moderate disability = Barthel score 50–70; mild or no disability = Barthel score 75–100.

[a] $p < 0.05$, [b] $p < 0.01$, compared with the placebo group; [c] $p < 0.05$, compared with the 0.5 g/day glycine group.

Table 6. Functional outcome at day 30: on-treatment analysis

Functional outcome	Placebo	Glycine		
		0.5 g/day	1.0 g/day	2.0 g/day
All patients				
Death	7/50 (14.0%)	7/49 (14.3%)	3/51 (5.9%)	5/50 (10.0%)
Severe disability	9/50 (18.0%)	6/49 (12.2%)	0/51 (0%)[b, c]	2/50 (4.0%)[a, c]
Moderate disability	20/50 (40.0%)	19/49 (38.8%)	8/51 (15.7%)[a, c]	10/50 (20.0%)
Mild or no disability	14/50 (28.0%)	17/49 (34.7%)	40/51 (78.4%)[b, c]	33/50 (66.0%)
Patients with mild to moderate stroke (OSS >40)				
Death	2/31 (6.5%)	2/29 (6.9%)	0/30 (0%)	1/30 (3.3%)
Severe disability	1/31 (3.2%)	1/29 (3.5%)	0/30 (0%)	0/30 (0%)
Moderate disability	16/31 (51.6%)	13/29 (44.8%)	3/30 (10.0%)[b]	4/30 (13.3%)[a]
Mild or no disability	12/31 (38.7%)	13/29 (44.8%)	27/30 (90.0%)[a]	25/30 (83.3%)
Patients with severe stroke (OSS ≤40)				
Death	5/19 (26.3%)	5/20 (25.0%)	3/21 (14.3%)	4/20 (20.0%)
Severe disability	8/19 (42.1%)	5/20 (25.0%)	0/21 (0%)[b]	2/20 (10.0%)
Moderate disability	4/19 (21.1%)	6/20 (30.0%)	5/21 (23.8%)	6/20 (30.0%)
Mild or no disability	2/19 (10.5%)	4/20 (20.0%)	13/21 (61.9%)[a]	8/20 (40.0%)

Severe disability = Barthel score 0–45; moderate disability = Barthel score 50–70; mild or no disability = Barthel score 75–100.

[a] $p < 0.05$, [b] $p < 0.01$, compared with the placebo group; [c] $p < 0.05$, compared with the 0.5 g/day glycine group.

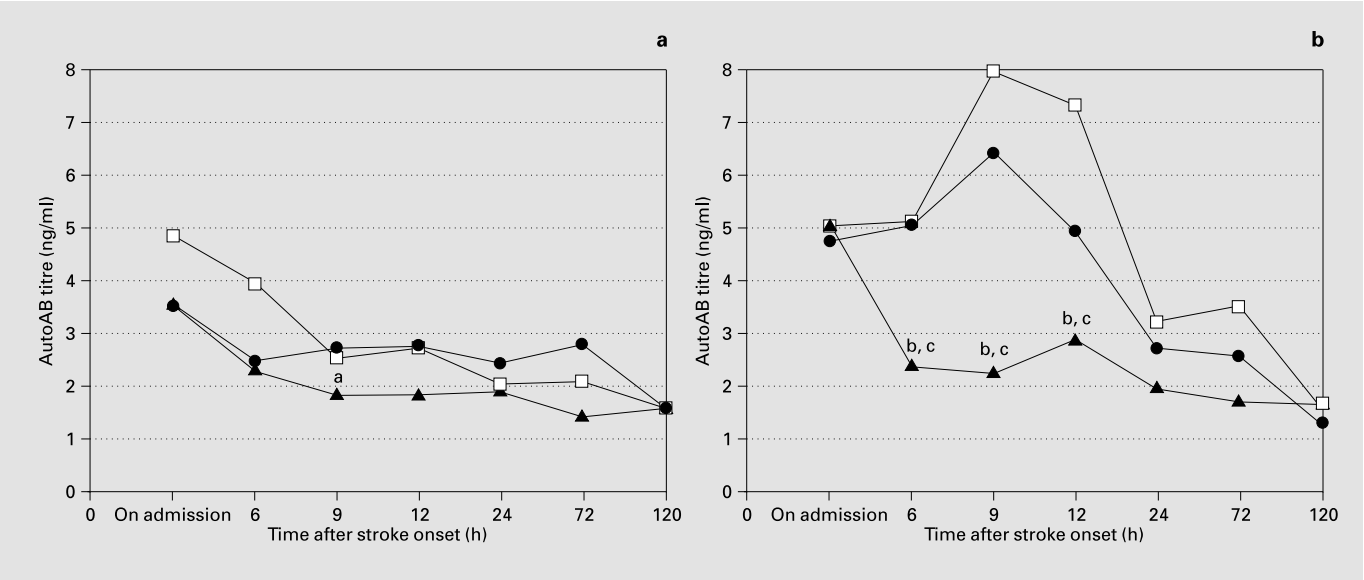

Fig. 2. Dynamics of autoAB titres to NMDA-BP in acute ischaemic stroke. □ = Placebo group; ● = 0.5 g/day glycine group; ▲ = 1.0–2.0 g/day glycine group. [a] $p < 0.05$, [b] $p < 0.01$, versus placebo; [c] $p < 0.01$, versus 0.5 g/day glycine. **a** Mild to moderate stroke. **b** Severe stroke.

Table 7. The dynamics of serum autoAB titres to NMDA-BP (ng/ml) in patients with ischaemic stroke in the territory of the carotid artery

Groups of patients	Placebo	Glycine	
		0.5 g/day	1.0–2.0 g/day
Mild to moderate stroke	31	29	60
On admission[1]	4.86 ± 0.8[a]	3.51 ± 0.9[a]	3.55 ± 0.4[a]
6 h	3.95 ± 0.7	3.45 ± 0.7	2.30 ± 0.8
9 h	2.54 ± 0.3	2.74 ± 0.8	1.85 ± 0.7[c]
12 h	2.72 ± 0.3	2.78 ± 0.3	1.84 ± 0.4
24 h	2.45 ± 0.3	2.05 ± 0.3	1.89 ± 0.2
72 h	2.80 ± 0.5	2.10 ± 0.4	1.42 ± 0.6
120 h	1.63 ± 0.1	1.59 ± 0.1	1.55 ± 0.4
Severe stroke	19	20	41
On admission[1]	5.04 ± 0.9[a, b]	4.75 ± 0.7[a, b]	5.10 ± 0.6[a, b]
6 h	5.10 ± 0.7[b]	5.05 ± 0.8[b]	2.40 ± 0.4[d, e]
9 h	7.90 ± 1.2[b]	6.41 ± 1.1[b]	2.23 ± 0.4[d, e]
12 h	7.30 ± 1.5[b]	4.95 ± 0.6[b]	2.90 ± 0.5[d, e]
24 h	3.20 ± 0.6	2.70 ± 0.3	1.95 ± 0.5
72 h	3.50 ± 0.5	2.55 ± 0.3	1.70 ± 0.1
120 h	1.67 ± 3	1.30 ± 0.4	1.66 ± 0.1

[a] $p < 0.01$, versus control; [b] $p < 0.01$, ANOVA, severe stroke versus mild to moderate stroke; [c] $p < 0.05$, [d] $p < 0.01$, ANOVA, 1.0–2.0 g/day glycine versus placeo; [e] $p < 0.01$, ANOVA, 1.0–2.0 g/day glycine versus 0.5 g/day glycine.
[1] Before the glycine treatment.

When outcome was evaluated with the Barthel index, the 1.0 and 2.0 g/day glycine groups were found to have a higher proportion of patients with good recovery (no or mild disability, i.e. a Barthel score >70) than the 0.5 g/day glycine group and the placebo group. A significant decrease was revealed in the number of patients with severe disability in the 1.0 g/day glycine group ($p < 0.01$ vs. placebo; $p < 0.05$ vs. 0.5 g/day glycine) and the 2.0 g/day glycine group ($p < 0.05$ vs. placebo; $p < 0.05$ vs. 0.5 g/day glycine; tables 5, 6).

After the neurological and functional assessment had shown that there was no significant difference between the 1.0 and the 2.0 g/day glycine groups, we used the 0.5 g/day and the 1.0–2.0 g/day glycine groups for the analysis regarding biochemical parameters.

Carried out within the first 6 h before starting the glycine treatment the immunoferent analysis of blood serum autoAB to glutamate NMDA receptors revealed increased levels of autoAB to NMDA-BP in all groups of patients with acute ischaemic stroke in comparison with the control group ($p < 0.01$). Initial levels of autoAB to glutamate NMDA receptors were significantly higher in patients with severe stroke. The predominance of increased levels in severe stroke patients remained for 12 h after the stroke onset as compared to the group with mild to moderate stroke in the placebo and 0.5 g/day groups ($p = 0.01$ and

Table 8. The dynamics of CSF concentrations of amino acids (μmol/l) in patients with ischaemic stroke in the territory of the carotid artery

Groups of patients	Placebo	Glycine	
		0.5 g/day	1.0–2.0 g/day
Mild to moderate stroke	31	29	60
Glutamate			
Within the first 6 h	26.30 ± 3.5[b]	25.50 ± 4.35[b]	21.96 ± 3.2[b]
Day 3	16.66 ± 1.84	17.88 ± 1.7	14.89 ± 1.16
Aspartate			
Within the first 6 h	79.88 ± 7.13[b]	82.88 ± 7.73[b]	90.38 ± 7.28[b]
Day 3	98.33 ± 1.73	80.63 ± 8.03	66.90 ± 5.03[d, f]
Glycine			
Within the first 6 h	24.21 ± 4.4	28.60 ± 5.72	35.91 ± 2.79
Day 3	29.93 ± 2.8	32.59 ± 4.92	34.16 ± 3.33
GABA			
Within the first 6 h	0.87 ± 0.49	0.97 ± 0.87	0.68 ± 0.29
Day 3	1.75 ± 0.87	1.65 ± 0.78	1.94 ± 0.68
Severe stroke	19	20	41
Glutamate			
Within the first 6 h	33.86 ± 5.17[b]	32.30 ± 4.15[b]	35.84 ± 3.47[b]
Day 3	38.49 ± 6.46	36.37 ± 5.92	23.32 ± 1.97[c, f, g]
Aspartate			
Within the first 6 h	70.05 ± 6.38[b]	65.25 ± 5.78[b]	59.78 ± 2.48[b]
Day 3	54.98 ± 4.88	56.48 ± 5.03	41.18 ± 3.08[c, e, g]
Glycine			
Within the first 6 h	27.53 ± 3.59	30.99 ± 3.86	40.03 ± 2.53
Day 3	36.44 ± 4.12	28.86 ± 4.39	28.99 ± 1.99[e]
GABA			
Within the first 6 h	0.097 ± 0.097[a]	0.097 ± 0.097[a]	0.19 ± 0.097[a]
Day 3	0.097 ± 0.097	0.29 ± 0.097	1.94 ± 0.49[d, e, h]

Concentrations are given as means ± SD. [a] $p < 0.01$, [b] $p < 0.001$, Mann-Whitney U test, versus control; [c] $p < 0.05$, [d] $p < 0.01$, Mann-Whitney U test, 1.0–2.0 g/day/glycine versus placebo; [e] $p < 0.05$, [f] $p < 0.01$, Wilcoxon matched-pairs signed-rank test, day 3 versus within the first 6 h; [g] $p < 0.05$, [h] $p < 0.01$, Mann-Whitney U test, 1.0–2.0 g/day glycine versus 0.5 g/day glycine.

$p = 0.01$, respectively). At the same time, first application of 1.0–2.0 g/day of glycine induced an early normalization of autoAB titres to NMDA-BP in those patients ($p < 0.01$ vs. placebo and $p < 0.01$ vs. 0.5 g/day glycine). Levels of autoAB titre in the 1.0–2.0 g/day glycine group remained significantly lower during the period from 6 to 12 h ($p < 0.01$) in comparison with both the placebo and 0.5 g/day glycine groups (table 7, fig. 2). In patients with mild to moderate stroke a tendency towards normalization of autoAB titres to NMDA receptors was demonstrated in all groups of the study by 9–12 h after the stroke onset (table 7, fig. 2).

Neurotransmitter amino acid analysis which was performed within the first 6 h after the stroke onset before starting the glycine treatment demonstrated a significant increase in levels of excitatory amino acids (aspartate, glutamate) versus control levels ($p < 0.001$), which corresponded to previous investigations [40, 49, 50]. A tendency was revealed towards elevation of glycine levels versus control ($p > 0.05$). GABA concentrations in CSF of patients with mild to moderate ischaemic stroke were shown to be untouched in comparison with the control group, while in patients with severe stroke GABA levels were significantly lower than control readings ($p < 0.01$). There were no significant differences in amino acid levels within the first 6 h between all groups of patients with acute ischaemic stroke (table 8).

On day 3, no significant differences were demonstrated between the placebo and the 0.5 g/day glycine groups with regard to excitatory and inhibitory amino

Table 9. The dynamics of CSF level of lipid peroxidation (nmol/ml) in patients with ischaemic stroke in the territory of the carotid artery

Groups of patients	Placebo	Glycine	
		0.5 g/day	1.0–2.0 g/day
Mild to moderate stroke	31	29	60
Within the first 6 h	3.03 ± 0.7	3.02 ± 0.55	2.82 ± 0.8
Day 3	3.95 ± 0.94	2.48 ± 1.2	1.48 ± 0.54*
Severe stroke	19	20	41
Within the first 6 h	3.29 ± 1.4	3.1 ± 1.3	2.86 ± 1.4
Day 3	5.54 ± 1.8	4.5 ± 0.5	3.42 ± 1.8*

* $p < 0.05$, Mann-Whitney U test, 1.0–2.0 g/day glycine versus placebo.

acid levels in CSF. Application of glycine in doses of 1.0–2.0 g/day was accompanied by statistically significant changes of amino acid concentrations (vs. the placebo and 0.5 g/day glycine groups) that were more pronounced in patients with severe stroke. There was a significant reduction in glutamate levels by 35% ($p < 0.05$ vs. placebo, $p < 0.01$ vs. day 1 and $p < 0.05$ vs. 0.5 g/day of glycine) and in aspartate levels by 31% ($p < 0.05$ vs. placebo, $p < 0.05$ vs. day 1 and $p < 0.05$ vs. 0.5 g/day of glycine) in CSF of patients with severe stroke, while GABA concentrations significantly increased by day 3 ($p < 0.01$ vs. placebo, $p < 0.05$ vs. day 1 and $p < 0.01$ vs. 0.5 g/day of glycine). In CSF of patients with mild to moderate stroke, application of glycine in doses of 1.0–2.0 g/day was accompanied by analogical tendencies in changes of amino acid concentrations, but significance was obtained only for dynamics of aspartate levels ($p < 0.01$ vs. placebo and $p < 0.01$ vs. day 1; table 8).

Determination of lipid peroxidation products revealed a slight tendency towards elevation of concentrations of TBARS ($p > 0.05$ vs. control group) within the first 6 h in all groups of patients with acute ischaemic stroke before starting the glycine treatment, without a significant difference between the groups. In patients with mild to moderate stroke the tendency to continuous increase in TBARS by day 3 was demonstrated in the placebo group, while in both glycine groups a reduction of concentrations of lipid peroxidation products was found that was significant in the 1.0–2.0 g/day glycine group ($p < 0.05$ vs. placebo). In patients with severe stroke the tendency to an increase in TBARS by day 3 was demonstrated in all groups of the study. However, in both glycine groups the rate of this elevation was shown to be significantly lower as compared to the placebo group: by 19.58% in the 1.0–2.0 g/day glycine group, by 45% in the 0.5 g/day glycine group and by 68.3% in the placebo group ($p < 0.05$, 1.0–2.0 g/day glycine vs. the placebo group; table 9).

Discussion

The present study confirmed the absence of side-effects and adverse events of the drug glycine, which corresponded to results of previous investigations [42] and could be connected with properties of glycine as a natural product of brain metabolism.

The intention-to-treat and on-treatment analysis demonstrated a tendency to improvement of neurological recovery and functional outcome in patients with acute ischaemic stroke, treated with 1.0–2.0 g/day glycine for 5 days after the event. A tendency to a decrease in mortality rate was also shown. The positive effects of glycine were more pronounced in severely affected patients.

The early normalization of the autoAB titre to NMDA-BP in the 1.0–2.0 g/day glycine group also prevailed in severely affected patients and corresponded to an accelerated restoration of altered neurological functions, which possibly reflected the improvement of the functional state of glutamate NMDA receptors under the influence of the drug glycine [51].

Neurotransmitter amino acid analysis confirmed the effects of glycine on glutamate and aspartate excitotoxicity. The glycine treatment in doses of 1.0–2.0 g/day induced not only a statistically significant reduction of glutamate and aspartate concentrations in CSF up to day 3 (vs. placebo and vs. day 1), but also a significant increase in GABA levels (vs. placebo and vs. day 1); thus, it provided evidence of a reduction of the imbalance between excitatory and inhibitory neurotransmitter systems. It is interesting that application of 1.0–2.0 g/day of glycine in severely affected patients was accompanied by a statistically significant reduction of CSF concentration of glycine up to day 3 after stroke onset. Perhaps the marked decrease in the CSF glycine level under the influence of the drug glycine reflected a tendency to normalization of amino acid metabolism in brain tissue (as a result of improved participation of glycine in anabolic cell processes).

As revealed in the 1.0–2.0 g/day glycine group, the significant reduction in TBARS levels in patients with mild to moderate stroke and the deceleration of increase in concentrations of lipid peroxidation products in severely

affected patients probably reflected not only neurotransmitter effects of the drug glycine but also its general metabolic properties.

In conclusion, the trial suggests that the sublingual application of 1.0–2.0 g/day glycine started within 6 h after the onset of acute ischaemic stroke in the carotid artery territory is safe and can exert favourable clinical effects. These results will be verified in further trials with a larger number of patients.

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