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Morphological and functional changes in rat hippocampal slice cultures after short-term oxygen-glucose deprivation

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Abstract

To study effects of short-term cerebral ischemia, hippocampal slice cultures were subjected to oxygen and glucose deprivation (OGD) followed by a period of normoxic reoxygenation. Propidium iodide staining, and MTT/formazan-assay were used to evaluate cell viability and metabolic activity. CA1 pyramidal cells were analyzed at the light- and electron microscopic levels. Cell damage was found to be insignificant during the first hour after 10 min OGD but profound following 4 h, showing delayed neuronal cell damage caused by short-term OGD. Our model can be used to characterize the mechanisms of cell damage caused by mild cerebral ischemia. These data might apply to further development of neuroprotective tools for the treatment of brain diseases.

Keywords: rat hippocampal slice cultures - short-term oxygen-glucose deprivation - cell viability - morphology

Introduction

Transient cerebral ischemia is known to lead to delayed neuronal dysfunction, which may occur in hours and days after the ischemic insult [1, 2]. Depending on the severity of the insult, such pathological condition can result in variable extent of cell damage or death. Strong ischemia has been shown to lead to quick pronounced neuronal destruction whereas mild insults might cause less remarkable cell damage, which is usually delayed [3]. Ischemia is now suggested to be a common risk factor of Alzheimer's and Huntington's diseases, epilepsy and other neurodegenerative disorders [4, 5].

The organotypic hippocampal slice culture (OHSC) is an appropriate model to study cell damage occurring in different neuropathological states, including ischemia. There are different ways to produce the ischemic damage in experimental conditions. The most common approach is to subject slice cultures to oxygen and glucose deprivation (OGD) for some duration. The time of deprivation used varies greatly [6–8]. Mostly, quite prolonged (30 min and more) deprivation is used in slice cultures; less numerous studies deal with effects of short-term OGD. The latter presumably induces more expanded development of neuronal cell injury resulting in delayed cell death, and this is supposed to provide a

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promising time interval for detailed studying and correction of the cellular and molecular mechanisms induced by OGD.

The aim of the present study was to evaluate the morphologic and functional changes in cells of organotypic hippocampal slice cultures exposed to short-term (10 min) combined oxygen and glucose deprivation followed by normoxic reoxygenation during 1 or 4 h.

Materials and methods

Hippocampal organotypic slice cultures

Organotypic hippocampal slice cultures were prepared from 7-day-old Wistar rats according to the technique described by Stoppini *et al.* [9]. Rat pups were decapitated, the brains were aseptically removed into ice-cold dissection medium consisting of 50% of Minimum Essential Medium (MEM), 10 mM Tris, 2 mM $NaHCO_3$, 12.5 mM HEPES, 15 mM glucose, 25% Hanks' balanced salt solution (HBSS), 100 U/ml penicillin and 100 μg/ml streptomycin, pH 7.3. Then the hippocampi were rapidly isolated, and 350 μm thick transversal slices were cut from the middle part of each hippocampus using McIllwain tissue chopper (England). The slices were transferred to sterile porous membrane inserts (Millicell, Bedford, MA), which were placed in a 6-well plate containing 1 ml culture medium/well (50% of MEM, 25% horse serum (HS), 25% HBSS, 5 mM Tris, 2 mM $NaHCO_3$, 12.5 mM HEPES, 15mM glucose, 100 U/ml penicillin and 100 μg/ml streptomycin, pH 7.2). Four hippocampal slices were placed on each insert and cultivated for 12-14 days at +35°C at air atmosphere with 5% CO_2. The culture medium was changed the next day after preparation of the slices and then twice a week.

Oxygen-glucose deprivation

The hippocampal slices were washed twice with warm deoxygenated glucose-free and serum-free medium (GSFM: 0,1 M phosphate buffered saline, PBS, 20 mM Hepes, 15 mM sucrose), then the culture medium was changed to 1 ml GSFM (+35°C) and cultures were transferred to a thermostatically controlled chamber, where air was quickly replaced by 95% N_2 / 5% CO_2. Following OGD (10, 30, 60 min), the cultures were washed with the regular culture medium (+35°C) and returned to the normal conditions in the incubator for 1 and 4 h (normoxic reoxygenation), and then analyzed. Untreated slice cultures were used as a control.

Propidium iodide (PI) staining

Cellular uptake of PI was used to estimate of cell damage in slice cultures. PI is a stable fluorescent dye entering cells with damaged cell membrane. Once inside a cell, PI interacts with DNA to yield a bright red fluorescence. PI (2 μM) was added to the cultures before the experimental treatment for 24 h. Slices in which PI fluorescence was not detected before OGD treatment were used in the experiment (the number of PI-stained slices was negligible). Grayscale images of PI stained cultures were taken after the 10 min OGD and following 1 h or 4 h of normoxic reoxygenation using a standard fluorescent microscope equipped with a rhodamine filter set. The area of PI-fluorescence in a region of fixed size within hippocampal CA1 area (magnification ×200) was calculated using Bioquant Classic 95 image analysis software (R&M Biometrics, Inc., USA).

LDH efflux assay

LDH is a stable cytosolic enzyme. LDH-efflux into the culture medium was determined as another marker of cell damage in slice cultures. Samples were obtained from culture media after the experimental influence. LDH-activity in the samples of culture medium was determined colorimetrically using kit G1780 (Promega, USA). The method is based on the conversion of tetrazolium salts into a red formazan product following alternate electron acception [10]. The color intensity of formazan is proportional to LDH-activity in the culture medium and to the number of damaged cells. Data are shown as percentage of whole slice culture lysis.

MTT / formazan-assay

The MTT/formazan-assay was used to assess the metabolic activity of the cultured slices [11.12]. Soluble yellow tetrazolium MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) is converted to insoluble blue formazan by metabolically active cells. Slices were incubated for 10 min with MTT (0.5 mg/ml) in phenol-free medium containing MTT. Subsequently, formazan was dissolved with a mixture of isopropanol/formic acids (95/5), and the optical density of the solution was measured at 545 nm with a Microwell Plate Reader (Sigma).

Table 1. Changes of LDH-activity changes after OGD. Data are shown in percentage of whole cell lysis.

	Immediately after OGD	1 h after OGD	4 h after OGD
Control	6.7 ± 1.1 (n=24)	8.8 ± 1.6 (n=14)	10.1 ± 2.2 (n=10)
OGD 10 min	5.8 ± 1.1 (n=19)	12.4 ± 4.0 (n=13)	32.1 ± 8.2 (n=10)
OGD 30 min	12.2 ± 2.4 (n=21)	45.4 ± 9.6 (n=14)	60.8 ± 12.4 (n=10)
OGD 60 min	19.5 ± 3.8 (n=19)	62.1 ± 9.9 (n=11)	64.2 ± 8.7 (n=10)

Light and electron microscopy

Slice cultures were fixed by mixture of 2.5% formalin and 2.5% glutaraldehyde in 0.1 M PBS. Then samples were washed three times in PBS, stained in 1% OsO_4 in 0,1 M PBS, pH 7.4, for 1 h, dehydrated in graded ethanol series followed by acetone, and flat-embedded in EPON (Fluka).

Semithin (1–2 μm) sections were cut, stained with toluidine blue and analyzed using a light microscope. Within the CA1 area, the numbers of normal, condensed and swollen pyramidal cells in a region of fixed size were analyzed in every slice (magnification ×200). To estimate the degree of cell condensation, the sizes of nuclear and somatic profiles of the CA1 pyramidal cells in control and experimental cultures were calculated using Bioquant Classic 95 image analysis software (R&M Biometrics, Inc., USA).

Ultrathin (40–50 nm thick) sections were cut with an ultratome (LKB 8800), stained with uranyl acetate and lead citrate and examined using a JEM100-CX transmission electron microscope.

Data analysis

Data from at least three independent experiments are presented as a means ± SEM, for $n \geq 9$. Statistical analyses were performed using Student's test. In all cases a p value ≤ 0.05 was considered significant for the statistics. Asterisks (*) indicate significant differences as compared to the control group.

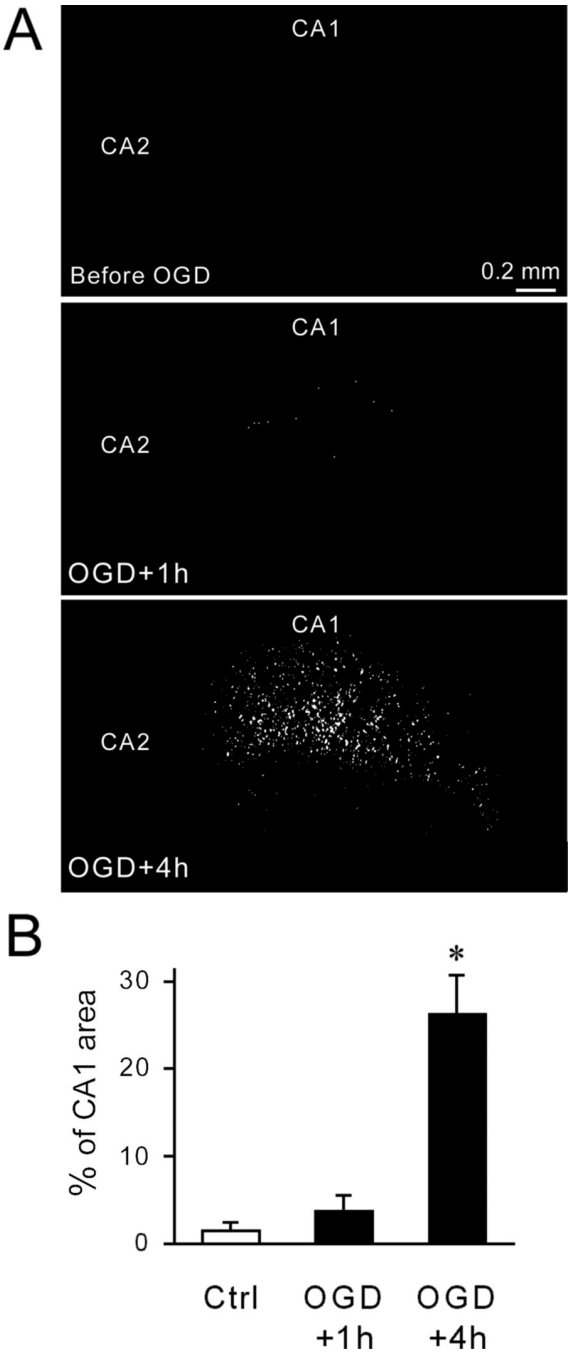

Fig. 1 Effects of 10-min OGD on cell viability in the hippocampal slice cultures. (**A**) Representative images of PI fluorescence in the CA1 area in the hippocampal slice culture. (**B**) Neuronal damage as the area of PI fluorescence within the hippocampal CA1 area.

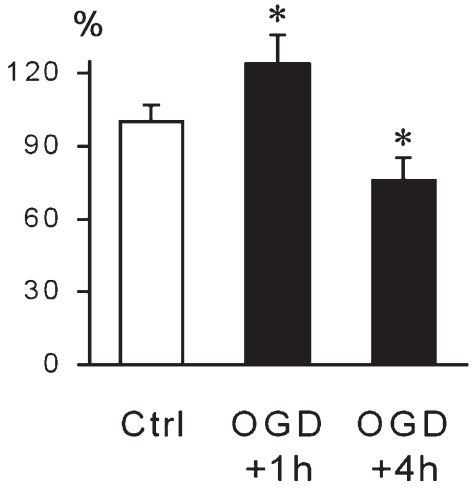

Fig. 2 Metabolic activity of the hippocampal slice cultures assessed by MTT/formazan-assay.

Results

To simulate ischemic damage *in vitro*, OGD followed by normoxic reoxygenation was used. Cell damage was estimated using propidium iodide (PI) staining and the measurement of LDH-activity in the culture medium. The analysis of LDH-activity after the exposure to OGD of different duration (10, 30, and 60 min) followed by reoxygenation showed clear time-dependence of cell damage on the duration of both deprivation and reoxygenation (Table 1). It was noted that the development of the effect was not immediate but postponed after OGD. 30-min and 60-min OGD resulted in pronounced cell damage during the first hour of normoxic reoxygenation, while the effect of 10-min OGD was more delayed and less marked.

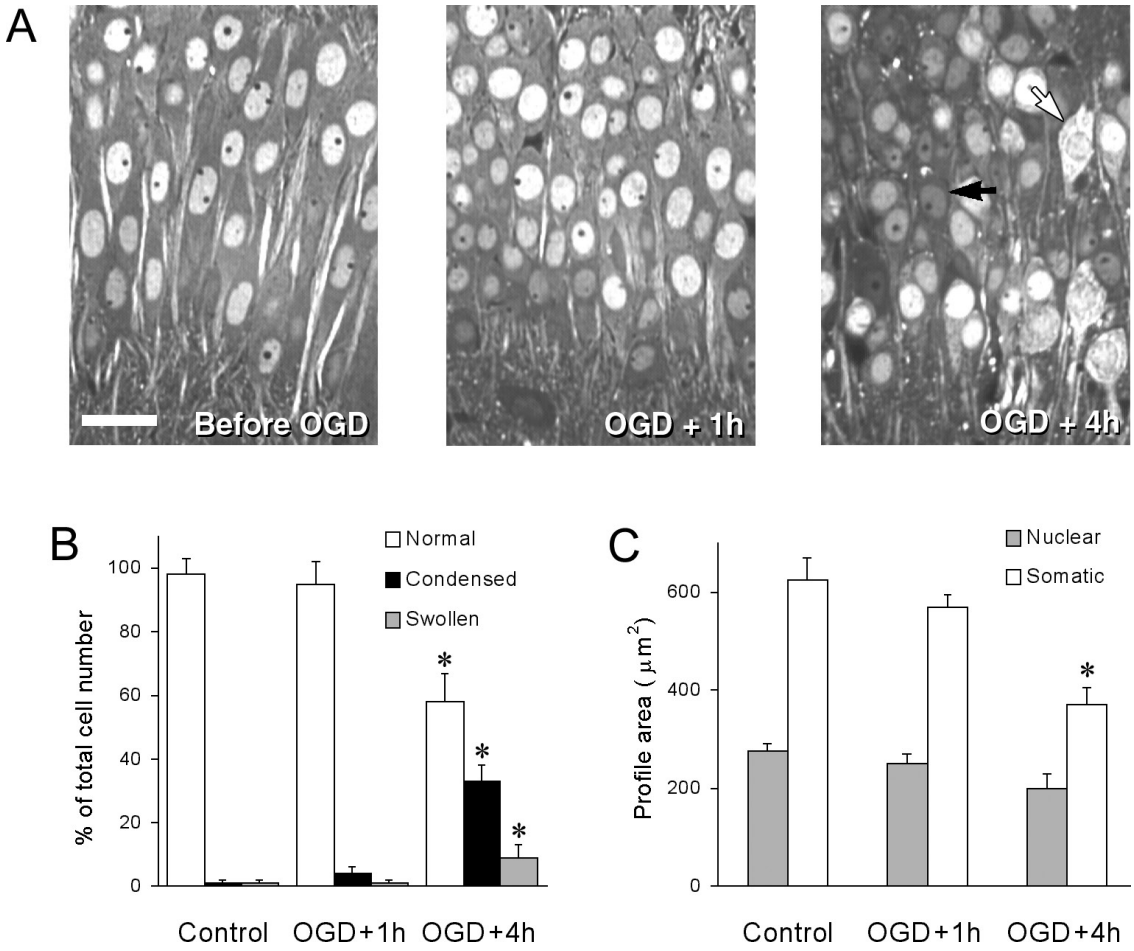

Fig. 3 Effects of 10-min OGD on the morphology of pyramidal cells in the hippocampal slice cultures (epoxy-embedded semithin sections). (**A**) Representative images of area CA1 in hippocampal slices. Scale bar 50 μm. On the right image, note dark shrunken (***black arrow***) and translucent (***white arrow***) pyramidal cells. (**B**) Different types of neuronal cell damage observed within the CA1 area. (**C**) Comparison of the effects of OGD on areas of nuclear and somatic profiles of the CA1 pyramidal cells.

The effects of 10-min OGD were examined in detail. LDH-activity in the culture medium increased significantly only after 4 h of post-OGD culturing. PI fluorescence also showed minor changes suggesting unaltered vulnerability of the cultures during by 1 h of reoxygenation, with the subsequent increase in PI fluorescence of pyramidal cells in the hippocampal CA1 area (Fig.1 A, B). Further, clear increase in the metabolic activity of the slices was determined by MTT/formazan assay at 1 h after 10-min OGD, whereas this parameter decreased by 4 h following the OGD (Fig.2).

Structural changes in CA1 area of hippocampal slice cultures were analyzed by light and electron microscopy. No significant signs of cellular destruction were found in the cultures at 1 h of reoxygenation, but profound neuronal cytoplasm condensation was observed following 4 h of culturing after 10-min OGD. Rarely, swollen pyramidal cells were found in CA1 area (Fig. 3 A,B). Morphometric analysis showed that the cell condensation is mostly associated with an evident decrease in the area of neuronal somatic profile, while nuclear condensation was less significant (Fig. 3C).

Electron microscopic analysis of the cultures subjected to OGD also revealed different degenerative structural changes in the soma of CA1 pyramidal cells within a slice, namely, condensation and swelling. Condensed cells had dark small mitochondria and, in some cases, fragmented nuclei (Fig. 4 B). Swollen neurons were found to have light swollen mitochondria and vacuolated cytoplasm (Fig. 4 C).

Discussion

There are several *in vitro* systems to study cell damage resulting from various neuropathological states, such as acute brain slices, dissociated cell cultures and organotypic slice cultures. The latter offers several advantages since they maintain the properties of the tissue, namely cytoarchitecture, cell types and layers, local neuronal connectivity, synaptic organization, receptor distribution, etc. [9, 13], and is considered to be a convenient model to study cell damage occurring in different neuropathological states, including ischemia.

To simulate events occurring in ischemia, OGD of various durations may be applied. In cultured hippocampal slices at least 30-min OGD has been previously applied to induce ischemic neuronal death [8, 14,15], and up to an hour and more [16, 17, 18, 19, 20]. Such long-term OGD is known to cause significant neuronal damage. Quite brief episodes of OGD are usually used to induce long-term potentiation-like events in CA1 pyramidal neurons of acute hippocampal slices [21, 22] and are not considered to be destructive. Recently, brief transient periods of OGD (up to 10 min) have been shown to cause rapid ultrastructural changes in synaptic apparatus in hippocampal slice cultures [3], presumably presenting morphological substrate for post-ischemic long-term potentiation [23]. Further, short-term episodes of OGD in cultured hippocampal slices are considered to be protective against the following prolonged anoxia [24, 25], and called "ischemic preconditioning". It should be also noted that at least two factors, deprivation and reoxygenation, may cause the tissue response [18, 26, 27].

The time course of the development of cell damage caused by OGD may vary greatly depending on the *in vitro* model used, quality of the cultures, and a multitude of other factors. Systematic studies of the time course of ischemic injury development depending on the duration of ischemic and post-ischemic episodes are not numerous.

A pilot study was undertaken to monitor the time course of injury induced by OGD, both short- and long-term. Based on our data of LDH efflux assay, damage produced in the cultures by OGD used did not differ significantly before 1 h of reoxygenation, while cell injury in the slice cultures was found to increase gradually later and to depend on the duration of the OGD as well as reoxygenation. Among the tested deprivations, 10-min OGD caused minimum and delayed destructive effects, although molecular mechanisms induced by OGD are presumed to have started. Thus we examined in detail the changes occurring in the slice cultures after this short OGD.

Pyramidal cells in CA1 area of hippocampal slice cultures were examined because they are known to be highly vulnerable to damage from hypoxia [1, 28, 29, 30]. Analysis of the cell degeneration using PI fluorescence confirmed that 10-min OGD resulted in minor changes of the vulnerability of the hippocampal CA1 area at 1 h of reoxygenation. Interestingly, clear activation of the cell metabolism level was revealed at 1 h of reoxygenation following 10-min OGD. The MTT/formazan assay was used to estimate metabolic activity of the cultured hippocampal slices.

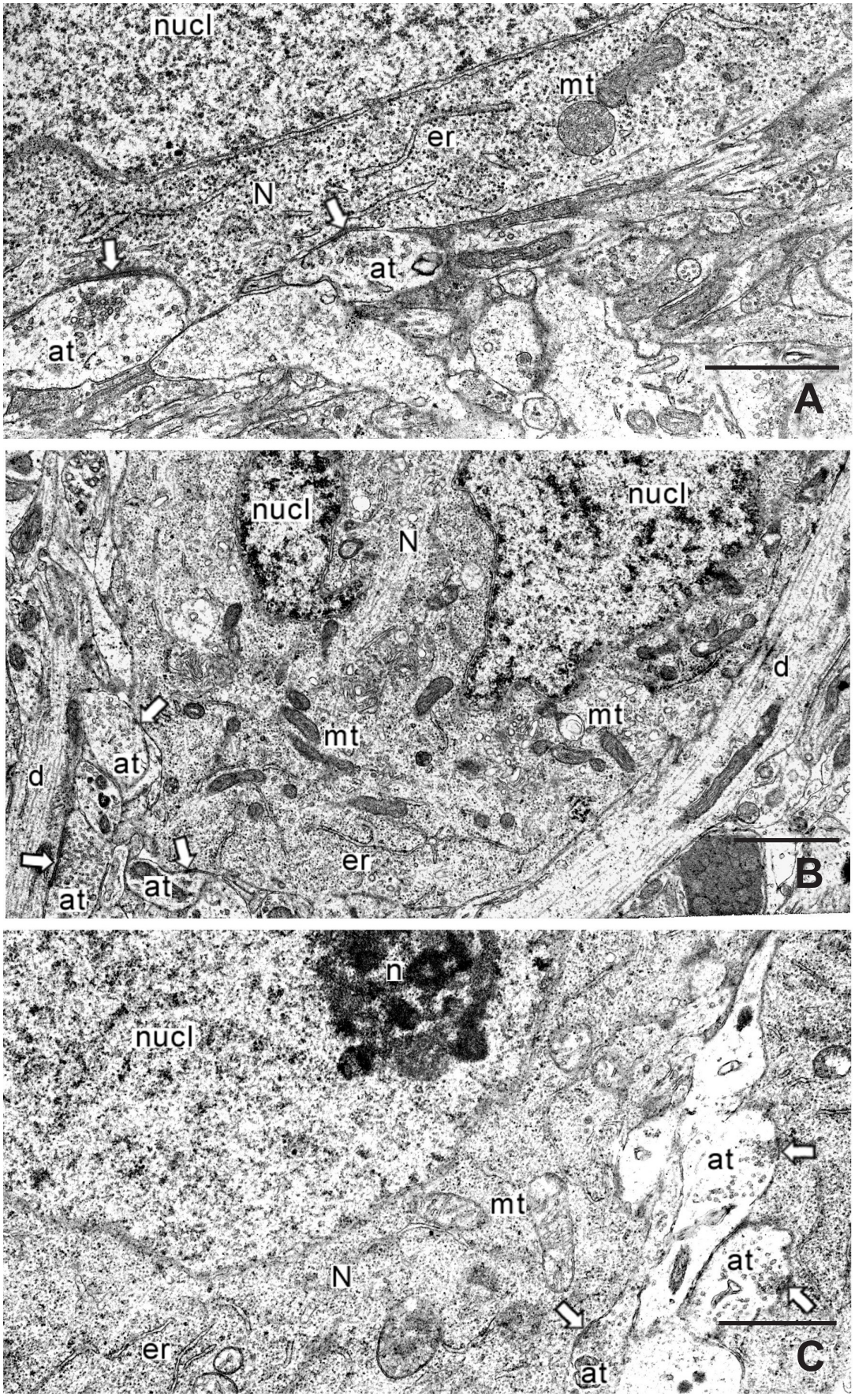
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Fig. 4 Representative electron micrographs of the pyramidal cells in the hippocampal CA1 area of the control slice cultures (**A**) and of the slices subjected to OGD: condensed (**B**), and swollen (**C**) neurons. Scale bar 1 μm. Note: (**B**) dark cytoplasm, shrunken and fragmented nucleus, numerous condensed mitochondria, and (**C**) numerous swollen mitochondria and axonal terminals. Abbreviations: ***N*** - neuronal soma; ***nucl*** - nucleus; ***n*** - nucleolus; ***at*** - axonal terminal; ***d*** - dendritic process; ***mt*** - mitochondria; ***er*** - endoplasmic reticulum; ***arrows*** indicate synaptic contacts.

During the assay, gradual accumulation of the formazan precipitate throughout a whole slice was readily visualized by focusing through the different cell layers. There might be several possible explanations for the phenomenon of the increase of metabolic activity during the first hour after OGD. Cerebral ischemia is known to induce rapid glial activation [31, 32]. Probably, related to the observed effect are the glial cells. On the other hand, as apoptotic death is considered to call for maintained energy level in the cell [33], the observed increase in cell metabolic activity might be caused by apoptosis-like events occurring in the cultures deprived of oxygen and glucose. Furthermore, additional energy is essential for structural remodeling of synaptic network, which has been shown to occur soon after anoxia-hypoglycemia [3]. Presumably, each of these processes can be involved in the increase of the slice metabolic activity at the first hour after OGD.

The cell metabolic activity decreased and PI fluorescence in CA1 pyramidal layer increased after 4 h and more of reoxygenation following 10-min OGD. This was found to correspond to the findings of structural and ultrastructural analyses showing different signs of destruction (presumably, apoptotic and necrotic) in the CA1 pyramidal cells. Our data demonstrated that 4 h of reoxygenation resulted in neuronal damage, which mainly appeared as cellular condensation.

10-min OGD applied to the hippocampal slice cultures, being comparatively mild, is supposed to induce both early adaptive and delayed destructive changes to neurons, and can be useful in studying mechanisms of delayed neuronal cell damage.

Recent evidence suggest that different forms of cell death might contribute to the ischemic neuronal cell death, although there is not a generally accepted view of either the essence or the terms. Some cell death concepts consider that different processes whereby cells die can be referred to as necrosis, apoptosis or others [1, 34, 35]. According to other concepts, the term apoptosis refers to the process followed by dying cells, while the term necrosis is related to the morphological alterations appearing after cell death, *i.e.* necrosis might be resulted from apoptosis [36–38]. We did not specifically aim our study to estimate apoptotic, oncotic, or necrotic changes in the hippocampal slices, but to assess cell damage in terms of viability and morphological alterations induced by OGD. Our data showed the increase in LDH level and PI fluorescence, and appearance of swollen pyramidal cells in CA1 area after OGD followed by reoxygenation, implying plasma membrane damage and probably reflecting oncotic or necrotic changes occurring in cells. Within the same slices, histological and ultrastructural alterations, observed in CA1 pyramidal cells (*e.g.*, cell shrinkage and disintegration, nuclear compaction), presumably indicate apoptotic-like events. These findings suggest that different forms of cell damage might be observed in the reported model simultaneously.

The reported *in vitro* OGD was found to reproduce the effects of short-term ischemia *in vivo* and is to be used in studying different aspects of mild brain ischemia and applied in the further development of neuroprotective tools for the treatment of brain diseases.

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