# Homocysteine Toxicity in Organotypic Cultures of Rat Retina

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Effects of homocysteine in toxic concentrations on retinal neurons were studied *in vitro*. In organotypic roller cultures of postnatal (8-12-day-old) and adult rat retina homocysteine caused multiple damage to neurons in the outer nuclear layer, in deep compartments of the inner nuclear layer, and ganglion cell layer.

**Key Words:** *retina; organotypic cultures; ganglion and amacrine cells; homocysteine; toxicity*

Homocysteine is a stimulatory amino acid, a cysteine analog; it causes activation of glutamate receptors and DNA injury, stimulates the production of free radicals and caspases, which causes apoptotic death of nerve cells [5,12-14].

Experimental and clinical data suggest that increased blood content of homocysteine (hyperhomocysteinemia) caused by deficiency of folic acid, vitamins $B_6$ and $B_{12}$, and genetic disorders in the content of cystathionine β-synthase leads to organic abnormalities in the vascular and nervous systems [7,10], including the retina [8,11,12]. It was shown *in vitro* that homocysteine caused death of cultured hippocampal, neocortical, and cerebellar neurons [3,4,6].

We studied the effect of homocysteine on organotypic cultures of the retina of postnatal (8-12-week-old) and adult rats.

## MATERIALS AND METHODS

Experiments were carried out with consideration for "Regulations for Studies on Experimental Animals" and requirements of ARVO (The Association for Research in Vision and Ophthalmology) to ophthalmological studies on animals.

Adult and 8-12-day-old rats were decapitated under deep ether narcosis. The heads were treated with 70% ethanol. Enucleation was carried out under sterile conditions. The retina was isolated after circular incision of the cornea and removal of the lens and vitreous body, washed twice in $Ca^{2+}/Mg^{2+}$-free Dulbecco phosphate buffer (DPB; Gibco) with 0.8% glucose. The retina was dissected in DPB with belied scalpel into rectangular fragments (1-2 $mm^2$). The fragments were transferred into nutrient medium (90% minimum Eagle medium (Gibco), 5% FCS (Gibco), 5% human placental serum, 0.8% glucose, 2 mM glutamine, 0.2 U/ml insulin, 25 mM HEPES buffer, 100 U penicillin, and 100 μg/ml streptomycin). Floating retinal sections were cultured in 25-ml flasks in 10 ml nutrient medium for 12 h in a thermostat (36.5°C) on a horizontal roller at constant rotation (60 rpm) [1]. Each flask contained sections of 4 retinas. One-third of the medium was replaced twice a week (or earlier, if needed).

On day 12 of culturing equal amounts of formed retinal bodies [1] were transferred from each flask into two new flasks in equal volumes of the same medium. DL-homocysteine (200 mM; Sigma) dissolved in culture medium to the final concentration of 20 mM was added into one flask. After 24-h culturing retinal bodies from both flasks were

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washed with DPB and fixed with modified Telesnitsky fixative (96% ethanol — 70%, 20% formalin, 10% glacial acetic acid, and 0.1% eosin Y) for 10-15 min. After fixation the retinal bodies were washed in 70% ethanol, dehydrated in three portions of isopropyl alcohol (isopropanol 99.9), 30 min in each portion, and transferred into melted (58°C) paraffin (Paraplast Plus, Sigma) for 2-3 h or overnight, after which the preparation was embedded in blocks using a fresh portion of paraffin [2]. Sections (8-μ) were mounted on gelatin-coated slides, deparaffinized, and stained by the method of Nissl with 0.1% Cresyl Violet dissolved in 0.1 M acetate buffer (pH 3.4) or Gill hematoxylin (Sigma, Cat. No. GHS-1-16) and 1% eosin Y (Sigma, Cat. No. E 4382).

## RESULTS

In control cultures pyknotic nuclei of solitary dead neurons were detected in the inner nuclear layer and ganglion cell layer (Fig. 1, *a*, *b*). Toxic concentrations of homocysteine caused numerous cell injuries in the retinal organotypic culture: Cresyl Violet, hematoxylin, and eosin staining detected pyknotic nuclei of dead neurons in the deep compartments of the inner nuclear layer and in the ganglion cell layer (Fig. 2, *a*, *c*), as well as in the photoreceptor layer (Fig. 2, *d*). Acute cytotoxic edema and lysis of the ganglion cell cytoplasmatic organelles developed at the initial stages of injury. This was followed by the development of extracellular edema of neurons and nuclear chromatin condensation with the formation of pyknotic nuclei (Fig. 2, *a*, *b*), indicating irreversible death of these neurons. Similar processes of neuronal injury and death were observed in deep compartment of the inner nuclear layer, in which amacrine cells were located, and in the photoreceptor layer (Fig. 2, *c*, *d*). The thickness of the retina decreased at later stages because of massive death of photoreceptors and neurons of the inner nuclear layer (Fig. 3).

These pathological changes in the neurons under the effect of homocysteine in toxic concentrations suggest that these cells died by the mechanism of acute cytotoxic edema and subsequent apoptosis. This is in line with the results of other studies of homocysteine retinotoxicity. In experiments on mice homocysteine injected into the vitreous body caused partial (about 23%) apoptotic death of ganglion cells, which was detected by the TUNEL method and confirmed by immunocytochemical test for caspase-3 [12]. Studies of retinotoxicity of other stimulatory amino acids (glutamate and its agonists N-methyl-D-aspartate and kainic acid) *in vivo* and *in vitro* gave similar results [9,15]. The sensitivity of the retinal amacrine and ganglion cells to cytotoxicity of glutamate and its agonists remains disputable. Studies of the toxicity of N-methyl-D-aspartate for mouse retina *in vivo* and *in vitro* showed that ganglion cells were not sensitive to the toxic effect of glutamate and its agonists, while amacrine cells were the first and main target for the cytotoxic

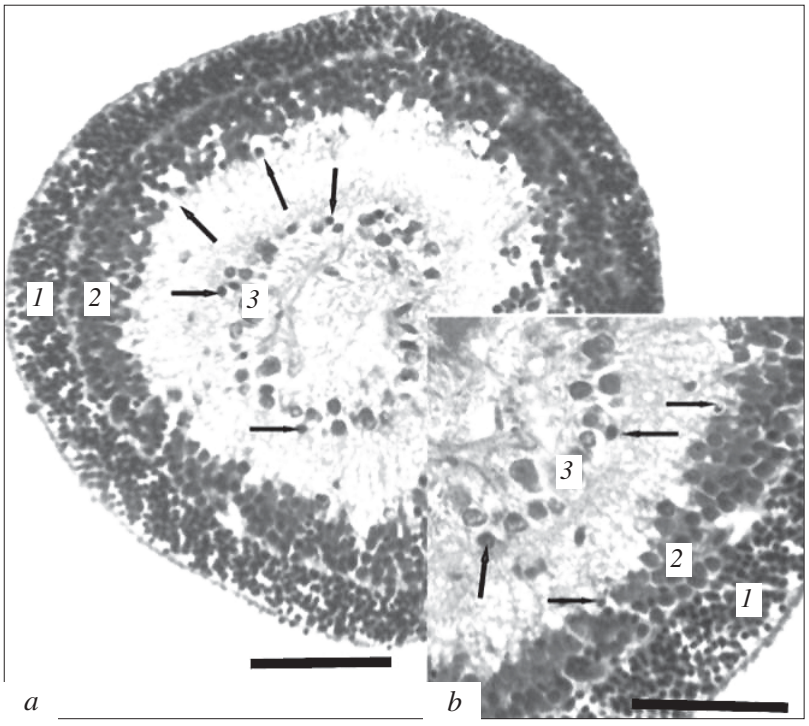


**Fig. 1.** Sections of retinal bodies. Roller organotypic culture of retinal sections from 8-day-old rat (12 days of culturing). *a*) control section of retinal body; *b*) fragment of retinal body at greater magnification. *1*) outer nuclear layer; *2*) inner nuclear layer; *3*) ganglion cell layer. Arrows show pyknotic nuclei. Combined staining with hematoxylin, eosin, and Cresyl Violet. Here and in Figs. 2, 3: 100 μ scale.

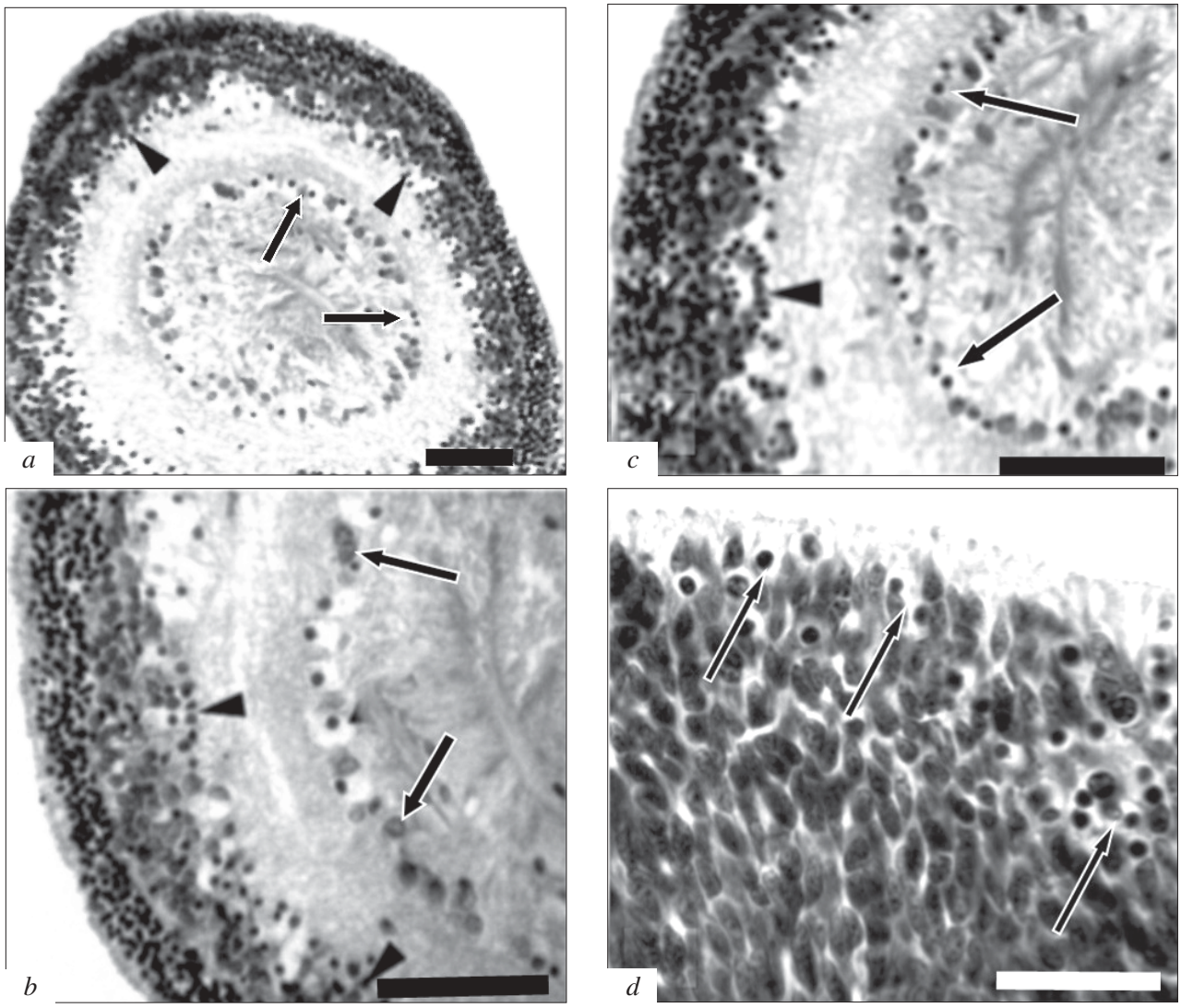


**Fig. 2.** Cell abnormalities in retinal bodies after 24-h exposure to 20 mM homocysteine. *a*) retinal body cross-section; *b*) fragment of a retinal body at greater magnification: acute cytotoxic edema of ganglion cells (arrows) and pyknotic nuclei in deep compartments of inner nuclear layer (triangles); *c*) neuron death in inner nuclear layer, pyknotic nuclei (triangles) and ganglion layer (arrows); *d*) pyknotic nuclei in outer nuclear layer (arrows). Combined staining by hematoxylin, eosin, and Cresyl Violet.

effect of stimulatory amino acids. The death of ganglion cells under the effect of stimulatory amino acids can result from primary death of amacrine cells and subsequent disorders in their synaptic and trophic bonds with ganglion cells [15]. This hypothesis is confirmed by selective and incomplete death of the ganglion layer cells under the effect of glutamate and its agonists, which was demonstrated previously [9,12,15] and confirmed in the present study. Evaluating the death of the ganglion layer neurons, we should remember that this layer contains about 50% "shifted" amacrine cells [15] and histological analysis of their death under the effect of stimulatory amino acids does not provide us reliable quantitative data on the state of damaged ganglion and amacrine neurons in the ganglion layer. This problem requires further research by immunocytochemical methods.

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## REFERENCES

1. I. V. Viktorov, A. A. Lyzhin, and O. P. Aleksandrova, *Byull. Eksp. Biol. Med.*, **137**, No. 4, 476-480 (2004).
2. I. V. Viktorov and S. S. Pashin, *Ibid.*, **136**, No. 7, 119-120 (2003).
3. J. P. Kim, J. Y. Koh, and D. W. Choi, *Brain Res.*, **437**, 103-110 (1987).
4. W. K. Kim and Y. S. Pae, *Neurosci. Lett.*, **216**, 117-120 (1996).
5. I. I. Kruman, C. Culmsee, S. L. Chan, *et al.*, *J. Neurosci.*, **20**, No. 18, 6920-6926 (2000).

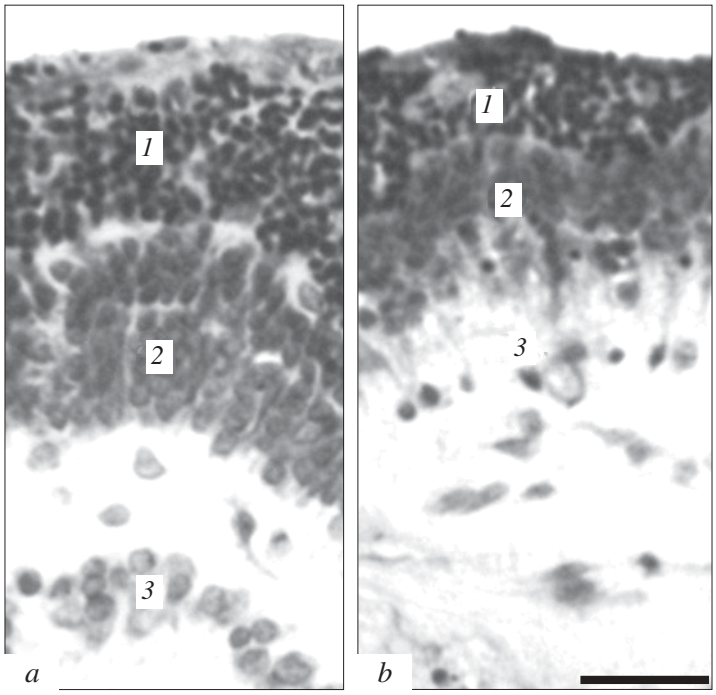


**Fig. 3.** Changed transverse size of the retina after cytotoxic homocysteine exposure. *a*) control culture; *b*) experimental culture (20 mM homocysteine). *1*) outer nuclear layer; *2*) inner nuclear layer; *3*) ganglion cell layer.

6. M. Langmeier, J. Folbergrova, R. Haugvicova, *et al.*, *Epilepsia*, **44**, No. 3, 299-304 (2003).
7. S. A. Lipton, W. K. Kim, Y. B. Choi, *et al.*, *Proc. Natl. Acad. Sci. USA*, **94**, 5923-5928 (1997).
8. H. C. Looker, A. Fagot-Campagna, E. W. Gunter, *et al.*, *Diabetologia*, **46**, No. 6, 766-772 (2003).
9. X. Luo, V. Heidinger, S. Picaud, *et al.*, *Invest. Ophthalmol. Vis. Sci.*, **42**, 1096-1106 (2001).
10. M. P. Mattson, *Ann. Intern. Med.*, **139**, 441-444 (2003).
11. M. P. Mattson and T. B. Shea, *Trends Neurosci.*, **26**, No. 3, 137-146 (2003).
12. P. Moore, A. El-Sherbeny, P. Roon, *et al.*, *Exp. Eye Res.*, **73**, 45-57 (2001).
13. J. W. Olney, M. T. Price, K. S. Salles, *et al.*, *Brain Res. Bull.*, **19**, No. 5, 597-602 (1987).
14. Q. Shi, J. E. Savage, S. J. Hufeisen, *et al. J. Pharmacol. Exp. Ther.*, **305**, No. 1, 131-142 (2003).
15. E. M. Ullian, W. B. Barkis, S. Chen, *et al.*, *Mol. Cell. Neurosci.*, **26**, 544-557 (2004).