The Role of Posthypoxic Reoxygenation in Destruction of Cultured Hippocampal Neurons

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UDC 616.831−005.4:547.631.4

Translated from *Byulleten' Eksperimental'noi Biologii i Meditsiny*, Vol. 115, № 2, pp.130−132, February, 1993
Original article submitted August 19, 1992

Key Words: *nerve cell culture; hippocampus; hypoxia; posthypoxic reoxygenation*

Recent experimental findings [3] indicate that neurodestructive processes in acute disorders of brain circulation occur not only during nerve cell blood supply arrest, but later too, in complete or partial bloodstream restoration associated with the recovery of oxygen supply to affected brain sites (that is, during posthypoxic reoxygenation). A cell culture of various structures of mammalian embryonal brain is one of the objects of investigation permitting controlled simulation and study of the mechanisms of hypoxic injury of neurons [5,8,11,13].

The aim of our study was to compare the rates of neuronal death in murine embryo hippocampal cell cultures in hypoxia and in subsequent posthypoxic reoxygenation.

The investigation was carried out within the framework of the State Program "Ishemic Damage and the Most Prevalent Brain Disorders: Mechanisms and Methods of Correction".

MATERIALS AND METHODS

Dissociated hippocampal cells of 17-19-day murine embryos were used for the preparation of primary cultures that were cultivated in accordance with the method described previously [9] on 20×22 mm slides covered with collagen with poly-L-lysine layered onto it [6]. The cultures were grown for 18-23 days in plastic petri dishes 40 mm in diameter, each containing 1 ml of nutrient medium, in a CO_2 incubator (95% air + 5% CO_2) at 35.5°C. In the hypoxia experiments the cultures were used on days 18-21 of *in vitro* development, for by this time they had attained a high degree of neuronal differentiation and were characterized by mature synaptic connections [9]. Ten to 12 cultures in petri dishes without covers were placed in hermetic chambers filled with a gas mixture of 95% nitrogen and 5% carbon dioxide. Polarographic measurements carried out over the course of the experiment detected no oxygen in the hypoxic chambers filled with this gas mixture. In the first series of experiments the cultures were exposed to hypoxia for 5 or 7 h at 35.5°C, after which, following a brief (10-15 sec) exposure in a sterile air stream in a laminar box, they were placed for 3 h in a CO_2 incubator with approximately 21% oxygen. In the second series of experiments, carried out in parallel with the first, the cultures were exposed to continuous hypoxia not followed by reoxygenation for 8 or 10 h at the same temperature. After the experimental exposures, the cultures from both batches were simultaneously fixed in a mixture of 100° alcohol, 40% formalin, and glacial acetic acid in a ratio of 7:2:1 and stained with cresyl violet (after Nissl) or with vanadium hematoxylin [1]. The neurodestructive

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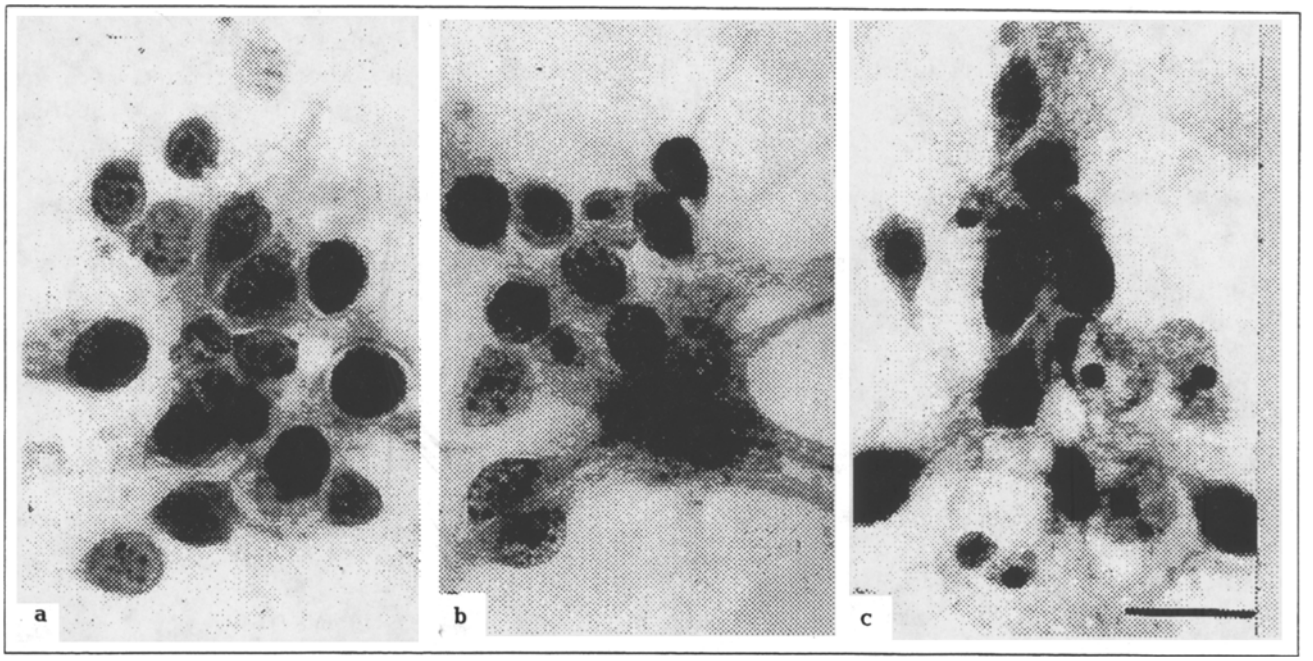

Fig. 1. Neurodestructive effects of hypoxia and subsequent reoxygenation in hippocampal cell cultures. 1) control culture; 2) culture after 10 h hypoxic exposure (without reoxygenation); 3) culture after 7 h hypoxia and 3 h reoxygenation. Vanadium hematoxylin staining. 20 days *in vitro*. Scale: 25 μ. Objective 40, ocular 10.

effect was assessed as described previously [2] in histologic preparations by estimating the percent share of dead neurons (in relation to their total counts) at 15 randomly chosen sites 1 mm^2 in size in each culture. In some experiments the rate of neuronal death was assessed by measurements of the lactate dehydrogenase (LDH) levels in the nutrient media of the intact cultures and of the cultures after the experimental exposures [10]. Student's *t* test was used in statistical processing of the results.

RESULTS

Analysis of the histologic preparations (Figs. 1, 2, *a*) showed that the number of dead neurons in the first group of cultures continuously exposed to hypoxia for 10 h was 27.1±1.3%. In a parallel experiment, when the cultures were exposed to hypoxia for 7 h and then were reoxygenated for 3 h, the number of dead neurons increased to 40.3±1.5% (Fig. 2, *a*). LDH activity in the nutrient medium obtained from the first group of cultures was found to be 13.2±0.8%. At the same time this activity significantly increased in the cultures exposed to 7 h hypoxia and 3 h reoxygenation, constituting 16.8±1.3% (Fig. 2, *b*).

The activation of neurodestructive processes in the posthypoxic reoxygenation period was observed even in cases where a shorter hypoxic exposure preceded this period. If hypoxia lasted 5 h, followed by 3 h reoxygenation in a CO_2 incubator, the number of dead neurons was 35.8±3.0%, whereas after 8 h hypoxia without subsequent reoxygenation this value was 21.2±1.8% (Figs. 2, *c*, *1-3*). Neuronal destruction was similarly increased (by 16%) in cases where after 5 h incubation in an oxygen-free atmosphere the cultures were kept for another 5 h not in a medium with a normal oxygen level (about 21%) but in a gas mixture containing only 5% oxygen. Note that after exposure of the cultures to such a gas mixture for 10-14 h without preliminary incubation in an oxygen-

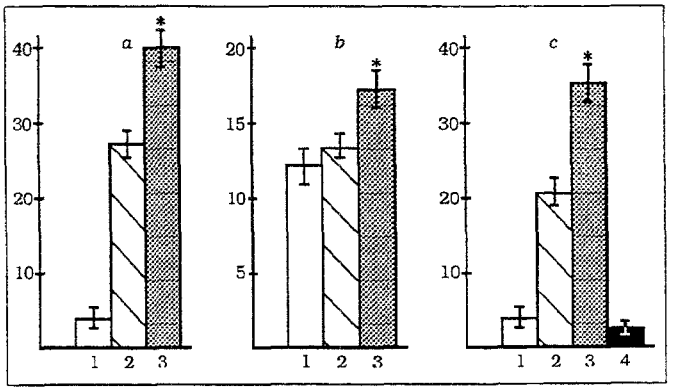

Fig. 2. Cultured hippocapmus neuron destruction values in hypoxia and subsequent reoxygenation. *a*, *b*: 1) control cultures (*a*: n=12; *b*: n=6); 2) cultures after 10 h hypoxia without reoxygenation (*a*: n=20; *b*: n=4); 3) cultures after 7 h hypoxia and 3 h reoxygenation (*a*: n=15; *b*: n=6). *c*: 1) control cultures; 2) cultures after 8 h hypoxia without reoxygenation (n=6); 3) cultures after 5 h hypoxia and 3 h reoxygenation (n=5); 4) cultures after 14 h incubation in gas mixture with 5% oxygen (n=9). Asterisk: a reliable difference from 2 (*a*: $p<0.001$; *b*: $p<0.05$; *c*: $p<0.001$). n) number of cultures examined. Ordinate: *a*, *c*) dead neurons (%); *b*) LDH activity (%).

free medium the fraction of dead neurons was only 2.3-0.7% (Fig. 2, *c, 4*), which was comparable to the control cultures kept in normoxia.

Our findings indicate that neurodestructive processes induced by hypoxic exposure of cultured hippocampal neurons run a more active course during posthypoxic reoxygenation than during a hypoxic period of the same length. This is true no matter how long the hypoxic period preceding reoxygenation, and can be observed even when the oxygen content in the environment during the posthypoxic reoxygenation period is under 5%. It should be mentioned that such hypoxia (5% oxygen) is not in itself destructive to the neurons, because under such conditions without previous incubation in an oxygen-free environment the results of such an exposure are similar to the results of spontaneous neuronal destruction observed in intact cultures.

Some scientists believe that one of the causes of neuron mortality during reoxygenation is the increased formation of free radicals and peroxide compounds which activate lipid peroxidation (LPO), leading to neuron membrane destruction [4,7,12]. The results of recent research [14] confirm this hypothesis; direct measurements demonstrated a marked increase in the production of free radicals and in the related LPO reactions in rat brain tissue during reoxygenation. Our previous findings on the protective effect of an antioxidant belonging to stereo-hindered phenols (U-18) added to hippocampal cultures after hypoxic exposure confirm LPO participation in the neurodestructive processes during this period [2].

The above results indicate that complete or partial reoxygenation in the posthypoxic period results in increased mortality of neurons as against that observed after continuing culture incubation under hypoxic conditions for the same period. Therefore, these data confirm the hypothesis that posthypoxic reoxygenation is an important factor aggravating neurodestructive processes initiated by hypoxic exposure and activated, possibly, by accelerated production of peroxide compounds after the restoration of the oxygen supply to the nerve cells.

The authors are grateful to A.N.Erin, Doctor of Biological Sciences, for the fruitful discussion of the results.

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