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Light and Electron Microscopic Observations in Nerve Cell Nucleolar Damage in Human Traumatic and Complicated Brain Injuries*

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Abstract

Objective: To study microscopically the nucleolar alterations induced by severe and complicated traumatic human head injuries using cortical biopsies taken during neurosurgical treatment. Material and methods: 10 cortical biopsies from different cortical regions were study by means of light microscopy, scanning-transmission electron microscopy, and transmission electron microscopy. Results: Pyramidal nerve cells examined with light microscopy thick sections, and scanning-transmission electron microscopy semithin sections exhibited apparent intact nucleolar structure, and ring-shaped nucleolar morphology. Ultrathin sections examined by transmission electron microscopy showed some populations of edematous non-pyramidal neurons exhibiting normal nucleolar substructure with well preserved subcompartments. Another groups of non-pyramidal neurons displayed a nucleolar homogenization process without distinction of nucleolar subcompartments. Other neuronal populations and perivascular astrocytes showed a frank nu-

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cleolar disassembly process. The nucleolar morphological alterations are discussed in relation with the traumatic brain injury, anoxic ischemic conditions of brain parenchyma, oxidative stress, calcium overload, glutamate and hemoglobin excitotoxicity, and caspase activation. Conclusion: The complicated traumatic brain injuries showed neuronal populations with apparent intact nucleolar structures, and other neuronal groups with homogenization, disassembly and fragmentation of nucleolar components.

KEYWORDS: nucleolar damage, nerve cells, brain injury, light microscopy, electron microscopy.

Observaciones de microscopía óptica y electrónica del daño nucleolar en las injurias cerebrales humanas traumáticas complicadas

RESUMEN

Objetivos: Estudiar microscópicamente las alteraciones nucleolares inducidas por traumas cerebrales humanos severos y complicados mediante biopsias corticales tomadas durante intervenciones neuroquirúrgicas. Material y Métodos: 10 biopsias corticales de diferentes regiones cerebrales fueron estudiadas mediante microscopía óptica, microscopía electrónica scanning y microscopía electrónica de transmisión. Resultados: Neuronas piramidales examinadas mediante secciones semifinas para microscopía óptica y para microscopía electrónica scanning mostraron estructuras nucleolares aparentemente intactas y morfología nucleolar en forma de anillo. Las secciones ultrafinas para microscopía electrónica de transmisión mostraron poblaciones de neuronas no-piramidales edematosas y estructuras subnucleolares normales y buena preservación de sus compartimientos. Otro grupo de neuronas no-piramidales mostraron un proceso de homogenización nucleolar sin distinción de los compartimientos subnucleolares. Otros grupos neuronales y astrocitos exhibieron un franco proceso de desensamblaje nucleolar. Las alteraciones nucleolares se discuten en relación con la injuria traumática cerebral, condiciones anóxico isquémicas del parénquima cerebral, stress oxidativo, sobrecarga de calcio, excitoxicidad por glutamato y hemoglobina,y

activación de caspasas. Conclusiones: Las injurias traumáticas cerebrales complicadas mostraron poblaciones neuronales aparentemente normales, otros grupos neuronales exhibieron homogenización, desensamblaje y fragmentación de los componentes nucleolares.

Palabras clave: daño nucleolar, células nerviosas, injuria cerebral, microscopía óptica, microscopía electrónica.

Introduction

Striking recent observations show that the nucleolus and its components are highly dynamic, and that the steady state structure observed by microscopical methods must be interpreted as the product of these dynamics processes (Rasika *et al*., 2006). The dynamics of nucleolar substructures seem to be regulated by cellular stress, apoptosis, senescence, and DNA damage (Zimber *et al*., 2004; Valero *et al*., 2006). Jenkins *et al*. (1981) firstly reported chromatin clumping and nucleolar condensation in ischemic neuronal injury. Daxnerova *et al*. (1995) described ischemia-reperfusion-induced nuclear and nucleolar damage in rabbit dorsal root ganglia neurons, and reported complete fragmentation and segregation of nucleolar subcompartments. Raghupathi *et al*. (1995) examined the molecular (genomic) circuits associated with the pathophysiology of traumatic brain injury, mainly the acute alterations in expression of immediate early genes, heat hock proteins and cytokines, and suggested the trauma-induced activation of multiple signal transduction pathways. Yakovlev *et al*. (2001) observed DNA fragmentation in knockout mice after traumatic brain injury and considered that endonucleases may be essential for chromatin degradation. Mc Keage *et al*. (2001) reported nucleolar damage induced by platinum drug neurotoxicity. Few reports have been published related with the nucleolar changes induced by severe head injuries. In previous papers (Castejón, 2004; Castejón and Arismendi, 2006; Castejón, 2008) we have analyzed the nucleus and nucleolar abnormalities and nerve cell death types in edematous cerebral cortex associated to congenital malformations, vascular anomaly, and brain trauma. In the present paper we report further systematic observations on nucleolar structural alterations in severe and complicated human brain traumatic injuries using correlative microscopy methodology. To the best of our knowledge such

observations have not being reported thus far using correlative microscopy of surgical biopsies of different cortical regions taken during neurosurgical treatment.

1. Material and methods

Cortical biopsies of 10 patients with clinical diagnosis of severe and complicated brain trauma were examined with the light microscope, scanning transmission electron microscope, and transmission electron microscope. The table 1 contains the clinical data and lists the cortical regions from which the cortical biopsies were taken during neurosurgical treatment. The neurosurgical study was performed and the cortical biopsies were taken according to basic principles of Helsinki Declaration. The informed consent of parents and relatives was obtained in each case under study. The research protocol was approved by the Ethical Committee of Biological Research Institute.

Sample processing for transmission electron microscopy (TEM)

Two to five mm thick cortical biopsies were immediately fixed in the surgical room in 4% glutaraldehyde-0.1M phosphate or cacodylate buffer, pH 7.4, at 4°C. After 2 hours glutaraldehyde-fixation period, the cortical biopsies were divided into approximately 1 mm fragments and observed under a stereoscopic microscope to check the quality of fixation of the sample, the glutaraldehyde diffusion rate, and the brownish coloration of the surface and deeper cortical regions, indicative of good glutaraldehyde fixation by immersion technique. The cortical slabs were also performed to assure optimal diffusion rate of glutaraldehyde and osmium tetroxide fixatives. Immersion in fresh glutaraldehyde solution of 1 mm slices was done for 2 hours. Secondary fixation in 1% osmium tetroxide-0.1M phosphate buffer, pH 7.4, was carried out for 1-2 hours at 4°C. Black staining of the cortical slices was also observed under a stereoscopic microscope to check osmium tetroxide diffusion rate and quality of secondary fixation. They were then rinsed for 5 to 10 minutes in phosphate or cacodylate buffer of similar composition to that used in the fixative solution, dehydrated in increasing concentrations of ethanol, and embedded in Araldite or Epon. Ultrathin sections, obtained with Porter-Blum and LKB ultrami-

TABLE 1. Neurosurgical Study

Case No.	Age and Sex	Clinical Data	Diagnosis	Brain edema	Cortical biopsy region	Evolution time of brain Injury
1. JP	14 y, M	Contusion and cave-in-fracture of frontal region, and transitory loss of consciousness	Contusion and cave-in fracture of frontal region.	severe	Left frontal cortex. Focal Region.	1 day
2. HRF	18 y, F	Severe frontal contusion and cave-in fracture in road accident, loss of consciousness and convulsive crisis	Severe frontal contusion	Severe	Left frontal cortex. Focal Region.	8 days
3. JRCR	69 y, M	Falling from his own height, chronic alcoholic patient presented headache, diminution of muscle strength of lower extremities and right arm, temporary loss of consciousness, dysarthria, and anisocoria.	Brain trauma. Left fronto-parieto-occipital subdural hematoma	Severe	Left parietal cortex. Perifocal Region.	16 days
4. JM	58 y, M	Road accident. Patient showing contusion and hematoma of left temporo-parietal region. Clouded sensorium, temporospatial disorientation and left mydriasis.	Brain trauma Left parieto-occipital. Subdural hygroma	Severe	Left parietal cortex. Focal Region.	19 days

TABLE 1. (*Continuation*)

Case No.	Age and Sex	Clinical Data	Diagnosis	Brain edema	Cortical biopsy region	Evolution time of brain Injury
5. OP	60 y, F	Head injury in traffic accident, fracture of both legs, state of coma, abolition of reflexes. Left mydriasis. After recovery showed disorders of behavior. (Post-traumatic confusional syndrome)	Brain trauma. Subdural hygroma	Severe	Right parietal cortex. Focal Region.	25 days
6. ANG	39 y, M	Loss of consciousness after falling from a running truck, headache. Left hemiparesis, and papilledema.	Brain trauma. Right parieto-temporal subdural hematoma	Severe	Right temporal cortex. Focal Region.	8 months
7. LCS	20 y, F	Frontal headache	Brain trauma. Left frontal Subdural hematoma	Severe	Left parietal cortex. Focal Region.	6 days
8. IJA	27 y, F	Patient hit with a stick on a fighting street. Brain trauma. Biparietal fracture. Reintervened by biparietal cranioplastic surgery.	Biparietal trauma.	Severe	Right parietal cortex. Focal Region.	8 months
9. ASCR	6 y, M	Falling from his own height. Skull trauma in right temporo-parietal region, tonic clonic convulsion, and disorders of behavior	Brain contusion. Right parieto-temporal. Subdural hematoma.	Severe	Right temporo-parietal cortex. Focal Region.	7 months
10. PDM.	21 y, M	Falling from a light post, coma, bilateral papilledema.	Brain trauma Right epidural hematoma.	Severe	Right temporal cortex	1 day

crotomes were stained with uranyl acetate and lead citrate and observed in a JEOL 100B transmission electron microscope at magnifications ranging from 36.000 to 60.000X. For each case, approximately 50 electron micrographs were studied. Digitalized images were Photoshop treated.

Light microscopy (LM)

For proper orientation during the electron microscope study and observation of nucleolar morphology, approximately 0.5 to 1μm plastic thick sections of frontal, parietal and temporal cortex were stained with toluidine blue and examined with a Zeiss photomicroscope.

2. Scanning-transmission electron microscopy of semithin sections

Scanning-transmission electron microscopy (STEM), which implies transmission electron microscopy (TEM) performed with a scanned, focused electron beam, allowed us to explore semithin sections of frontal, temporal and parietal cortex in a raster-like form. A fine electron probe was passed across the semithin specimens and the intensity of the transmitted electron signal was measured using one or more electron detectors. An image was then built up point by point, just as in a conventional scanning electron microscope. The obtained images were correlated with optical microscope images, and consecutively compared with ultrathin sections for TEM.

3. Results

Light microscopy

Examination of plastic thick sections of frontal, parietal and temporal cortex showed some populations of pyramidal and non-pyramidal neurons exhibiting apparently intact nucleolar structure (figure 1).

Another groups of nerve cells displayed a nucleolus depicting a ring shape structure (figure 2).

Due to the low resolution power of light microscope we used semithin sections for scanning-transmission electron microscope in order

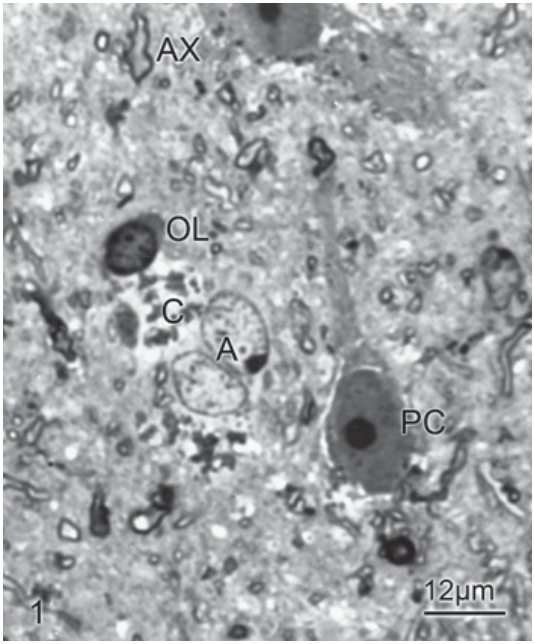

FIGURE 1. Case No. 1. Contusion and fracture of frontal region. Left frontal cortex. Photomicrograph showing a swollen pyramidal nerve cell (PC) exhibiting a round nucleolus. A capillary (C) shows a perivascular dense oligodendrocyte (OL), and swollen astrocytes (A). Note the degenerated myelinated axons (AX).

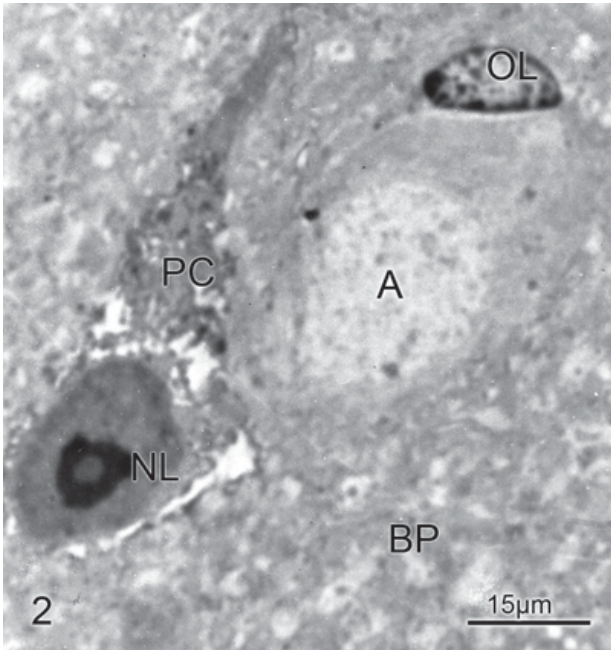

FIGURE 2. Case No. 3. Brain trauma. Left fronto-parieto-occipital hematoma. Photomicrograph showing a swollen and vacuolated pyramidal nerve cell (PC) exhibiting a ring-shaped nucleoulus (NL). A swollen astrocyte (A), and an oligodendroglial cell (OL) also are seen. Note the status spongiosus of brain parenchyma (BP).

to obtain further details of nucleolar components. However, a similar image to that obtained with the light microscopy was observed (Figure 3), revealing that both microscopical techniques lack the adequate resolution to study nucleolar component alterations.

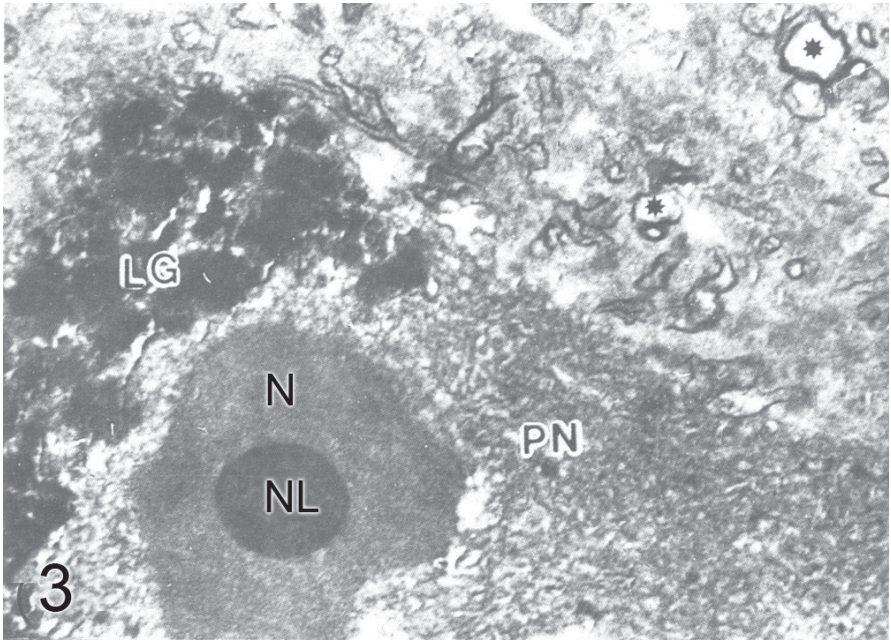

FIGURE 3. Case No. 2. Severe frontal contusion. Left frontal cortex. Scanning-transmission electron micrograph of a semithin section showing an edematous pyramidal nerve cell exhibiting at the nucleus (N) an intact round and electron dense nucleolus (NL). Note the heavy accumulation of lipofucsin granules (LG), and the swollen and degenerated myelinated axons (asterisks).

The examination of ultrathin sections with conventional transmission electron microscope showed some edematous pyramidal and non-pyramidal neurons with an apparently normal nucleolar structure and well preserved subcompartments, being the nucleolar dense fibrillar and granular components, and the fibrillary centers clearly distinguished (Figure 4).

Some populations of swollen and vacuolated non-pyramidal neurons showed a nucleolar homogenization process, in which the nucleolar substructures were not distinguished (Figure 5).

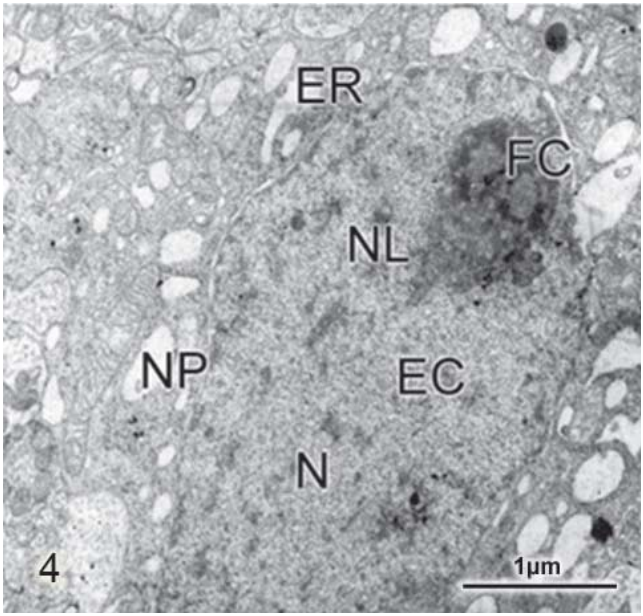

FIGURE 4. Case No. 6. Brain trauma. Subdural haematoma. Right parietal cortex. Transmission electron micrograph of an edematous non-pyramidal neuron (NP) showing at the nuclear level (N) an irregularly outlined and skrunken nucleolus (NL), in which two fibrillary centers (FC) appear surrounded by the dense fibrillary components. The euchromatin (EC) appears less electron dense. The cytoplasm exhibits a vacuolated endoplasmic reticulum (ER).

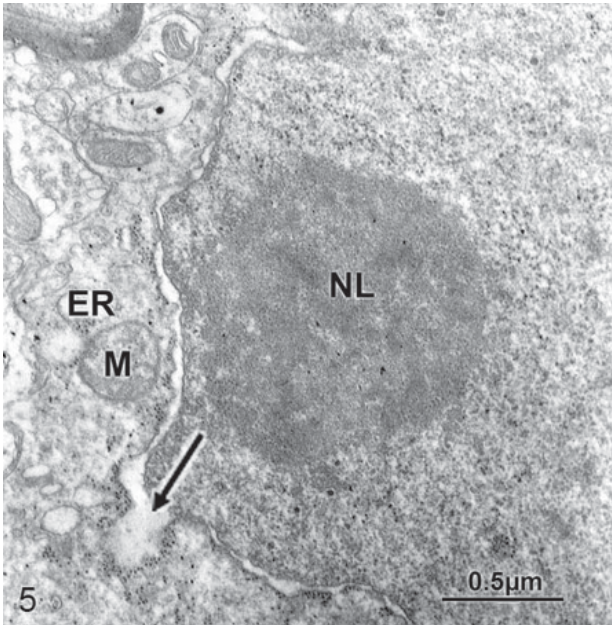

FIGURE 5. Case No. 3. Brain trauma. Left fronto-parieto-occipital subdural hematoma. Left parietal cortex. Edematous non-pyramidal neuron showing a nucleolar (NL) homogenization process in which the nucleolar subcompartments are not distinguished. Note the dilated nuclear envelope (arrow), the swollen mitochondria (M), and endoplasmic reticulum (ER).

Another groups of non-pyramidal neurons displayed a nucleolar disassembly process, in which the compact structure of nucleolus began to disaggregate (Figures 6 and 7).

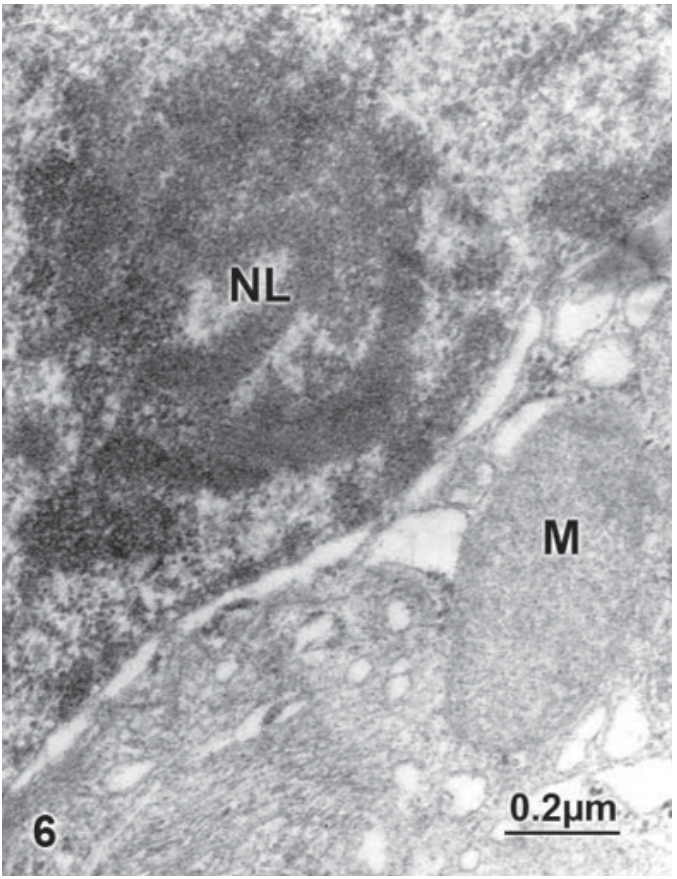

FIGURE 6. Case No. 10. Brain trauma. Right epidural hematoma. Right temporal cortex. edematous non-pyramidal neuron showing an irregularly outlined nucleolus (NL), in which the nucleolar subcompartments are not clearly distinguished. Note the degenerated mitochondria (M).

Some swollen perivascular astrocytes and oligodendroglial cells also exhibited a fragmented nucleolus exhibiting an irregularly outlined dense granular and fibrillar components, and absence of fibrillary centers (Figures 8 and 9).

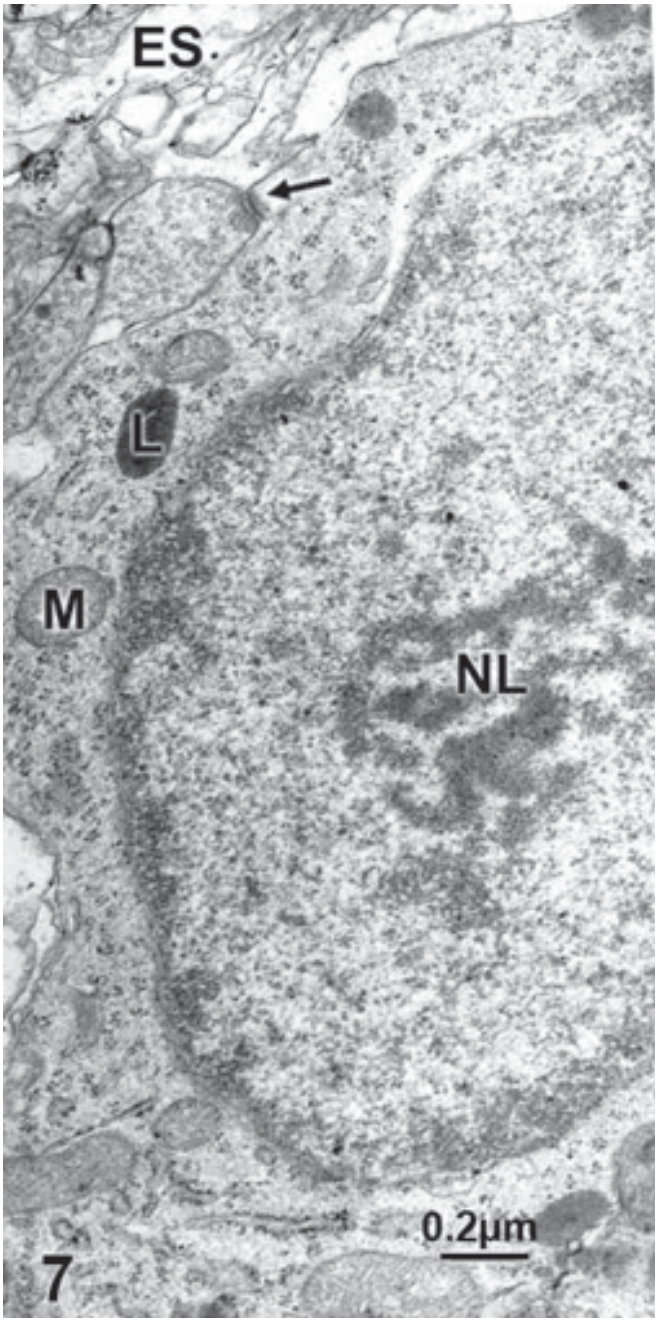

FIGURE 7. Case No. 1. Brain trauma. Frontal contusion. Left frontal cortex. Edematous non-pyramidal neuron exhibiting a nucleolar (NL) disassembly process. The swollen mitochondria (M), a dense lysosome (L), an axospinodendritic contact (arrow), and the neighboring enlarged extracellular space (ES) also are seen.

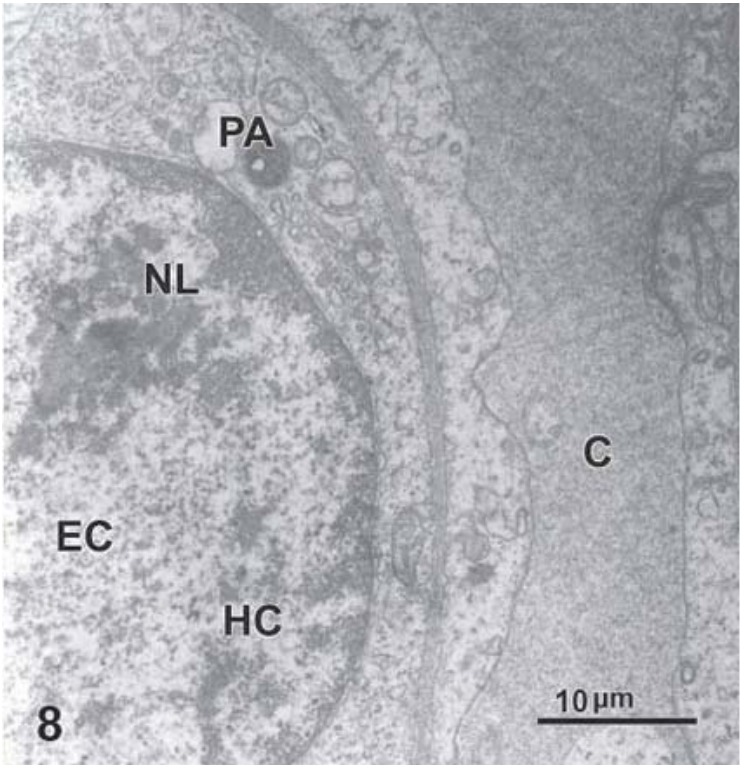

FIGURE 8. Case No. 9. Brain trauma. Right parieto-temporal subdural hematoma. Right temporal cortex. Perivascular astrocyte (PA) applied to the basement membrane of a cortical capillary (C) showing a disassembly process featured by an irregularly outlined nucleolus (NL) with a distorted arrangement of granular and fibrillar dense components.

4. Discussion

In the present paper we have shown some populations of pyramidal and non-pyramidal neurons exhibiting at light microscopy and scanning-transmission electron microscopy levels intact nucleolar morphology. Besides, at transmission electron microscopy level the nucleolar substructures, such as the nucleolar dense fibrillar and granular components, and the fibrillary centers could be clearly distinguished, suggesting that in spite of severity of brain traumatic injury and the degree of brain edema the nucleolar substructures are apparently unaltered. The nucleolar components constitute sites of transcription of ribosomes, and the dense fibrillar com-

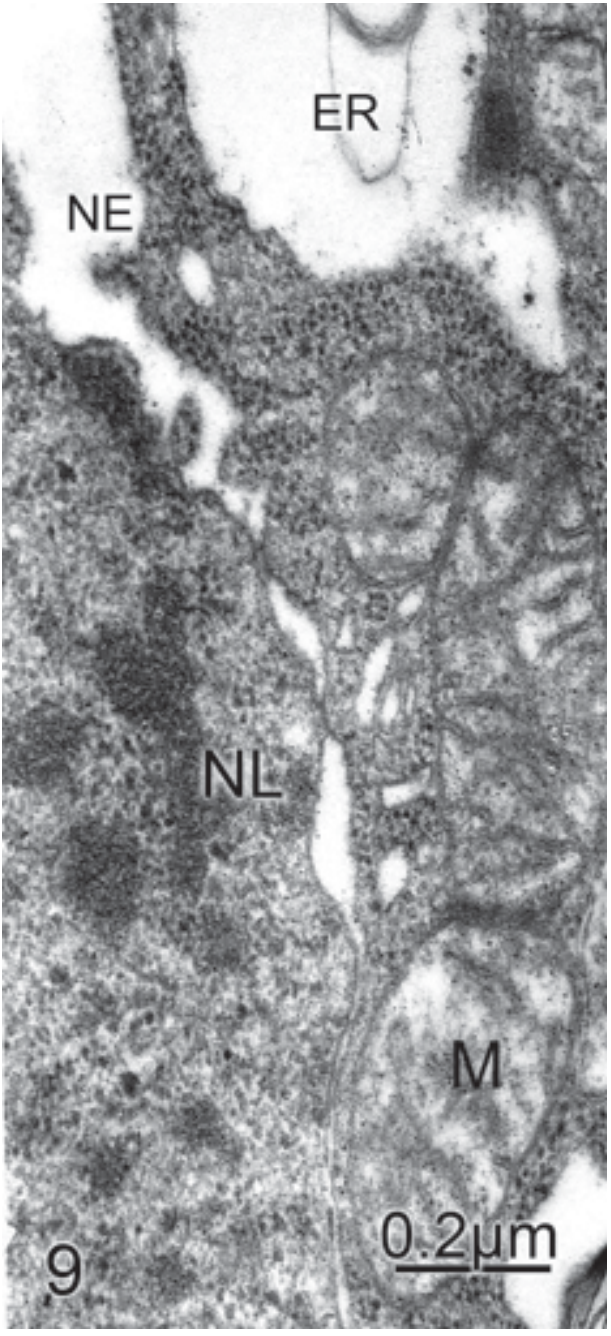

FIGURE 9. Case No. 10. brain trauma. Right epidural haematoma. Right temporal cortex. Swollen oligodendroglial cell showing a fragmented nucleolus (NL). Note the swollen mitochondria, and the distended nuclear envelope (NE) and endoplasmic reticulum (ER).

ponent has been related with transcription of rDNA, and the subsequent early steps of ribosome biosynthesis (Trentani *et al*., 2003; Schwarzacher and Wachtler, 1993). In the different cortical biopsies studied in our laboratory we also found nucleolar fragmentation as previously reported in premature aging process (Comal, I. (1999), in rat perinatal asphyxia (Kastner *et al*., 2003 Kastner), and ischemic dorsal root ganglia neurons (Daxnerova *et al*., 1995). The nucleolar alterations herein described in severe and complicated traumatic brain edema seem to be primarily due to the physical intensity of traumatic brain injury, and secondarily to the associated anoxic-ischemic conditions of brain parenchyma (Castejón *et al*., 2001). Nucleolar alterations following ischemia have been reported and in complete cerebral ischemia [Jenkins *et al*.,1981), and in rabbit lumbosacral ganglionic neurons (Daxnerova *et al*., 1995). Nucleolar alterations have also been reported in inflammatory injury of peripheral nerve endings (Navacues *et al*., 2004).

The nucleolar disassembly and fragmentation processes seem to be related with the nerve cell death types observed in the human traumatic edematous cerebral cortex: apoptosis, the continuum oncotic-apoptotic process, and necrosis. This latter conceptualized as a postmortal stage (Castejón and Arismendi, 2006). The nucleolus may undergo irreversible disassembly in apoptosis, thus allowing redistribution of nucleolar proteins, which may be cleaved or be extruded into the cytoplasm culminating in the formation of apoptotic blebs containing different nucleolar proteins (Soldani *et al*., 2006)

The following biochemical abnormalities are presumably involved in nucleolar alterations, such as oxidative stress and peroxidative damage (Traystman, *et al*., 1991; Evans, 1993; Ginsberg *et al*., 1998; Annunziato *et al*., 2003), increased level of intracellular calcium (Paschen, 2000; Morley *et al*,1999), glutamate excitotoxicity (Nicholls *et al*., 1999; Paschen, 1996), and caspase activation (Karamaris *et al*., 2000; Annunziato *et al*., 2003).

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