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Cysteine-induced hypoglycemic brain damage: an alternative mechanism to excitotoxicity

V. Gazit[1], **R. Ben-Abraham**[2], **R. Coleman**[3], **A. Weizman**[4], and **Y. Katz**[1,5]

[1] Laboratory for Anesthesia, Pain and Neural Research, The Bruce Rappaport Faculty of Medicine, Technion-Israel Institute of Technology, Haifa, Israel
[2] Departments of Anesthesiology and Intensive Care, Tel Aviv Sourasky Medical Center, Tel Aviv, Israel
[3] Department of Anatomy and Cell Biology, The Bruce Rappaport Faculty of Medicine, Technion-Israel Institute of Technology, Haifa, Israel
[4] Research Unit, Geha Psychiatric Hospital and Felsenstein Medical Research Center, Petah Tiqva, and Sackler Faculty of Medicine, Tel Aviv University, Tel Aviv, Israel
[5] Department of Anesthesiology, HaEmek Medical Center, Afula, Israel

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Summary. Central neural damage caused by L-cysteine (L-Cys) was first reported more than 30 years ago. Nevertheless, the exact mechanisms of L-Cys-mediated neurotoxicity are still unclear. Preliminary study in mice demonstrated that, following L-Cys injection, animals developed tachypnea, tremor, convulsions, and death in conjunction with documented hypoglycemia. The aim of the present study was to further investigate the mechanism of L-Cys-mediated hypoglycemic effect and neural damage. Neonatal ICR mice ($n = 6$) were injected with L-Cys (0.5–1.5 mg/g body weight [BW]), and their blood glucose and insulin levels were determined up to 90 min following the injection. Experiments were repeated in chemically (streptozotocin [STZ]) pancreatectomized animals. Brain histology was assessed. Mice injected with L-Cys exhibited dose-dependent neurotoxicity and higher mortality as compared with controls. L-Cys (1.2–1.5 mg/g BW) caused severe hypoglycemia (glucose <42 mg/dl) ($P < 0.001$). In STZ-treated (diabetic) animals, L-Cys (1.5 mg/g BW) increased plasma insulin levels 2.3-fold and decreased serum glucose levels by 50% ($P < 0.01$). Brain histology revealed destruction of as much as 51% of hippocampal neurons in the L-Cys-treated mice but not in the glucose-resuscitated animals. These findings suggest that L-Cys injection can cause pronounced hypoglycemia and central neural damage which is glucose reversible. Since L-Cys is chemically different from the other excitatory amino acids (glutamate and aspartate), L-Cys-mediated neurotoxicity may be connected to its hypoglycemic effect.

Keywords: Excitatory amino acids – Cysteine – Glucose – Brain damage

1. Introduction

Reports in the late 1960s on the potential toxicity of the food supplement monosodium glutamate led to vast research on the possible toxicity of other amino acids. Olney and Ho (1970) were the first to report on brain damage caused by oral intake of glutamate, aspartate, or L-cysteine (L-Cys) in mice. Their finding paved the way for a new era in neuroscience dealing with N-methyl-D-aspartate (NMDA) receptor-mediated neurotoxicity. Indeed, the amino acids mentioned were later grouped as excitatory acids by virtue of their potential to stimulate brain NMDA receptors, leading to cell apoptosis and neural damage. Nevertheless, whereas glutamate- and aspartate-related neuroexcitability is well established, that of L-Cys remains less defined (Olney et al., 1990). Unlike the other excitatory amino acids, L-Cys does not contain an omega-side chain, which constitutes the side chains of the other linear excitatory amino acids (glutamate and aspartate). This side chain facilitates the entrance of aspartate and glutamate through the blood-brain barrier (Olney et al., 1990; Nunn et al., 1991). In addition, L-Cys neuroexcitatory properties differ from those of glutamate, as examined microelectrophoretically in spinal neurons Olney (1990).

In a preliminary study, we reported on the ability of L-Cys to cause tachypnea, tremor, convulsions, and death following subcutaneous injection in mice (Gazit et al., 1997). This physiological response resembled that of hypoglycemia and was subsequently verified in blood analysis. The aim of the present study was to further

investigate the mechanism of L-Cys-mediated hypoglycemic and neurotoxic effects.

2. Materials and methods

Experiments were performed on ICR (Institute for Cancer Research, USA) mice. The animals were housed in plastic cages ($60 \times 40 \times 20$ cm) under reversed-cycle lighting (lights on 1900-0700 h) in a temperature- and humidity-controlled animal facility, with free access to food and water in the home cage. The study was approved by the Animal Care and Use Committee of the Technion Faculty of Medicine.

ICR mice 4–6 days old and weighing 5–8 g were injected subcutaneously with L-Cys (0.5, 1.0, 1.2, and 1.5 mg/g body weight [BW]). A control group was similarly injected with normal saline and returned to their cages for recovery. Breast-feeding was withheld throughout the study to avoid the complicating effect of food intake on blood glucose level. All mice were observed for clinical, biochemical, and histological changes.

2.1. Blood glucose assay

At zero time and 5, 10, 15, 25, 35, 45, 60, and 90 min following L-Cys injection, blood samples taken from the tail vein were checked for glucose using an Accutrend sensor with disposable measuring strips (Roche Diagnostics, Indianapolis, IN, USA); the strips were read 40 s after immersion in blood.

2.2. Insulin measurements

At zero time and 5, 10, 15, 25, 35, 45, 60, and 90 min after L-Cys (1.5 mg/g BW) or saline injection, mice ($n = 5$) were decapitated, their brains were removed, and blood was collected into centrifuge tubes. Then samples were centrifuged at 3,000 rpm for 10 min at 25°C. After serum collection, insulin levels were measured using a radioimmunoassay kit (Linco Research, Inc., St. Charles, MO, USA).

2.3. Injections of streptozotocin

In order to induce chemical pancreatectomy and a diabetic state, streptozotocin (STZ) injection was used (Shah et al., 1995). Two-month-old ICR mice weighing 25–30 g were given a single intraperitoneal injection (150 mg/kg BW) of STZ (Sigma Israel) dissolved in buffered citrate, pH 4.5. Blood glucose levels were measured on day 7 following the injection. To examine the effects of L-Cys on blood glucose and serum insulin levels, mice were injected with a single dose of L-Cys (1.5 mg/g BW) subcutaneously, and blood glucose and plasma insulin levels were measured at zero time and 5, 15, 30, 45, 60, and 90 min thereafter. Mice in the control group were injected with buffered citrate only.

2.4. Brain histology and morphometric studies

The L-Cys (1.2 and 1.5 mg/g BW), and L-Cys (1.5 mg/g BW) plus glucose (3 mg/g BW twice apart 25 min after L-Cys administration) or control neonatal mice were sacrificed at 30, 60, and 90 min and after 24 h, 48 h, and 3 days. Mice were decapitated, and their brains were removed and washed with ice-cold phosphate-buffered saline (PBS), pH 7.4. Meninges and connective tissue were removed, and the brains were stored in neutral buffered 4% formaldehyde. Then brains were washed with PBS, put in 70% alcohol overnight, and embedded in paraffin. Brain sections were cut into 6-μm-thick cross-sections at the coronal level 3.6 mm caudal to the bregma. Blocks were mounted on 1% gelatin-coated slides. Brain specimens were deparaffinized using xylene or ethanol for 5 and 2 min, respectively. Specimens were put into distilled water for 10 min and then incubated in hematoxylin and eosin in a Coplin jar for 20 min and 8 s, respectively, at room temperature. The specimens were washed again with ethanol and xylene, dehydrated, and covered with a glass coverslip (Gavrieli et al., 1992). Results of the undamaged neurons are presented as a percentage of the sham-operated group value.

2.5. Statistical analysis

Differences among groups ($n = 6$) were analyzed using one-way analysis of variance, and after hoc analysis was performed by the Newman-Keuls multiple comparison tests. Data are presented as means ± S.E.M. Statistical significance was defined at $P < 0.05$.

3. Results

3.1. Animal survival

Ten minutes after injection of high-dose L-Cys (1.2 and 1.5 mg/g BW), mice exhibited hyperventilation, sweating, tremor, and convulsions, and some died. These symptoms were accompanied by severe hypoglycemia (see below).

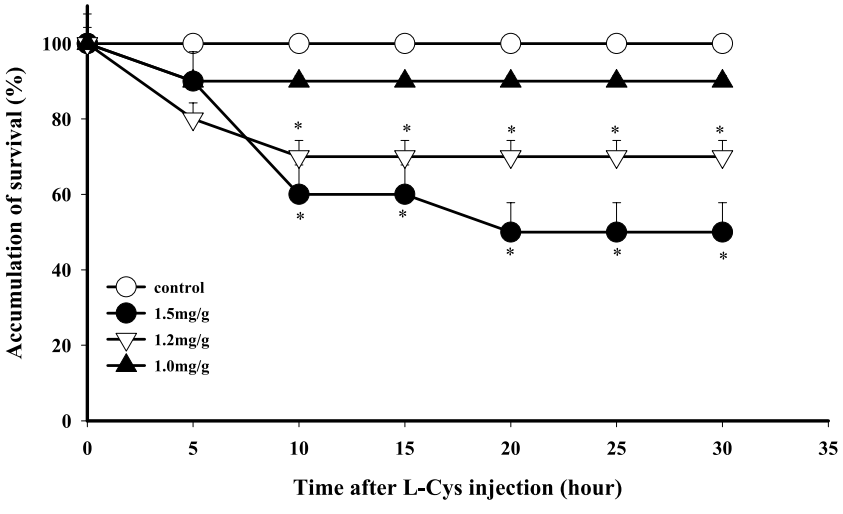


Fig. 1. Effect of various concentrations of L-Cys on mouse survival. Animal survival was inversely correlated with L-Cys concentration, e.g., in the 1.5 mg/g BW dose, mortality was 50%. In the lower range of L-Cys injection and control, almost all mice survived. $^{*}P < 0.001$ versus control

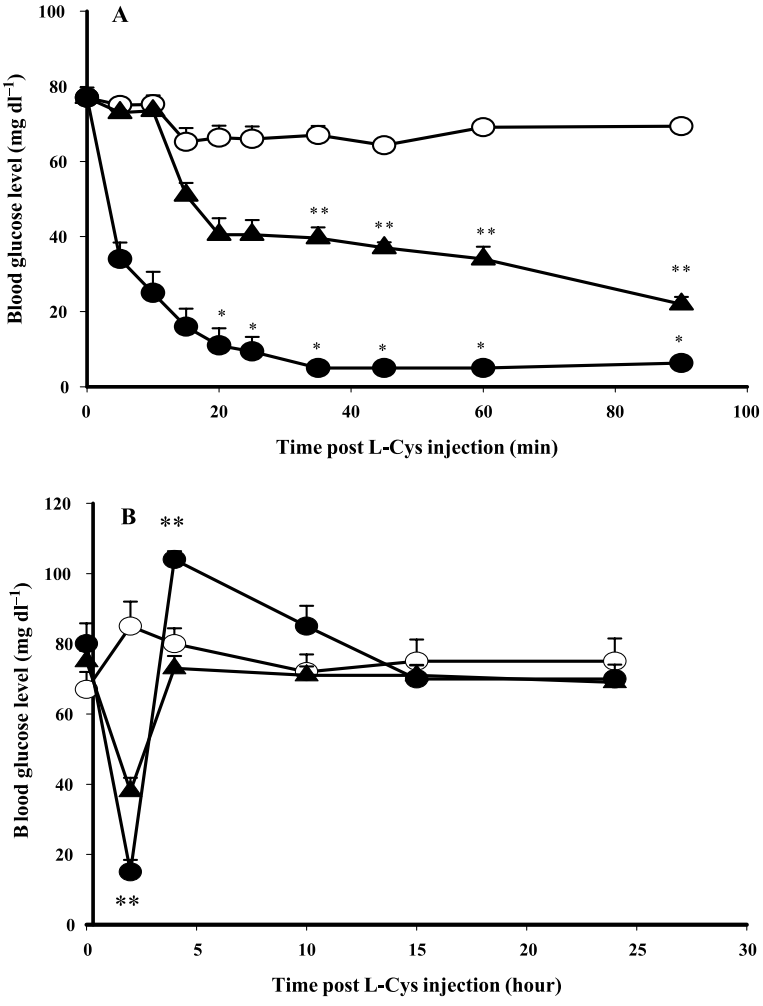


Fig. 2. Dose-response curve of L-Cys and its hypoglycemic effect. **A** After L-Cys injection, blood glucose levels started to decrease to the hypoglycemic range (<42 mg/dl; $^{*}P<0.01$, $^{**}P<0.001$ versus control). The most significant hypoglycemic effect was observed 30 and 60 min after L-Cys injection (1.5 and 1.2 mg/g BW respectively) ($P<0.001$). **B** Blood glucose levels in the surviving mice were normal 24 h following L-Cys injection. ○, control; ●, L-Cys 1.5 mg/g; ▲, L-Cys 1.2 mg/g

Figure 1 depicts the survival rate of mice as a function of L-Cys administration. Mouse survival was inversely related to the amount of L-Cys injected. Twenty-four-hour survival rate following L-Cys injection at doses of 1.2 and 1.5 mg/g was 70% and 50% versus 100% in control ($n=6$ per group; $P<0.001$). Injection of L-Cys at lower doses (<1 mg/g BW) did not affect survival rates.

3.2. Effect of L-Cys injection on blood glucose levels

Figure 2 shows blood glucose concentration as a function of time after L-Cys injection in the surviving mice. L-Cys at doses of 1.2 and 1.5 mg/g significantly decreased blood glucose levels (from 70–80 mg/dl to values in the hypoglycemic range, i.e., <40 mg/dl). Figure 2b shows a rebound hyperglycemic effect that was observed in the L-Cys (1.5 mg/g)-injected mice 4 h after-injection ($n=6$; $P<0.001$). Blood glucose levels were normal at 24 h after L-Cys injection.

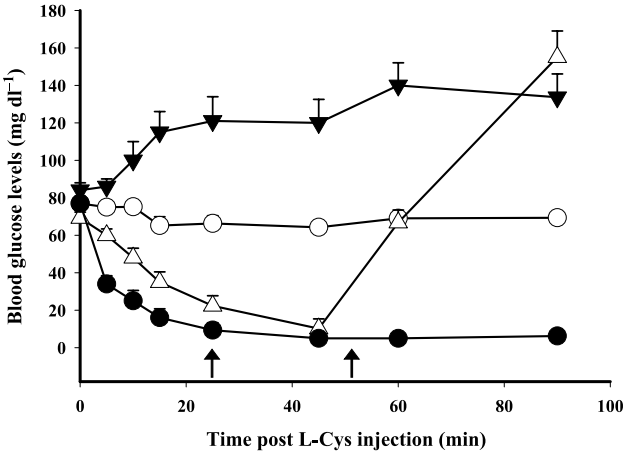


Fig. 3. Reversal of L-Cys-induced hypoglycemic effect. The administration of glucose (3 mg/g BW) twice 25 min apart to the L-Cys-injected (1.5 mg/g BW) mice successfully reversed the hypoglycemia. $P<0.001$ versus control. ○, control; ●, L-Cys 1.5 mg/g; ▼, glucose 3 mg/g; △, L-Cys 1.5 mg/g plus glucose 3 mg/g. Arrow indicates time for glucose administration

The L-Cys-induced hypoglycemia was attenuated by two subcutaneous injection of glucose 3 mg/g BW given 25 and 50 min after L-Cys (1.5 mg/g) injection (Fig. 3).

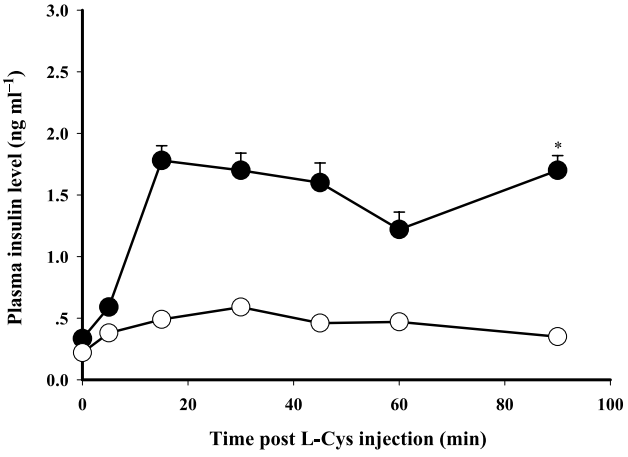


Fig. 4. Plasma insulin levels after administration of L-Cys. The injection of L-Cys (1.5 mg/g BW) caused a rise in serum insulin levels as compared with control ($n = 6$; $P < 0.001$). ○, control; ●, L-Cys 1.5 mg/g

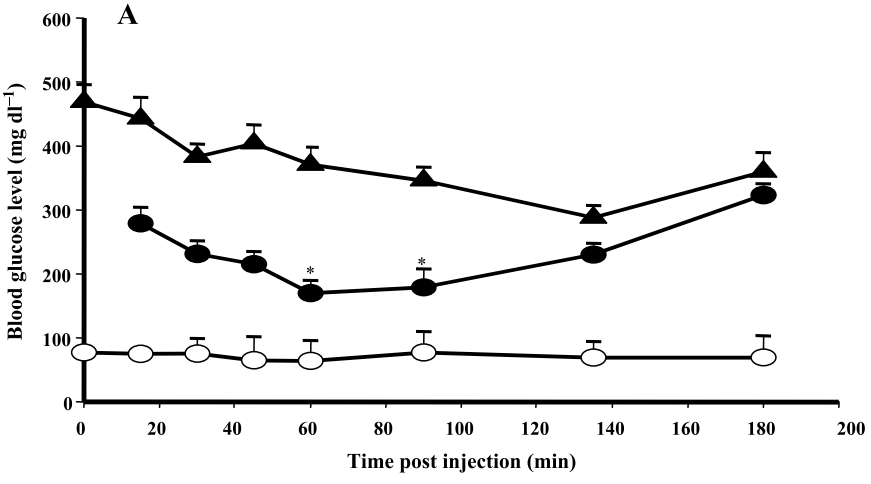
The second administration of glucose (3 mg/g) 50 min after L-Cys injection significantly increased blood glucose levels 2-fold as compared with control ($P < 0.001$) (Fig. 3).

3.3. Effect of L-Cys on plasma insulin levels

As early as 10–15 min after L-Cys injection (1.5 mg/g), plasma insulin levels significantly increased to a level above 1.8 ng/ml, versus control levels, which were 0.5 ng/ml ($n = 6$; $P < 0.001$) (Fig. 4).

3.4. Effect of L-Cys on blood glucose and insulin levels in STZ-treated mice

Following STZ injection, mice became diabetic (hyperglycemia >250 mg/dl). Sixty minutes after injection of L-Cys (1.5 mg/g) into the STZ-treated mice, blood glucose levels decreased to below 250 mg/dl, returning to baseline values after 3 h (Fig. 5A). Figure 5B shows that



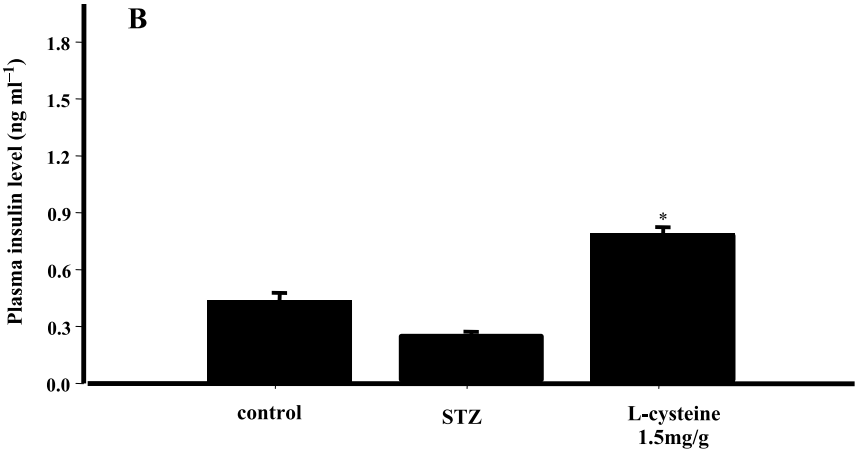


Fig. 5. Effect of L-Cys on levels of blood glucose **A** and plasma insulin **B** in STZ-treated mice. **A** Sixty minutes after injection of L-Cys (1.5 mg/g BW) to the STZ-treated mice, blood glucose levels decreased significantly below the diabetic baseline values as appears in time zero ($P < 0.01$), attaining baseline values again after 3 h. **B** In the STZ-treated mice, plasma insulin levels were increased as compared with control 60 min following L-Cys (1.5 mg/g BW) injection ($P < 0.001$). ○, control; ●, STZ + L-Cys 1.5 mg/g; ▲, STZ 150 mg/g

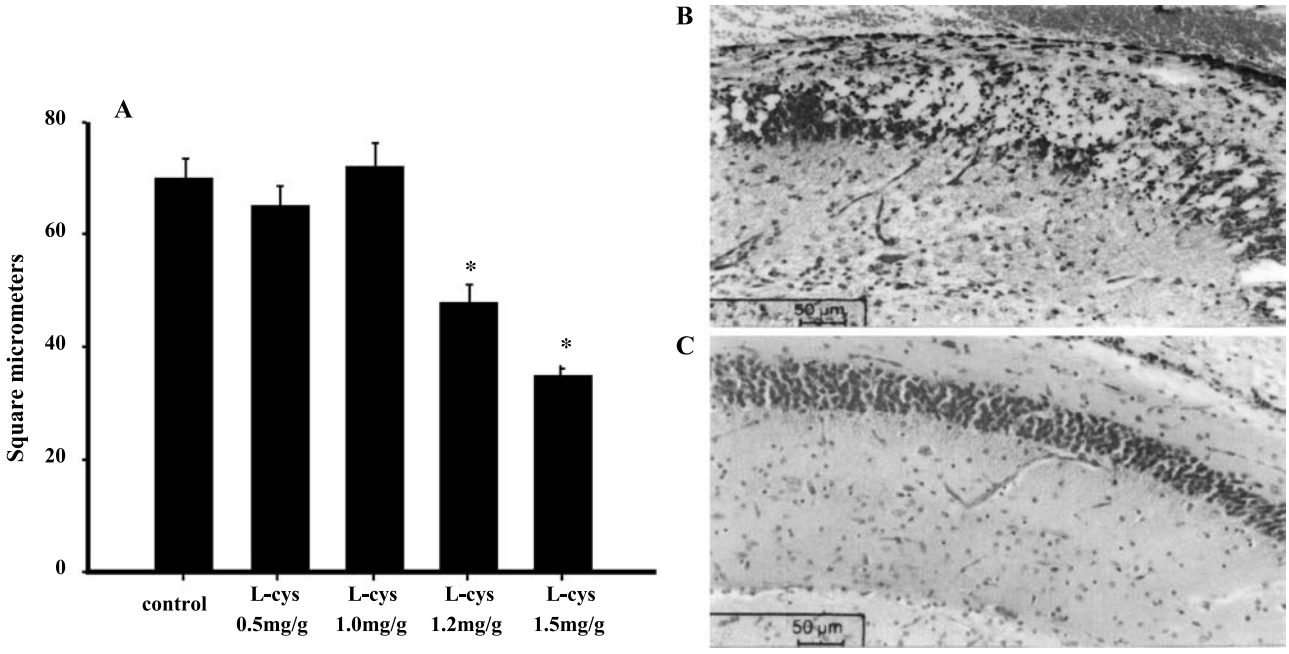


Fig. 6. Effect of L-Cys on neuron viability in hippocampal regions after L-Cys administration. **A** L-Cys induced neural degeneration in a dose-response manner. $^{*}P < 0.001$ versus control. **B** Hippocampal region of 4-day-old mouse after L-Cys (1.5 mg/g BW) injection. **C** Necrotic areas can be seen as compared with control. ×50

in the STZ-treated mice, plasma insulin levels were reduced as compared with control. The administration of L-Cys (1.5 mg/g) caused a 2.3-fold increase in plasma levels of insulin 60 min after-injection.

3.5. Brain histology

The administration of L-Cys at doses of 1.2 and 1.5 mg/g destroyed 33% and 51% of hippocampal neurons, respectively (Fig. 6A), compared with control, as observed in brain slices examined 48 h after-injection. Other brain regions (cerebellum, hypothalamus, and amygdala) damage was less pronounced. The neuropathological damage as demonstrated by the hematoxylin and eosin staining was expressed as severe necrosis and edema, especially in the hippocampal regions CA1 and CA3 (Fig. 6B). This damage was more pronounced in brains of mice that were exposed to the higher L-Cys dose as compared with the lower dose and control (Fig. 6C). In contrast, animals resuscitated with glucose after L-Cys administration exhibited no morphological changes in brain tissue.

4. Discussion

In this study we demonstrated that the injection of L-Cys in high doses to neonatal mice caused pronounced hypoglycemia, physiological deterioration (i.e., tremor deteriorating to convulsions and death), and histologically manifested central neural damage. The hypoglycemia following L-Cys injection was dose-dependent and, in conjunction with the physiological deterioration, reversed by glucose injections or spontaneously in the surviving animals. In addition, animals exposed to L-Cys and resuscitated with glucose did not exhibit morphological changes in the brain.

L-Cys is a known insulin secretagogue *in vitro* (Ammon et al., 1986); as blood glucose levels decreased appreciably for up to 90 min following injection, plasma insulin levels increased. Interestingly, in mice made diabetic by chemical pancreatectomy using STZ, the administration of L-Cys also reduced blood glucose levels. These combined findings suggest that L-Cys may possess an insulin-independent peripheral glucose-lowering effect. However, STZ does not destroy all pancreatic beta islet cells hence some insulin secretion capability is still preserved not excluding a retained endogenous insulin activity. Furthermore, we checked animals 7 days following the STZ injection. It is known that longer period should last before the full toxic effect of STZ on the pancreatic islets is manifested (Movassat and Portha, 1999). As it is known that partial we postulate that the pronounced hypoglycemia observed might be responsible

for the L-Cys-induced central neural damage, which was prominent in the hippocampal regions and has also been reported by others (Olney and Ho, 1970; Olney et al., 1972). It is well known that hypoglycemic shock can cause irreversible brain damage (Sieber and Traystman, 1992), which can be seen in light microscopy as neural necrosis spreading from the cortex to more deeply located brain structures (Auer et al., 1984). The histological damage in the L-Cys-treated mice was pronounced in the hippocampal regions, where hypoglycemia is known to produce its destructive effects (Auer and Siesjo, 1988).

Previous studies reported that the administration of high doses of L-Cys to infant mice caused brain damage similar to that produced by glutamate (Ammon et al., 1986). This type of damage was attributed to the neuroexcitatory potential of L-Cys, but its potent hypoglycemic effect was ignored (Shah et al., 1995). L-Cys penetrates the blood-brain barrier only poorly, due to its lack of an acidic omega-side chain as is present in the potent neuroexcitatory amino acids glutamate and aspartate. Prominent brain histological damage, following L-Cys injection, that was observed in our study, occurred in the hippocampal region. Indeed, brain histological damage that follows L-Cys injection has been described as differently located and more extensively widespread than that which follows the administration of glutamate or aspartate (Olney et al., 1972; Lehmann et al., 1993). These findings may exclude the notion that direct neurotoxic excitatory mechanism may trigger L-Cys-induced neural damage. However, other contradictory evidence suggest the existence of dedicated sodium- and energy-dependent, carrier-mediated system for L-Cys which nevertheless could contribute to L-Cys neurotoxicity (Tayarani et al., 1987).

In this study we did not assess possible correlations between the severity of the physiological disturbances, various doses of L-Cys, hypoglycemia and brain cellular damage. Also, the exact type of damaged cells (i.e., neuronal, glial) was not determined. Further studies with cell specific markers such as NeuN or GFAP to confirm phenotype of cell death are warranted.

It may be concluded that L-Cys-induced physiological deterioration and brain damage is attributable to its potent hypoglycemic effect, without exclusion of neuroexcitatory potential. Inasmuch, other postulated mechanisms for L-Cys-induced neurotoxicity such as generation of cysteine alpha-carbamate, a toxic analog of NMDA, generation of toxic oxidized cysteine derivatives, generation of free radicals, and generation of the neurotoxic catecholamine derivative, 5-S-cysteinyl-3,4-dihydroxyphenylacetate have been proposed (Janaky et al., 2000). However, the interactions between L-Cys and NMDA receptor are very complex and are dependent on the chosen concentration and investigational and may be on other factors as well (Puka-Sundvall et al., 1995). Thus, the contributory role of L-Cys-induced hypoglycemia alongside these neurotoxic mechanism should be further investigated.

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Authors' address: Yeshayahu Katz, Department of Anesthesiology, HaEmek Medical Center, 18101 Afula, Israel,
Fax: +972-4-649-4361, E-mail: ykatz18@hotmail.com