Effect of \( S \)-adenosyl-\( L \)-methionine on rat brain oxidative stress damage in a combined model of permanent focal ischemia and global ischemia-reperfusion

M.A. Villalobos\(^a\), J.P. De La Cruz\(^b,\ast\), M.A. Cuerda\(^a\), P. Ortiz\(^b\), J.M. Smith-Agreda\(^a\), F. Sánchez De La Cuesta\(^b\)

\(^a\)Department of Anatomy, School of Medicine, University of Málaga, 29071 Málaga, Spain
\(^b\)Department of Pharmacology and Therapeutics, School of Medicine, University of Málaga, 29071 Málaga, Spain

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Abstract

We analyzed the effects of \( S \)-adenosyl-\( L \)-methionine (SAM) on tissue oxidative status in a combined model of permanent focal ischemia and global reperfusion in the rat brain. The production of thiobarbituric acid reactive substances (TBARS) was measured under basal conditions and after induction with ferrous salt as an indicator of brain lipid peroxidation. Total, oxidized and reduced glutathione were measured as indicators of the antioxidant defense capacity of brain tissue. Mitochondrial reduction of tetraphenyl tetrazolium (TPT) was quantified morphometrically. Results obtained in vitro showed that incubation with SAM reduced lipid peroxidation, with a maximum inhibition of 65.12\( \pm \)5.99\% after incubation with 1 mmol/l; glutathione production was not significantly modified. In the brain ischemia-reperfusion model, TBARS production increased and glutathione content decreased, and mitochondrial reduction of TPT decreased significantly after ischemia-reperfusion in areas dependent on carotid circulation. The administration of 50 mg/kg SAM per day for 3 days led to the inhibition of brain lipid peroxidation and increased total glutathione production. These changes were accompanied by an increase in mitochondrial capacity to reduce TPT. We conclude that SAM reduces oxidative damage in the rat brain in an experimental model of ischemia-reperfusion.

Theme: Disorders of the nervous system

Topic: Ischemia

Keywords: \( S \)-Adenosyl-\( L \)-methionine; Lipid peroxidation; Glutathione; Oxidative stress; Ischemia-reperfusion

1. Introduction

Oxygen-derived free radicals play an important role in the pathophysiological processes of damage during brain ischemia and reperfusion, both during ischemia and when the tissue supply of oxygen is restored [5,6,24]. Changes caused by ischemia-reperfusion can include an increase in oxidant factors, impairment of endogenous antioxidant systems, or both. For example, earlier work reported significant increases in the levels of lipid peroxides in the cell membrane, decreases in the activities of enzymes such as superoxide dismutase (SOD) and catalase, and impaired effectiveness of the glutathione system [1,6,18,22,29].

These effects have led to studies designed to identify drugs with antioxidant activity in models of ischemia-reperfusion, especially agents able to inhibit lipid peroxidation [2,11–15,17,37]. However, another possibility might be to analyze the role of drugs that increase glutathione production. One such drug — \( S \)-adenosyl-\( L \)-methionine (SAM) — is produced by the body and participates in many physiological processes, mainly those involving methylation or transsulfuration. It is through this latter pathway that SAM produces intracellular glutathione [27].

\( S \)-adenosyl-\( L \)-methionine has shown antioxidant effects in kidney and liver tissue in several models of ischemic or toxic damage [16,27], and in some normal rat and human
organs [8,9,16]. In a study of the rat brain we recently showed that SAM modulates cellular oxidative status, mainly by inhibiting lipid peroxidation and enhancing the glutathione system [10]. The present study was designed to determine whether these pharmacological effects also occurred in an experimental model of ischemia-reperfusion in the rat brain.

2. Materials and methods

2.1. Drugs and reagents

S-Adenosyl-l-methionine (sulphoadenosylmethionine paratoluenesulphate, SAM) was obtained from Boehringer Ingelheim España S.A. (Barcelona, Spain). All other reagents were obtained from Sigma Chemical Co. (St. Louis, MO, USA).

2.2. Experimental model

The experiments were done in groups of 10 male Wistar rats aged 12–14 weeks and weighing 200–300 g. All experiments were carried out in accordance with the European Communities Council Directive of 24 November 1986 (86/609/EEC) regarding the handling of experimental animals. The rats were deprived of food overnight. On the following day they were anesthetized with an intramuscular injection of droperidol (0.66 mg/kg) and phentanyl (0.012 mg/kg). A combination model of ischemia and reperfusion was produced as described previously [11,37] by electrocoagulation of the superficial branches of the left middle cerebral artery, followed by bilateral occlusion of the common carotid arteries for 1 h and restoration of circulation for a period of 2 h. The limits of the area in which vascular electrocoagulation was performed were the frontal bone in the front, the occipital bone in the back, the zygomatic arch and coronoid process described above without sucrose at a proportion 1:10 w/v.

In addition, 20 sham-operated rats were used for in vitro experiments. The brains were dissected into the same portions specified above. Lipid peroxidation experiments (n=10) were done in fractions enriched in cell membranes (see below), and glutathione (n=10) was measured in 2-mm brain slices (see below), corresponding to the same brain sections as were used in the lipid peroxidation experiments. In each experiment, membrane concentrates were incubated with concentrations of SAM ranging from 10⁻⁶ to 10⁻³ M for 15 min at 37°C. Each experiment was carried out in duplicate.

2.3. In vitro experiments

Lipid peroxidation was determined by quantifying the reaction products with thiobarbituric acid (TBARS, thiobarbituric acid-reactive substances) under basal conditions and after induction with ferrous salts [7]. The fraction enriched in cell membranes was obtained from brain tissue with the method of Bossman and Hemsworth [3]. Briefly, the tissue was diluted (1:10 w/v) in buffer containing NaCl 0.1 M, KCl 5×10⁻³ M, CaCl₂ 3.1×10⁻³ M, MgSO₄ 1×10⁻³ M, glucose 4.9×10⁻³ M, NaHCO₃ 2.4×10⁻² M, KH₂PO₄ 5.5×10⁻⁴ M, and sucrose 0.32 M. The sample was homogenized and centrifuged at 4°C for 15 min at 1000×g, and the supernatant was removed. This fraction was centrifuged at 4°C for 20 min at 12 000×g, and the resulting pellet was resuspended in the buffer described above without sucrose at a proportion 1:10 w/v (with respect to the fresh weight).

To determine lipid peroxides the tissue was divided into aliquots of 850 μl, and 100 μl of dilution buffer were added to one tube to measure basal lipid peroxidation. To the other tubes were added 100 μl of 100 μmol/l ferrous sulfate and ascorbic acid (FeAs) to measure induced lipid peroxidation. All samples were shaken and incubated at 37°C for 45 min, and 500 μl of 0.5% thiobarbituric acid in 20% trichloroacetic acid were then added. The samples were then shaken again and incubated at 100°C for 15 min, then centrifuged at 4°C at 2000×g for 15 min. Spectrophotometric absorbance was determined in the supernatant at 532 nm (Perkin Elmer C-532001 spectrophotometer, IL, USA). Blank samples were processed in the same way except that they were incubated at 4°C. The final results were expressed as μmol of TBARS per mg of protein, determined with the Lowry method [25]. The results were obtained as nmol of TBARS per mg of protein, according
to a standard curve prepared with malondialdehyde-bis-diethyl-acetal. Each determination was carried out in duplicate.

2.5. Determination of total glutathione levels

Glutathione levels were determined by spectrophotometry according to the technique described by Hissin and Hill [20]. Briefly, 200 mg of tissue were homogenized in 4 ml of 0.1 M sodium phosphate buffer (pH 8.0) with 25% phosphoric acid. The mixture was centrifuged at 13 000× g for 15 min at 4°C, and the supernatant was collected. Two spectrophotometry cuvettes per sample were prepared with 1.8 ml sodium phosphate buffer, 100 μl supernatant of the sample, and 100 μl of a concentration of 6 mg/ml o-phthalaldehyde. The preparations were shaken and incubated for 15 min at 4°C, and a spectrophotometric reading was then obtained at an excitation wavelength of 350 nm and an emission wavelength of 440 nm. The results were compared against a standard curve for commercial glutathione processed as described above, and were expressed as μmol TG/g tissue.

To find the percentages of total glutathione (TG) in oxidized and reduced forms, 200 μl of the sample supernatant were incubated with 8 μl 4-vinylpyridine for 1 h at room temperature; the sample was then processed as described above to determine oxidized glutathione (GSSG). Reduced glutathione (GSH) was determined by subtracting GSSG from TG. Each determination was made in duplicate.

2.6. Measurement of mitochondrial reduction of TPT

After TPT perfusion, the brain was cut transversely into 2-mm sections (section 1, frontal neocortex; sections 2–5, neocortex and midbrain; section 6, cerebellum, and section 7, brainstem-mesencephalon) with a vibratome (Capdem Instruments Ltd., USA). Brain sections were incubated with a 2% TPT solution in 0.06 M phosphate buffered saline at 37°C for 30 min and then photographed.

Mitochondrial reduction of TPT was quantified according to the intensity of the red color using a Sparc Station 20 image analyzer (SUN Microsystem, Palo Alto, CA, USA) and Visilog 5.0 software (Noesis, Lesulis, France), with a resolution of 512×512 pixels and a scale of 256 grey values screening, ranging from 0 (black, maximal reduction of TPT) to 255 (white, minimal reduction of TPT). For each measurement a control sample from a rat not used in any experiment was used to obtain the 100% reference value.

Grey values were measured in the following areas [33]: cortex, nucleus caudatus-putamen, hypothalamus, hippocampus, gyrus dentatus, mesencephalon and pons. All determinations were made in all of the slices in which these areas were identified, in both hemispheres.

2.7. Statistical analysis

All data in the text, tables and figures are means±S.E.M. of the results in each group. Statistical analyses were done with SPSS software for personal computers. Distribution of the results was normal, and the ANOVA test with post hoc Bonferroni transformation was used. A P value of <0.05 was considered significant.

3. Results

3.1. In vitro experiments

Incubation of different rat brain tissues with increasing concentrations of SAM led to a concentration-dependent inhibition of lipid peroxide production (Fig. 1). The concentration of SAM that inhibited TBARS production by 50% in different brain tissue fractions (IC50) was in the range of 10−4 M for the midbrain and brainstem-mesencephalon, and higher than 10−3 M in the cortex and cerebellum (Table 1).

Glutathione production by brain tissues from different areas was not significantly modified by incubation with any of the concentrations of SAM we tested (maximum increase: 2.8±0.3%).

3.2. Ex vivo experiments

3.2.1. Effect of SAM in sham-operated rats

In sham-operated animals the administration of 50 mg SAM/kg during 3 days did not significantly modify lipid peroxide production under basal conditions or after induction with 100 μmol/l FeAs (Fig. 2). The concentration of ferrous salt that led to 50% maximal TBARS production (EC50) did not change in any of the brain areas studied after the intraperitoneal administration of SAM (Table 2).

Brain concentrations of TG were significantly higher in the cortex and midbrain of sham-operated rats given SAM than in untreated animals; there was no significant difference in the other brain areas studied (Fig. 3, upper panel). The percentage of glutathione in oxidized form did not differ significantly in any of the brain areas (Fig. 3, lower panel).

S-adenosyl-l-methionine led to no significant changes in mitochondrial reduction of TPT in any group or in any brain area analyzed.

3.2.2. Effect of experimental ischemia-reperfusion

In animals that were subjected to experimental ischemia-reperfusion, noninduced lipid peroxide production was significantly higher than in control rats in the cortex from both hemispheres and in the midbrain (Fig. 2A). The changes in the other brain areas were not significant. Ferrous salt-induced lipid peroxide production (Fig. 2B) was significantly higher in the right cortex and midbrain,
Fig. 1. Production of thiobarbituric acid-reactive substances (TBARS) induced by 100 μmol/l ferrous sulfate and ascorbic acid after incubation with increasing concentrations of S-adenosyl-L-methionine (SAM) in membrane concentrates from different areas of the rat brain. RCtx, right cortex; LCtx, left cortex; MB, midbrain; Cbl, cerebellum; BS-Msc, brainstem-mesencephalon. Each value is mean±S.E.M. of 10 experiments.

and was significantly lower in the left cortex, in comparison with sham-operated rats given saline. The values of EC$_{50}$ for ferrous salt-induced lipid peroxide production were significantly lower in the cortex and midbrain in animals from the ischemia-reperfusion group (Table 2).

Brain levels of glutathione were lower in the left and right cortex and midbrain from animals in the ischemia-reperfusion group than in sham-operated rats (Fig. 3, upper panel). The percentage of glutathione in oxidized form was greater in the cortex, midbrain and cerebellum of rats from the experimental group (Fig. 3, lower panel).

Mitochondrial capacity to reduce TPT was diminished in the left and right cortex and in the left dentate gyrus, hippocampus and caudate nucleus putamen (Table 3). Fig. 4 shows three representative examples of the third brain slice (rostrocaudally) from three rats in the sham-operated, saline-treated group, three in the ischemia-reperfusion, saline-treated group, and one animal in the ischemia-reperfusion, SAM-treated group. The area of permanent ischemia appears in panel B (compare with control animal, panel A). In the example shown, the morphological changes are most evident in the cortex and caudate nucleus putamen.

3.2.3. Effect of SAM in experimental ischemia-reperfusion

Treatment with 50 mg/kg SAM per day for 3 days in rats subjected to cerebral ischemia-reperfusion significantly inhibited noninduced lipid peroxide production in the left cortex and midbrain (Fig. 2A), and also inhibited ferrous salt-induced peroxide production in the right cortex, midbrain, cerebellum and brainstem-mesencephalon (Fig. 2B). Induced peroxide production in the left cortex was significantly greater after SAM treatment in comparison to animals given saline. The EC$_{50}$ values for ferrous salt-induced lipid peroxide production were significantly higher in all brain areas in rats given SAM (Table 2).

Total glutathione content was significantly greater in all

| Table 1 |
|---|---|---|
| Mean values of ferrous-induced (100 μmol/l) thiobarbituric acid-reactive substances (TBARS), concentrations of SAM that inhibited 50% of TBARS production (IC$_{50}$), and percentages of inhibition of TBARS production after incubation with 1 mmol/l of SAM$^a$ | | |
| **TBARS** (nmol/mg protein) | **IC$_{50}$ SAM** (μmol/l) | **% Inhibition at SAM 1 mmol/l** |
| Right cortex | 11.45±1.03 | >1000 | 38.35±4.02 |
| Left cortex | 12.88±0.79 | >1000 | 40.38±5.11 |
| Midbrain | 12.82±1.05 | 623±79.12 | 56.00±5.73 |
| Cerebellum | 9.64±0.81 | >1000 | 46.73±5.20 |
| Brainstem-mesencephalon | 14.60±1.20 | 503±68.93 | 65.12±5.99 |

$^a$ Each value is mean±S.E.M. of 10 experiments.
Fig. 2. Production of thiobarbituric acid-reactive substances (TBARS) without induction (panel A) and after induction with 100 μmol/l ferrous sulfate and ascorbic acid (panel B) in different brain areas from sham-operated rats (Sham) and rats subjected to combined permanent focal ischemia and global reperfusion (IR), then treated with saline or 50 mg/kg S-adenosyl-L-methionine daily for 3 days (SAM). RCtx, right cortex; LCtx, left cortex (permanent ischemia); MB, midbrain; Cbl, cerebellum; BS-Msc, brainstem-mesencephalon. *P < 0.05 in comparison to saline-treated rats; †P < 0.05 in comparison to sham-operated, saline-treated rats. Each value is mean ± S.E.M. of five experiments.

Table 2

Mean concentrations (µmol/l) of the ferrous sulfate–ascorbic acid combination that produced 50% of the maximal production of thiobarbituric acid-reactive substances (TBARS) (EC50) in the ex vivo experiments

<table>
<thead>
<tr>
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<th>Sham-operated rats</th>
<th>Ischemia-reperfusion</th>
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<tr>
<td></td>
<td>SAM</td>
<td></td>
</tr>
<tr>
<td>Right cortex</td>
<td>28.69±1.66</td>
<td>25.19±1.85</td>
</tr>
<tr>
<td>Left cortex</td>
<td>24.55±1.85</td>
<td>24.20±2.90</td>
</tr>
<tr>
<td>Midbrain</td>
<td>17.31±0.71</td>
<td>23.12±2.15</td>
</tr>
<tr>
<td>Cerebellum</td>
<td>16.51±1.08</td>
<td>17.08±1.93</td>
</tr>
<tr>
<td>Brainstem-mesencephalon</td>
<td>18.06±1.32</td>
<td>19.92±3.27</td>
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<tr>
<td></td>
<td>11.26±0.83†</td>
<td>29.15±1.58*</td>
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<tr>
<td></td>
<td>8.39±0.72†</td>
<td>52.08±3.14*</td>
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<tr>
<td></td>
<td>9.27±0.87†</td>
<td>51.00±2.98*</td>
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<tr>
<td></td>
<td>17.93±1.66</td>
<td>26.37±1.98*</td>
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<td>15.90±0.93</td>
<td>73.00±5.12*</td>
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*SAM: 50 mg/kg/day×3 days (i.p.).
†P < 0.05 with respect to saline, †P < 0.05 with respect to saline-treated sham-operated rats. Each value is mean ± S.E.M. of five experiments.
brain areas in rats given SAM (Fig. 3, upper panel). The percentage of oxidized glutathione was significantly lower in this group in all brain areas except the brainstem-mesencephalon, where the difference failed to reach statistical significance.

Mitochondrial reduction of TPT, which was clearly impaired in animals subjected to ischemia-reperfusion and treated with saline, was significantly greater in animals treated with SAM in the cortex, caudate putamen, hippocampus and dentate gyrus (Table 3). In this table, an increase in grey values means a decrease in the intensity of TPT reduction, and vice versa. Panel C in Fig. 4 shows a representative brain slice from a SAM-treated rat; the area of cortical damage is clearly smaller than in the brain slice from a saline-treated rat shown in panel B.

4. Discussion

Our results show that SAM has a clear antioxidant effect in brain tissue that had been subjected to experimental ischemia-reperfusion; the effect we observed was much greater than in normal brain tissue.

The in vitro results show that incubation with increasing concentrations of SAM reduced the lipid peroxidation induced with ferrous salt, but did not significantly modify glutathione content in rat brain tissue. We have found two published reports on a possible in vitro effect of SAM on lipid peroxidation; Evans et al. [14] showed a little effect of SAM on lipid peroxidation, except at high concentrations (5 mmol/l); the second was our previous study [10], in which the level of inhibition of TBARS production in forebrain membranes (54.8% in samples incubated with 1000 μmol/l) was similar to that found in the present study.

The ex vivo administration of SAM in sham-operated animals failed to modify lipid peroxide production, but significantly increased total glutathione content in brain tissues. These findings are compatible with those of our earlier experiments [10], in which the chronic administration of SAM in normal rats increased glutathione levels. The percentage of glutathione in oxidized form was not significantly different after SAM treatment, a logical finding since in the absence of oxidative tissue damage, the glutathione defense system is not activated. However, a different study [16] showed that chronic administration of SAM to normal rats reduced lipid peroxidation and increased the rate of glutathione production in liver tissue. The present results therefore show that the systemic administration of SAM increases the brain glutathione pool in rats.

The experimental model used in this study and in earlier research that analyzed different antioxidant substances [11,37] creates two different situations in rat brain tissue. In the area of permanent ischemia, ischemic damage per se can be analyzed. In the rest of the brain tissue, which undergoes ischemia and reperfusion, free radicals produce evident tissue damage [6]. These effects were confirmed by the biochemical findings: in the area of permanent ischemia we found that noninduced lipid peroxides had accumulated, perhaps as the degradation products of membrane phospholipids as a result of the lack of oxygen [30]. However, the capacity to actively produce lipid peroxides in response to stimulation with ferrous salts was diminished in the area of permanent ischemia, possibly because this biochemical phenomenon requires cellular integrity. Ischemia leads to loss of cellular integrity, as
Fig. 4. Brain slices from three different animals representing the control group (sham-operated rats, A), ischemia-perfusion and saline treatment (B), and ischemia-reperfusion with S-adenosyl-l-methionine treatment at 50 mg/kg i.p. daily for 3 days (C). Tetraphenyl tetrazolium staining, third section rostrocaudally.
shown by the clear decrease in mitochondrial capacity to reduce TPT. In contrast, in the areas subjected to ischemia and reperfusion, both induced and noninduced lipid peroxides were increased as a result of the oxidative sensitization caused by tissue reoxygenation. This also leads to functional damage because the mitochondrial reduction of TPT was also decreased in areas of ischemia-reperfusion.

Of interest is our observation that this damage occurred in the cerebral cortex and midbrain, but was not evident in the cerebellum and brainstem-mesencephalon. This is logical, as these latter two areas depend to a large extent on the vertebral vascular tree [33], which was not altered in our experimental model. In the same way, glutathione levels were reduced in the ischemic area, such as demonstrated previously by Kinouchi et al. [22] and Mizui et al. [29]. However, these authors did not observe any changes in other areas than cortex, after middle cerebral artery; probably in our experiments, the electrocoagulation of all the superficial branches of the middle cerebral artery included the corticostriatal artery, which gives blood to the middle brain.

When oxidative damage to the brain has occurred, the administration of SAM clearly had an antioxidant effect, decreasing the degree of lipid peroxidation and increasing the level of glutathione to a much greater extent in areas dependent on the carotid vascular tree. These findings show that the antioxidant effect of SAM is greater in the presence of tissue oxidative stress (as in the areas subjected to ischemia-reperfusion), and is less evident under normal conditions of tissue oxidation, as in our in vitro experiments, in sham-operated animals, or in areas subjected to ischemia-reperfusion but dependent on the vertebral rather than the carotid vascular tree.

An interesting observation when we induced lipid peroxidation with ferrous salts was that SAM had opposite effects in the parts of the cortex subjected to permanent ischemia and ischemia followed by reperfusion. In the former area TBARS production increased, whereas in the latter it was inhibited. The reason for this difference may be that the tissue is prevented from responding to ferrous salts by a biochemical deficit in the area of permanent ischemia. The presence of SAM, which improves the tissue antioxidant status, may have ‘reactivated’ the mechanism of biochemical reaction. In the area of ischemia-reperfusion, under conditions of excessive lipid peroxide production, SAM may have inhibited this biochemical mechanism.

The antioxidant effect of SAM was translated as an evident improvement in brain tissue vitality, as shown by the significantly higher mitochondrial capacity to reduce TPT in areas where this capacity had been impaired, including the area of permanent ischemia.

Our results indirectly support the findings of Kobayashi et al. [23], who reported that SAM restored the deficit in glucose utilization by the brain in rats after brain ischemia. They also support the findings of Matsui et al. [28], who showed that SAM diminished neuronal death in layer CA1 of the hippocampus in rats subjected to brain ischemia. In this connection, Rao et al. [34] found that SAM improved the efficacy of the blood–brain barrier after damage caused by brain ischemia. However, in these three studies the dose of SAM was 100–120 mg/kg, twice as high as the dose we used.

In a different experimental model, Nakano et al. [31] showed that SAM decreased tissue damage in the liver after ischemia-reperfusion, a finding that Kaneshiro et al. [21] attributed to the increase in the glutathione pool in ischemic liver tissue (among other mechanisms).

The mechanisms by which SAM palliates the damage caused by ischemia-reperfusion in the brain have not yet been explained, although several hypotheses have been put forward. The brain may behave in a manner similar to other organs, favouring transmethylation reactions [36], although exogenous SAM does not take part in this enzymatic pathway in the brain [39]. In this connection, Carney et al. [4] reported a mechanism in tissues with impaired methylation (as in ischemia) in which homocysteine is remethylated to produce new SAM molecules, which in turn enhance the activity of brain methylation pathways. Our findings provide further evidence of such a mechanism: the antioxidant effects of SAM were greater under conditions of ischemia than in normal brain tissue.

Another hypothesis was proposed by Trovarelli et al. [36], who worked with a rat model of brain ischemia and showed that SAM prevented damage to choline phospholipids in the cell membrane. According to these authors, this effect was responsible for the improved neuronal transport and receptor functioning. Pavia et al. [32] found that SAM increased the number of muscarinic receptors in normal rats under chronic treatment. A related theory was proposed by Hirata et al. [19] and Strittmatter et al. [35], who found that SAM increased erythrocyte membrane fluidity and favoured cell flexibility, an effect that in turn aided brain microcirculation.

Mabe et al. [26] showed that SAM normalized ATP levels that had been lowered in a model of brain ischemia; this effect improved the supply of energy to the neurons, and thus neuronal functioning. This mechanism was cited by Yatsugi et al. [38] to explain their finding that SAM improved memory impairments in rats subjected to brain ischemia.

An additional factor that cannot be ruled out is a hypothetical neuroprotective effect of SAM based on its demonstrated antioxidant action, as other antioxidant substances have been shown to have a neuroprotective effect [2,11–16,37]. These results are in agreement with Kinouchi et al. [22] and Mizui et al. [29], who demonstrated the importance of glutathione in rats and SOD-1 transgenic mice after permanent focal cerebral ischemia.

In conclusion, our findings support the notion that restoration of brain oxidative status may be an important mechanism in the ability of SAM to protect tissues from...
oxidative damage in a model of brain ischemia-reperfusion.

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