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Long-term neurobehavioral and histological damage in brain of mice induced by L-cysteine

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Abstract

We investigated whether structural central neural damage and long-term neurobehavioral deficits after L-cysteine (L-Cys) administration in mice is caused by hypoglycemia. Neonatal ICR mice were injected subcutaneously with L-Cys (0.5–1.5 mg/g body weight [BW]) or saline (control). Blood glucose was measured. At 50 days of age, mice were introduced individually into an eight-arm maze for evaluation of spatial memory (hippocampal-related behavior). Times for visiting all eight arms and number of entries until completion of the eight-arm visits (maze criteria) were measured. The test was repeated once daily for 5 days. In situ terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) assay was used for detection of brain damage. As early as 20 min and up to 2 h postinjection, animals treated with L-Cys doses higher than 1.2 mg/g BW developed hypoglycemia and looked ill. Several animals convulsed. Long-term survivors required more time, in a dose-dependent manner, to assimilate the structure of the maze, and animals treated with L-Cys (1.5 mg/g BW) exhibited TUNEL-positive changes in the hippocampal regions. All these changes were reversible by coadministration of glucose. We conclude that L-Cys injection can cause pronounced hypoglycemia associated with long-term neurobehavioral changes and central neural damage in mice. Since L-Cys is chemically different from the other excitatory amino acids (glutamate and aspartate), the long-reported L-Cys-mediated neurotoxicity may be connected to its hypoglycemic effect.

Keywords: Neurotoxicity; Behavior; Brain damage; Eight-arm maze

1. Introduction

L-Cysteine (L-Cys), administered subcutaneously or orally in high doses to neonatal mice, can cause cerebral damage, sometimes culminating in death, similar to what has been observed with the prototypical excitatory amino acids glutamate and aspartate (Olney and Ho, 1970). Since L-Cys is chemically different from the other excitatory amino acids and the pattern of the L-Cys-induced brain damage is different, several mechanisms have been suggested as responsible for its neurotoxicity. Among them, generation of cysteine α-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 (Janaky et al., 2000).

It has also been reported that L-Cys can cause tachypnea and tremor, followed by convulsions, after subcutaneous injection in mice (Gazit et al., 1997). This physiological response resembled that of hypoglycemia, which subsequently was verified in blood analysis. Glucose is a primary energy source for the human central nervous system. Because the brain is especially dependent on a continuous delivery of glucose, it cannot survive more than a few minutes of glucose deprivation (Lancet, 1989). Accordingly, severe neural damage can occur following even a short period of hypoglycemia (Auer, 1986).

The aim of the present study was to investigate whether L-Cys-mediated hypoglycemia might be involved in the pathophysiology of the long-term neurodegenerative changes that occur following its administration.

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2. Materials and methods

2.1. Animals

Experiments were performed on ICR (Institute for Cancer Research, USA) mice. The animals were housed in plastic cages (60 × 40 × 20 cm) under normal lighting (lights on 0700–1900 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 4–5 days old and weighing 4–6 g were divided into equal groups ($n = 6$) and 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. After injection, animals were observed for physical and behavioral signs of stress, and blood samples (taken from the tip of the animal tail) were analyzed for glucose using Accu-Check glucometer (Roche Diagnostics, Mannheim, Germany) measured.

2.2. TUNEL assay

Forty-eight hours after L-Cys treatment, mice were decapitated and their brains removed and fixed in neutral buffered 4% formaldehyde overnight. Brains were washed with phosphate-buffered saline (PBS), dehydrated through increasing concentrations of alcohol, cleared in xylenes, and embedded in paraffin. Then blocks were cut into 3-μm-thick cross sections, at a coronal level 3.6 mm caudal to the bregma, which were mounted on 1% gelatin-coated slides. Slides were deparaffinized using xylene and ethanol for 5 and 2 min, respectively, and washed twice with PBS, pH 7.5, for 5 min. Then brain damage was assessed with the in situ terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) assay (Gavrieli et al., 1992), performed with the ApopTag peroxidase in situ apoptosis detection kit (Intergen, Purchase, NY). Rat mammary glands (Intergen) that had undergone programmed cell death following lactation were used as a positive control.

2.3. Apparatus: The eight-arm maze

Our maze was based on a modification by Pick and Yanai (1983) of the radial maze developed by Olton and Samuelson (1976). The maze was constructed on a circular base 76 cm across, made of gray Plexiglas. Eight arms of equal length (spreading out from a central platform), also made of gray Plexiglas, were attached to the base and covered with a transparent circular cover 77 cm wide. The width of all the walls was 0.5 cm, and there was a depression 1 cm deep located 1.5 cm before the end of each arm. At the beginning of the test, each mouse was lowered individually onto the central platform through a 6-cm-wide opening in the center of the transparent cover over the platform.

2.4. Eight-arm maze cognitive test

Another animal cohort was used for the cognitive tests. After subcutaneous injection with L-Cys (0.5–1.5 mg/g BW) or saline (control) at the age of 4 or 5 days, mice were put into a cage together with their mother (six mice in each cage) until weaning. At the age of 43 days (1 week before the start of the experiment), animals were subjected to a 1-week regimen of water deprivation that consisted of water access ad libitum for 30 min once a day only. This length of time was sufficient for the mice to drink as much as they wanted. The water deprivation continued throughout the period of the experiment. Food was supplied ad libitum. On the eighth day of water deprivation (at age 50 days) and throughout the study period, each mouse was introduced once daily into an eight-arm maze through an opening in the center. During habituation, mice were left in the maze for 10 min without reinforcement of water, during which time the first eight entries of the mice into the eight-arm maze were noted. Over the following 5 days of the test, mice received reinforcement of water drops (50 μl administered by micropipette) in the depression at the end of each arm. The animals were left in the apparatus until they had entered all the eight arms, one after the other, or until they had made 16 entries. Animals were considered to have reached maze criteria if there were eight correct entries out of the possible eight arms for two consecutive days (Laviola et al., 1992). Thus, it was possible to obtain two different scores: (1) the number of correct entries during the first eight attempts on each day of testing and (2) the number of days it took to reach criteria.

2.5. Statistical analysis

Data are presented as means ± S.E.M. Differences among the groups and the brain regions at baseline were analyzed by one-way analysis of variance, followed by Newman–Keuls post hoc test. In all experiments, $n = 6$ or more. Statistical significance was defined at $P < .05$.

3. Results

3.1. L-Cys and blood glucose

As early as 20 min and up to 2 h postinjection, treated animals looked ill. Whereas normoglycemia was preserved in the control animals, a significant reduction in blood glucose levels (below 40 mg/dl) was measured in the L-Cys-injected mice (Table 1). Coadministration of glucose (3.0 mg/g BW) to the L-Cys (1.5 mg/g BW)-injected animals restored blood glucose levels to normal. The

Table 1
Blood glucose levels as a function of time after L-Cys administration ($n = 6$ per group)

Treatment (mg/g BW)	Glucose (mg/dL)			
	30 min	60 min	120 min	240 min
Control	70 ± 5	75 ± 3	70 ± 3	70 ± 5
L-Cysteine (1.2)	40 ± 5	38 ± 3	35 ± 5	ND
L-Cysteine (1.5)	15 ± 3	10 ± 4	10 ± 4	ND
Glucose (3.0) with L-cysteine (1.5)	23 ± 6	110 ± 10	100 ± 5	90 ± 5

At time zero, blood glucose level was 78 ± 5 mg/dL in all treated groups. ND, not determined.

general physical condition of the surviving animals treated with L-Cys appeared normal in every respect when compared with the saline-treated group. There were no obvious differences between the two groups in body hair, motor function, food and water intake, or growth rate.

3.2. Brain damage

Fig. 1 shows that 48 h after the injection of L-Cys (1.5 mg/g BW), significant TUNEL-positive changes in the hippocampal regions had occurred. When glucose was given concomitantly, no changes were observed compared with control.

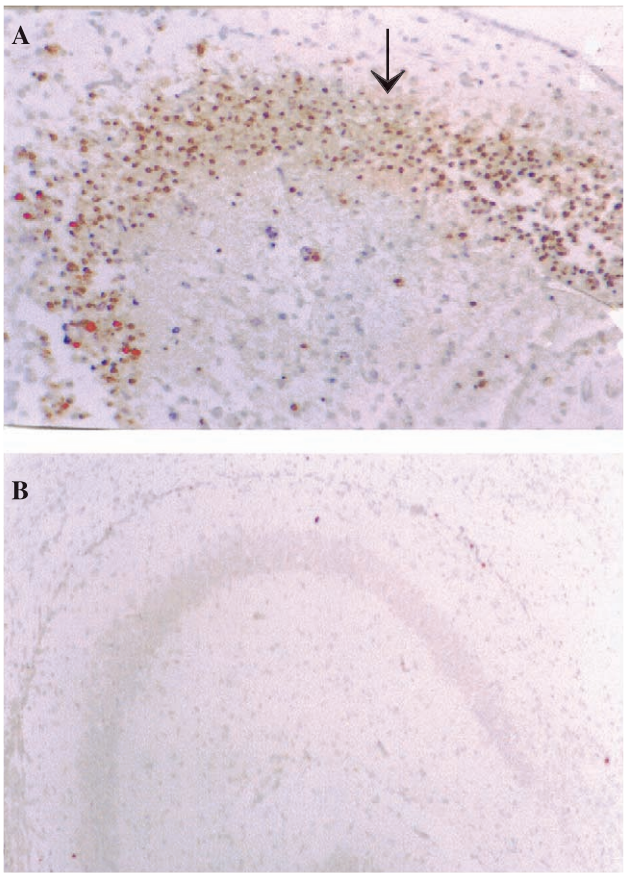

Fig. 1. (A) Hippocampal regions (CA1 and CA3) of neonatal mouse 48 h after L-Cys injection (1.5 mg/g BW). Arrow indicates significant DNA fragmentation in brown-colored cells (TUNEL stain, × 50). (B) Normal hippocampus in control neonatal mouse 48 h after saline injection (TUNEL stain, × 50).

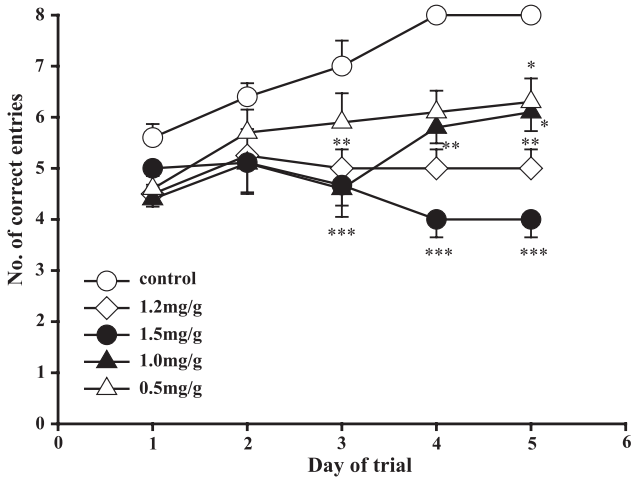

Fig. 2. Number of correct entries into eight-arm maze after L-Cys injection in various doses. Note that animals injected with higher dose of L-Cys (1.2 or 1.5 mg/g BW) scored poorly as compared with controls (** $P < .01$, *** $P < .001$, respectively). Injection with L-Cys at lower doses (0.5 or 1.0 mg/g BW) resulted in better performance, but not as good as that in controls (* $P < .05$).

3.3. Maze experiments

Control animals succeeded in reaching the maze criteria by Day 2 of the experiment. In contrast, animals treated with L-Cys exhibited, in a dose-dependent manner, a significantly lower number of correct entries during the first eight attempts as compared with control (Fig. 2). Coadministra-

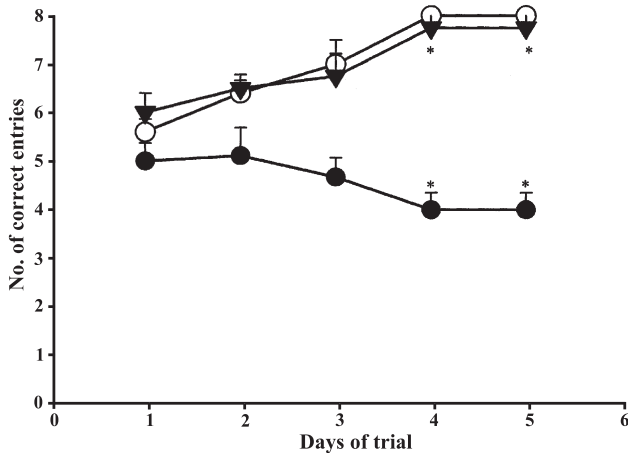

Fig. 3. Coadministration of glucose to L-Cys-treated mice. When mice received concomitantly two repeated doses of glucose (3 mg/g BW) after the injection of L-Cys (1.5 mg/g BW), no reduction in number of correct entries into the maze occurred as compared with the mice treated with L-Cys (1.5 mg/g BW without glucose) (* $P < .001$). Open circles: controls; solid circles: L-Cys (2.5 mg/g BW); solid triangles: L-Cys (1.5 mg/g BW) + glucose (3.0 mg/g BW).

Table 2
Number of days to reach maze criteria as a function of different treatments ($n = 6$ per group)

Treatment (mg/g BW)	Criteria (days)	P value
Control	2.7 ± 0.7	
L-Cysteine (0.5)	3.6 ± 0.7	NS
L-Cysteine (1.0)	3.7 ± 0.2	NS
L-Cysteine (1.2)	4.8 ± 0.2	< 0.01
L-Cysteine (1.5)	5.9 ± 0.2	< 0.001
Glucose (3.0) with L-cysteine (1.5)	2.8 ± 0.8	NS

tion of glucose in animals injected with 1.5 mg/g BW of L-Cys prevented functional deterioration in all animals (Fig. 3). Table 2 summarizes data concerning the days needed for the animals to reach the maze criteria. In a dose-dependent manner, animals injected with L-Cys (1.2 or 1.5 mg/g BW) required 1.78- or 2.2-fold more days, respectively, to accomplish the maze criteria as compared with control. In contrast, glucose coadministration resulted with the same performance in the maze experiment as control.

4. Discussion

In this study, we demonstrated the dose-dependent ability of L-Cys, when injected subcutaneously into neonatal mice, to cause long-term neurobehavioral deficits (as evaluated by use of the eight-arm maze to test learning and memory) and apoptotic hippocampal neuronal damage. These deficits were manifested only when L-Cys was administered at concentrations higher than 1.2 mg/g/BW. Below that, it took one more day for the mice to reach maze criteria but this trend was found to be insignificant. In addition, histological damages were pronounced only at the high L-Cys concentrations.

Mouse behavior and spatial memory in a radial maze is linked to its hippocampal integrity (Olton, 1977). Therefore, we chose the eight-arm maze model because of its proven ability to measure these hippocampal-related mental functions (Olton and Samuelson, 1976). The eight-arm maze has also proved useful to study neuropharmacological changes caused by administration of different drugs (Olton, 1977; Olton and Samuelson, 1976; Pick and Yanai, 1983). The results of this study demonstrated that the observed neurobehavioral deficit matched the morphological changes observed microscopically as TUNEL-positive staining in the hippocampus. Although detection of DNA fragments in situ using the TUNEL assay is applied to investigate active cell death (apoptosis), it is now well-established that DNA fragmentation is common to different kinds of cell death and thus, its detection in situ should not be considered a specific marker of apoptosis (Grasl-Kraupp et al., 1995). Hence, we cannot conclude that our L-Cys-induced morphological brain changes are apoptotic in nature as they could be related to necrosis or autolysis. Behavioral effects related to hippocampal lesions have been previously documented in mice using assessments of functions, such as food hoarding from a source outside the home base, tendency to displace food pellets from a tube inside the home cage (burrowing), and reduction of directed exploration (rearing and head dipping) (Deacon et al., 2002). These models may have aided to the interpretation of our findings regarding neurodegenerative conditions that induce hippocampal pathology.

The neurobehavioral deterioration and the simultaneously occurring morphological changes were successfully abrogated by coadministration of glucose, which attenuated the L-Cys glucose-lowering effect. Indeed, mice treated with L-Cys but concomitantly receiving glucose scored similarly to controls in their performance on the maze and did not exhibit apoptotic changes in the brain.

Neurons are known to be highly dependent on a constant supply of glucose to maintain their integrity and function (Boyle et al., 1995). Numerous publications have demonstrated that hypoglycemia, even clinically unnoticed, can cause long-term developmental and neurological deficits (Lancet, 1989; Sieber and Traystman, 1992). Repeated moderate hypoglycemic events are reported to have a potentially deleterious effect similar to less frequent episodes of protracted hypoglycemia (Lucas et al., 1988; Olney et al., 1990). Thus, the L-Cys-induced hypoglycemic effect in itself may be contributory to its reported neurotoxicity.

It is well known that hypoglycemic shock can cause irreversible brain damage, which can be seen in light microscopy as neural necrosis spreading from the cortex to more deeply located brain structures (Auer et al., 1984a,b). The histological damage in the L-Cys-treated mice was concentrated mainly in the hippocampal region, where hypoglycemia is known to produce its destructive effects (Auer and Siesjö, 1988). Previous studies have reported that administration of high dose of L-Cys to infant mice caused brain damage similar to that caused by glutamate (Olney and Ho, 1970). This type of damage has been attributed to L-Cys-related neuroexcitatory effect, which is caused by excessive activation of *N*-methyl-D-aspartate receptors, but its potent hypoglycemic effect has apparently been overlooked (Olney et al., 1990). However, the basic chemical formula of L-Cys differs in lacking the omega-side chain that is present in the potent neuroexcitatory amino acids glutamate and aspartate, rendering it more penetrable through the blood–brain barrier (Olney and Ho, 1970; Olney et al., 1990). The observed brain damage following L-Cys injection was anatomically localized in the hippocampal region in contrast to the more extensive and widespread damage that can follow the administration of glutamate or aspartate (Lehmann et al., 1993; Olney et al., 1972).

In this study, we did not monitor brain electrical activity to demonstrate flattening of the electroencephalogram, a known critical determinant of structural brain damage as a result of the hypoglycemia (Auer et al., 1984a,b). However, we have demonstrated that L-Cys-induced physiological

deterioration and brain damage may be partially related to its glucose-lowering potential. The chemical dissimilarities and different pattern of histological damage caused by L-Cys as compared with other potent excitatory amino acids support this contention.

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