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Pathology of nerve cell membranes in complicated and severe human brain traumatic injuries. An electron microscopic study using cortical biopsies.

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ABSTRACT

Cortical biopsies of ten patients with severe and complicated brain traumatic injuries were examined with transmission electron microscope to study the ultrastructural damage of nerve cell membranes. The non-pyramidal neurons, astrocytes and oligodendrocytes showed plasma membrane fragmentation and areas of focal necrosis, enlargement and degranulation of rough and smooth endoplasmic reticulum cisterns, Golgi complex membrane fragmentation, and irregular dilation and disassembly of nuclear envelope. The degenerated myelinated axons showed invaginations and fragmentation of axolemmal membrane, and myelin sheath vacuolization. Synaptic disassembly and disruption and disassembly of interastrocytary gap junctions were also found. Disruption of neuronal Ca^{2+} homeostasis, activation of phospholipases and calpain, peroxidative stress, hemoglobin cytotoxicity, glutamate cytotoxicity, and ischemia of brain parenchyma are discussed in relation with the nerve cell membrane damage.

KEYWORDS: nerve cell membranes, brain trauma, electron microscopy.

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Introduction

Tissue surrounding hematomas, traumatic lesions, infective zones, and certain tumors undergo autocatalytic peroxidation (Cohadon, 1984), a lipidic disorder which greatly altered membrane functions. An interaction between lipid peroxidation and calcium was earlier formulated by Brauglher et al. (1985) in the pathogenesis of neuronal injury. Membrane damage during situations of acute or subacute cerebral aggression has been studied using experimental models (Cohadon, 1984; Saatman et al., 1996; Homayoun et al., 1997; Paschen and Doutheil, 1999; Hu et al., 2000; Mengesdorf et al., 2002). Castejón (1995) describes the synaptic degeneration in traumatic human brain edema. Povlishock (1986, 1992 a, b) found axolemmal damage in traumatically induced axonal damage. Glial membrane damage of glial axonal junction after diffuse axonal injury was reported by Maxwell et al. (1988). Traumatic brain injuries produce damage to nodal axolemma (Genarelli et al., 1993), and a widespread derangement to the neuronal cytoskeleton (Castejón, 1985; Hayes et al., 1995, Castejón and Acurero, 2004), delayed phospholipids degradation (Homayoun et al., 1997), and calpain-mediated spectrin breakdown (Saatman et al., 1996, 2003), leading to ischemic neuronal death. Glutamate excitotoxicity-induced damage of plasma membrane occurs in transient global cerebral ischemia and in traumatic brain injuries (Paschen, 1996).

The most obvious structural membrane damage, which is identified post-mortem by neuropathologists, may not be the most reliable alteration with regard to clinico-pathological alteration (Pat and Brodhun, 1999). Therefore, we have used human cortical biopsies taken during the neurosurgical treatment and immediately processed for transmission electron microscopy in the surgical room for obtaining well preserved brain material, and a detailed knowledge of ultrastructural damage of nerve cell membranes.

Torp et al. (2000) described association of fibrillar beta amyloid with neuronal membrane surface in aged dog brains. Similar findings were also reported by Yamaguchi et al. (2000) in hereditary cerebral hemorrhage with amyloidosis-Dutch type, Alzheimer disease, and non-demented aged subjects. Haik et al. (2000) demonstrated that the putative transmembrane domains of prion protein induce neurotoxicity, and destabilize nerve cell membranes. Castejón and Castejón (2000) described the oligodendroglial cell changes in traumatic human brain edema.

Glutamate excitotoxicity-induced damage of plasma membrane occurs in transient global cerebral ischemia (Paschen, 1996), and in traumatic brain injuries. Peroxidative damage to cell membranes has been found following cerebral ischemia (Ginsberg et al., 1988; Traystman et al., 1991; Choi, 1993; Evans, 1993, Boldyrev et al., 2000, Pashen et al., 2001). Transient global cerebral ischemia triggers suppression of protein synthesis, a process controlled by endoplasmic reticulum function (Paschen and Doutheil, 1999; Paschen and Frandsen, 2001; Mengesdorf et al., 2002, Paschen, 2003).

Traumatic brain injuries involve direct mechanical damage, which may be aggravated by secondary insults such as ischemia (Engel et al., 2005). Membrane damage has been postulated as critical factor in mediating axonal degeneration and nerve cell death (Shi, 2004, Castejón and Acurero, 2004)

Protein aggregation analyzed by electron microscopy and laser-scanning confocal microscopy has been reported after focal brain ischemia (Hu et al., 2000). It has been suggested that nitric oxide may contribute to ischemia-induced cell injury acting upon endoplasmic reticulum, calcium homeostasis, protein synthesis, and energy metabolism (Doutheil et al., 2000).

Damaged of nerve cell plasma membranes, cytoskeleton, rough and smooth endoplasmic reticulum membranes, lysosomal limiting

membrane, and outer and inner mitochondrial membranes have been reported by Castejón and Castejón (2004), Castejón (2004), Castejón and Arismendi, and Castejón and Acurero (2004) in moderate and severe edema associated to congenital hydrocephalus, brain trauma and brain tumors. Recent studies have suggested that cholesterol, an important component of membranes that controls their physical properties and functions, plays a critical role in neurodegenerative diseases. Enrichment of neuronal plasma membrane with cholesterol protects cortical neurons from apoptosis induced by soluble oligomers of the Abeta (1-40) peptide. Conversely, cholesterol depletion renders cells more vulnerable to the cytotoxic effects of the Abeta soluble oligomers (Sponne et al., 2004). The binding of Abeta to membrane lipids facilitates Abeta fibrillation, which in turn disturbs the structure and function of membranes, such as membrane fluidity or the formation of ion channels (Verdier et al., 2004).

Recent reports also indicate that dysfunction of endoplasmic reticulum, which not only mediates proteins processing, but also regulates intracellular calcium homeostasis and cell death signal activation, occurs at an early stage after ischemia, and might be the initial step of apoptotic cascades in neurons (Hayashi and Abe, 2004). Singleton et al. (2002) and Singleton and Povlishock (2004) reported plasma membrane disruption in diffuse brain injury. Luo and Shi (2004) have found that acrolein, a byproduct of oxidative stress and lipid peroxidation, inflicts severe axolemmal disruption. The membrane damage is likely mediated by reactive oxygen species and lipid peroxidation, which are elevated after acrolein exposure. Shi (2004) has also reported axolemmal disruption in guinea pig spinal cord following compression. Kurnellas et al. (2005) have described plasma membrane calcium ATPase deficiency in multiple sclerosis and spinal cord injury, as a potential mechanism of neurodegeneration. Farkas et al. (2006) demonstrated mecanoporation or disruption of neuronal plasma membrane induced by diffuse traumatic brain injury. Thompson et al. (2006) described opening of neuronal gap junction hemichannels following ischemia after stroke. Yi et al. (2006)

have found after traumatic brain injury an increase in complexing I and complexing II, considered respectively markers of inhibitory and excitatory synapses. Sokka et al. (2007) reported kainic acid- induced disintegration of endoplasmic reticulum in hippocampal neurons. Tang et al. (2010) showed significant increase of AQP4 expression in astrocytic membranes following Intracerebral hemorrhage in AQP4(+ / +) mice. AQP4 deletion aggravated neurological deficits and brain edema contents of whole hemorrhagic ipsilateral hemisphere.

Sharma et al. (2011) reported at the ultrastructural level, perivascular edema together with neuronal, glial and endothelia cell damages is frequent in the brain areas showing albumin leakage. Damage to both pre- and post-synaptic membrane and myelinated axons is also common. Cullen et al. (2011) observed increased membrane permeability in a sub-population of cells in culture immediately upon deformation. Alterations in cell membrane permeability, however, were transient and biphasic over the ensuing hour post-insult, suggesting initial membrane damage and rapid repair, followed by a phase of secondary membrane degradation.

More recently, Harris et al. (2012) found altered cellular metabolic status after traumatic brain injury (TBI), with specific compounds proposed to reflect edema, excitotoxicity, neuronal and glial integrity, mitochondrial status and bioenergetics, oxidative stress, inflammation, and cell membrane disruption.

In the present paper we analyze the ultrastructural alteration of nerve cell plasma membrane, cytomembranes, synaptic membranes, and interastrocytary gap junctions in cortical biopsies of patients with complicated brain traumatic injuries. The brain parenchyma of these patients exhibits moderate and severe edema and sustained anoxic-ischemic conditions (Castejón et al., 2001).

1. Material and Methods

Samples of cerebral cortex of ten patients with traumatic complicated head injury were used in the present study. Cortical biopsy was performed during surgery according to the basic principles of the Helsinki Declaration. Clinical data, diagnosis, biopsy region and degree of brain edema appear listed in Table No. 1. Two to five mm thick cortical biopsies were immediately fixed at the surgical room in 4% glutaraldehyde-0.1M phosphate or cacodylate buffer, pH 7.4 at 4°C. Later, they were divided into 1mm fragments and immersed in a fresh, similar solution for periods varying from 2-72h, followed by secondary fixation in 1% osmium tetroxide-0.1M phosphate buffer, pH 7.4 for 1h. They were then rinsed 5 to 10 min in a buffer similar to that used in the fixative solution, dehydrated in increasing concentrations of ethanol and embedded in Araldite or Epon. For light microscopy thick sections of approximately 0.1 to 1 μ were stained with toluidine blue and examined with a Zeiss photomicroscope. Ultrathin sections obtained with a Porter-Blum and LKB ultramicrotomes, were stained with uranyl acetate and lead citrate and examined in a JEOL 100B electron microscope. Observations were made using intermediate magnifications ranging from 30-90.000 X.

Table 1. Neurosurgical Study

Case No.	Age and Sex	Clinical Data	Diagnosis	Edema	Cortical Biopsy and Site of Injury	Evolution Time of Brain Injury
1.JP (CCG29)	14 y, M	Contusion and cave-in-fracture of frontal region, transitory loss of consciousness	Contusion and cave-in fracture of frontal region.	severe	Left frontal cortex. Focal Region.	1 day

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2.HRF (CCH17)	18 y, F	Severe frontal contusion cave-in fracture in road accident, loss of consciousness. Convulsive crisis.	Severe frontal contusion	Severe	Left frontal cortex. Focal Region.	8 days
3.JRCR (CCH31)	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, anisocoria.	Brain trauma. Left frontoparietal occipital subdural hematoma	Severe	Left parietal cortex. Focal and Perifocal Regions.	16 days
4.JM (CCH21)	58 y, M	Road accident. Patient showing contusion and hematoma of left temporo-parietal region. Clouded sensorium, temporospatial disorientation. Left mydriasis.	Brain trauma Left parietooccipital subdural hygroma	Severe	Left parieto temporal cortex. Focal Region.	19 days
5 . OP (CCH30)	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 (CCH18)	39 y, M	Loss of consciousness after falling from a running truck, headache. Left hemiparesis, papilledema.	Brain trauma. Right parieto temporal subdural hematoma	Severe	Right temporo parietal cortex. Focal Region.	8 months
7. LCS (CCH64)	20 y, F	Frontal headache	Brain trauma. Left frontal Subdural hematoma	Severe	Left parietal cortex. Focal Region.	6 days

8. IJA CCH61	27 y, F	Patient hit with a stick on a fighting street. Brain trauma. Biparietal fracture. Reintervenied by biparietal craneoplastic surgery.	Biparietal trauma.	Severe	Right parietal cortex. Focal Region.	8 months
9. ASCR (CCH22)	26 y, M	Falling from his own height. Skull trauma in right temporo-parietal region, tonic clonic convulsion, and disorders of behavior	Brain trauma. Right parieto temporal. Subdural hematoma. Brain contusion.	Severe	Right temporo parietal cortex. Focal Region.	7 months
10. PDM. (CCH27)	21 y, M	Falling from a light post, coma, bilateral papilledema.	Brain trauma Right epidural hematoma.	Severe	Right temporal Cortex. Focal Region.	1 day

2. Results

In severe edema of traumatic brain injuries complicated with subdural hematoma, the non-pyramidal neurons, astrocytes and oligodendrocytes showed plasma membrane fragmentation and areas of focal necrosis, enlargement and degranulation of rough and smooth endoplasmic reticulum cisterns, Golgi complex membrane fragmentation, and irregular dilation and disassembly of nuclear envelope. The degenerated myelinated axons show invaginations of the axolemmal membrane and formation of endocytic vesicles. The myelin membranes appear separated forming large intraperiod vacuoles. (Figs.1 to 5).

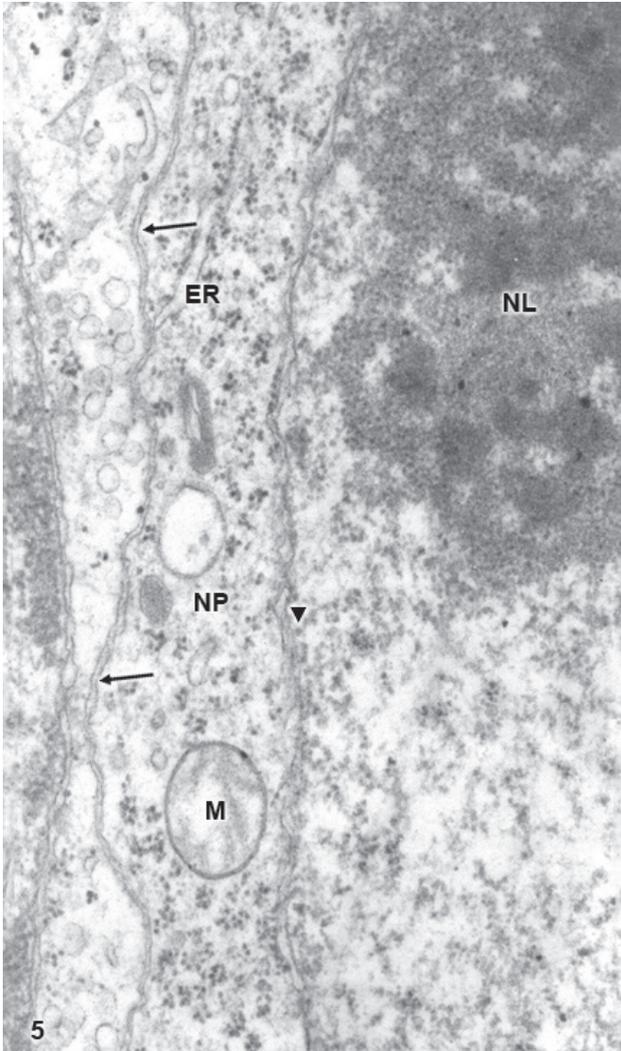


FIGURA 1. Brain trauma. Left frontal hematoma. Non-pyramidal neuron in an area of moderate perifocal edema bearing a continuous plasma membrane (arrows), a non dilated endoplasmic reticulum cistern (ER) and nuclear envelope (arrowhead), and a swollen mitochondrion (M). Note the well preserved nucleolar substructures (NL). X 30.000.

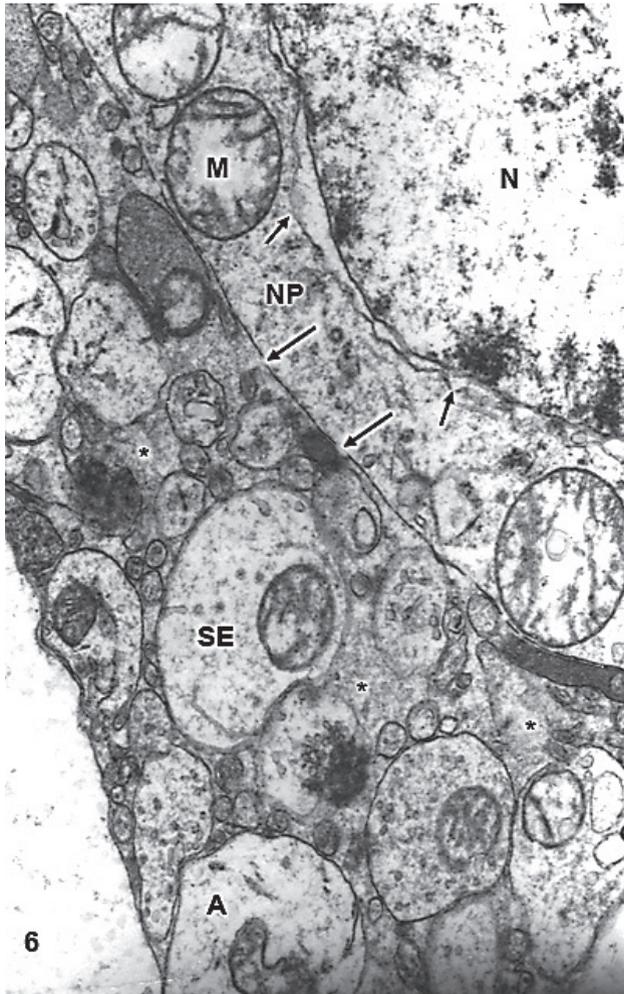


FIGURA 2. Brain trauma. Subdural hematoma. Left parietal cortex. Severe edema. Non-pyramidal neuron (NP) displaying disrupted plasma membrane (long arrows), irregularly dilated nuclear envelope (short arrows), and swollen mitochondria (M). In the neighboring neuropil, the asterisks label the enlarged extracellular space containing proteinaceous edema fluid, which separates degenerated synaptic endings (SE), and swollen astrocytic processes (A). Note the swollen nucleoplasm (N). X. 30.000.

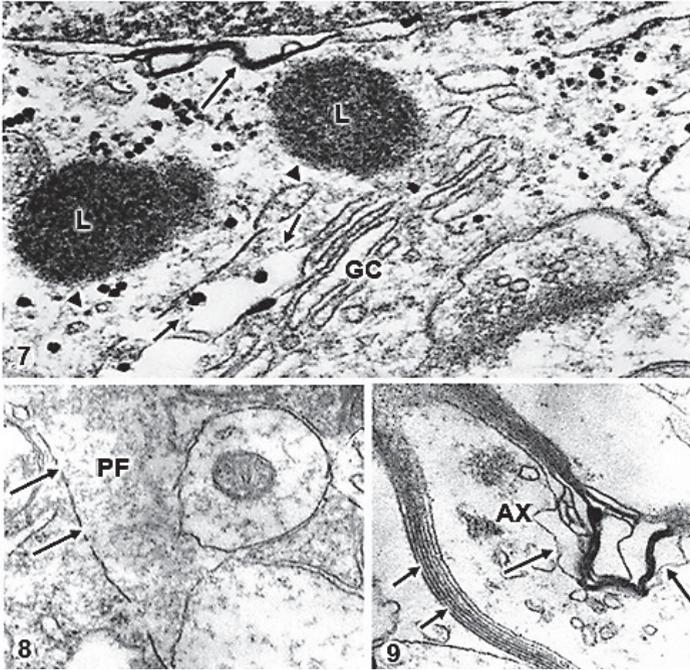


FIGURA 3. Brain trauma. Subdural hematoma. Right parietal cortex. Non-pyramidal neuron showing focal necrosis of nuclear envelope (long arrow) and of smooth Golgi complex (GC) membranes (short arrows). The lysosomes (L) show a discontinuous globular limiting membrane (arrowheads). X 75.000.

FIGURA 4. Severe frontal contusion. Left frontal cortex. Non-pyramidal neuron showing a high electron dense and fragmented plasma membrane (arrows) in contact with the proteinaceous edema fluid (PF) occupying the enlarged extracellular space. X 60.000.

FIGURA 5. Brain trauma. Right parieto-temporal hematoma. Right parietal cortex. Severely edematous neuropil showing a degenerated myelinated axon (AX). The myelin sheath lamellar arrangement appears disrupted and forming intramyelinic vacuoles (long arrows). Note the formation of axolemmal endocytic vesicles (arrowheads), and the apparently normal and compact arrangement of a segment of myelin sheath at the opposite side (short arrows). X 60.000.

The swollen and clear astrocytes display marked degenerative changes induced by brain edema in comparison with those exhibited by neurons. Areas of focal necrosis and fragmented limiting plasma membrane, over-distended rough endoplasmic reticulum cisterns with extended degranulated areas, and vacuoles of smooth endoplasmic reticulum with necrotic limiting membrane are found (Fig. 6).

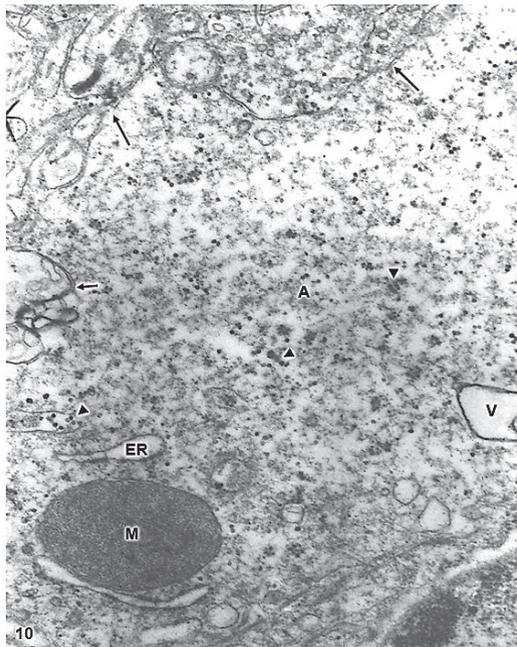


FIGURA 6. Brain trauma. Swollen and clear astrocyte cell (A) depicting fragmented plasma membrane (long arrows) and areas of focal necrosis (short arrow), degranulated and dilated rough endoplasmic reticulum cisterns (ER), glycogen granules (arrowheads), and a vacuole (V) limited by necrotic membrane. Note the degenerated and dense mitochondrion (M). X. 60.000.

Oligodendroglial cells also show marked edematous changes featured by lacunar enlargement of rough endoplasmic reticulum and nuclear envelope, detachment of membrane bound ribosomes, and discontinuous plasma membrane (Fig. 7).

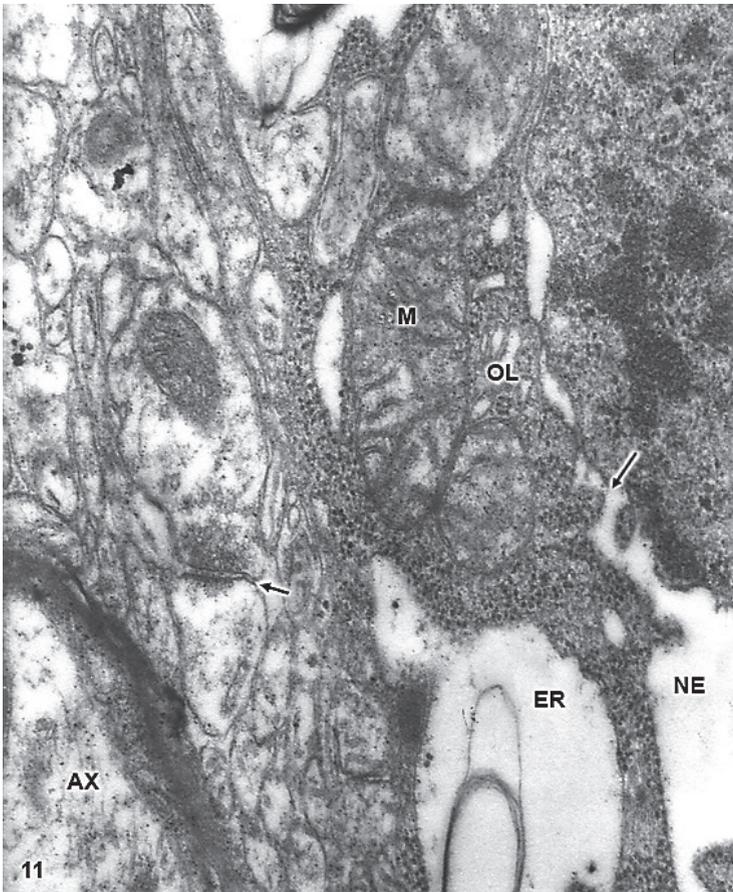


FIGURA 7. Brain trauma. Hydropic oligodendrocyte (OL) showing over-distended perinuclear (PN) and endoplasmic reticulum (ER) cisterns, disassembly of nuclear envelope (NE), and nuclear pores (long arrow). Note the swollen mitochondria (M). The neighboring neuropil exhibits degenerated axodendritic synapse (short arrow) and myelinated axon (AX). X 60.000.

In severe edematous regions of complicated traumatic brain injuries, synaptic disassembly occurs featured by wide separation of pre- and post synaptic membranes and lost of perisynaptic astrocytic glial ensheathment (Fig. 8).



FIGURA 8. Brain trauma. Subdural hematoma. Left parietal cortex. Severely edematous neuropil showing synaptic disassembly and disappearance of pre- and postsynaptic densities (long arrow) of an axospinodendritic contact. The short arrow labels another spine synapse exhibiting partial vestiges of the postsynaptic density, and irregular profile of presynaptic membrane (arrowhead). Note the expansion of extracellular space (asterisks), and the detachment of perisynaptic astrocytic cytoplasm (A). X. 60.000.

2. The damage of interastrocytary gap junctions

In severe traumatic brain injuries, astrocytic gap junction disruption and disassembly are observed. Gap junction disassembly is characterized by wide separation of astrocytic end- feet confronted membranes (Fig. 9).

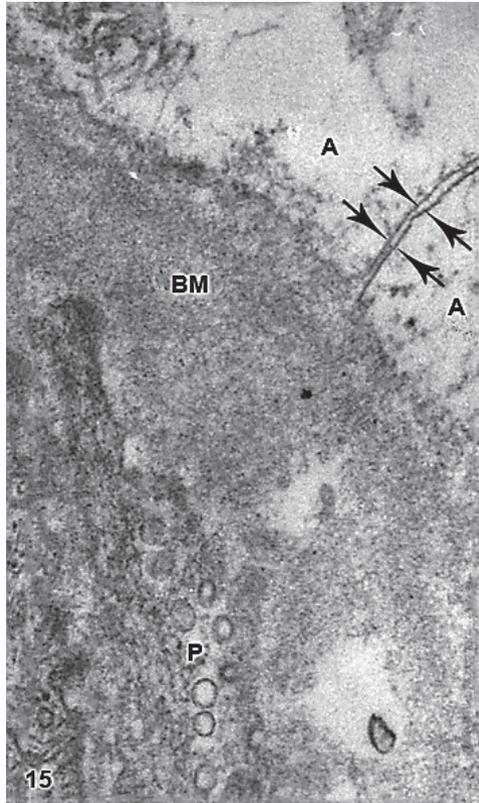


FIGURA. 9. Brain trauma. Subdural parieto-temporal hematoma. Left parietal cortex. Severe edematous region showing disassembly of interastrocytic gap junctions featured by wide separation of confronted astrocytic (A). end-feet membranes (arrows) Note the swollen and vacuolated basement membrane (BM), and the increased vesicular transport in the pericytal cytoplasm (P). X 90.000.

3.The changes of endothelial cell luminal membrane of cerebral capillaries

In severe and complicated traumatic brain injuries, the endothelial cell luminal membrane of brain capillaries undergo profound

activity changes that characterize the increased cerebrovascular permeability. Such changes are increased formation of micro- and macropinocytotic vesicles and clathrin-coated vesicles, deep invaginations and formation of incomplete transendothelial channels, and emission of pseudopods to form endothelial vacuoles (Fig.10).

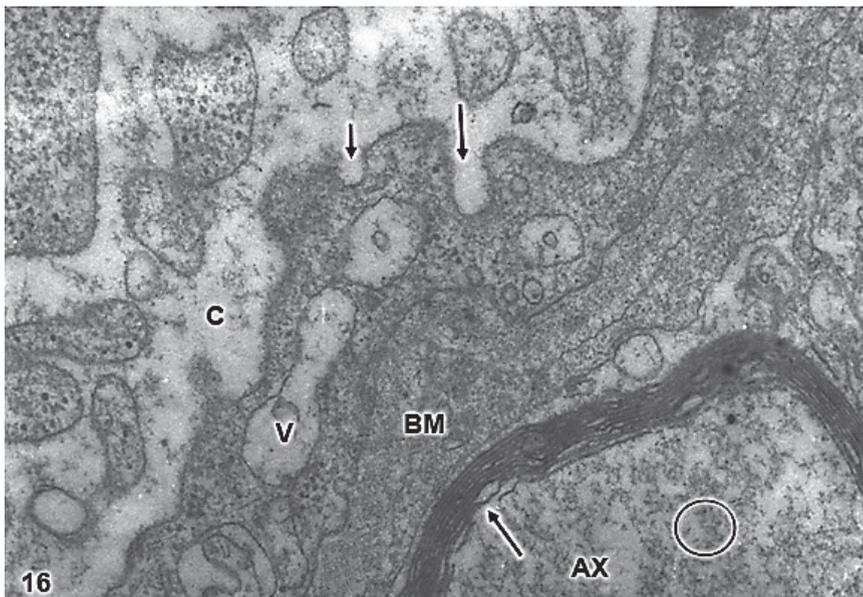


FIGURA 10. Brain trauma. Right epidural hematoma. Right temporal cortex. Capillary (C) showing the increased activity of endothelial cell luminal membrane, which exhibits the formation of pinocytotic vesicle (short arrow), deep invagination (long arrow), and formation of endothelial vacuoles (V). Note the swollen basement membrane (BM), and the degenerated myelinated axons (AX) in the neighboring neuropil. The arrow indicates the disrupted axolemmal membrane, and the circle the granular degeneration of neurofilaments. X 36.000.

4. Discussion

The present paper reports the fragmentation of nerve cell plasma membrane, enlargement and degranulation of rough endoplasmic reticulum, fragmentation of Golgi complex membranes, disassembly of nuclear envelope, damage of myelin sheath membranes, and synaptic disassembly.

Traumatic damage of axolemmal membrane was earlier reported by Castejón (1985), Genarelli et al. (1993), Erb and Povlishock (1998), and Maxwell et al. (1999). Singleton and Povlishock (2004). More recently Farkas et al. (2006) and Castejón (2008) also demonstrated disruption of neuronal somatic plasmalemma in diffuse traumatic brain injury, and in severe and complicated traumatic brain injuries.

4.1. Endoplasmic reticulum dysfunction

The endoplasmic reticulum is a subcellular compartment playing a fundamental role in the folding and processing of newly synthesized membranes and secretory processes, reactions which are strictly calcium/dependent (Lodish and Kong, 1990). Severe disruption of neuronal Ca^{2+} homeostasis leading to a lethal Ca^{2+} overload as occurs in brain ischemia, can initiate a cascade of destructive processes which lead to the death of neurons during cerebral ischemia (Morley et al., 1999; Paschen, 2000). Endoplasmic reticulum function is disturbed in many acute and chronic diseases of the brain, such as Parkinson's and Alzheimer's diseases (Paschen and Frandsen, 2001). Therefore endoplasmic reticulum dysfunction is basically important to elucidate the pathogenetic mechanisms of neurodegeneration.

4.2. Biochemical and molecular basis of nerve cell plasma membrane damage

Peroxidative stress has been implicated in mechanism leading to neuronal cell injury (Evans, 1993; Choi, 1993; Paschen et al., 2001;

Boldyrev et al., 2000). One source of free radicals in ischemic cells is arachidonic acid released by membrane phospholipids under the action of Ca^{2+} -activated phospholipase A_2 (Keuhl and Egan, 1980). Several oxygen radical species besides superoxide radicals are produced following hypoxia. Superoxide radicals have been shown to change phospholipid and protein structure. Hydroxyl radicals are the most reactive and are known to initiate lipid peroxidation and protein oxidation (Ginsberg et al., 1988; Siesjo et al., 1989; Traystman et al., 1991; Spuler et al., 1996; Wilberger, 1996). Peroxidation of polyunsaturated fatty acids damages cell membranes and disrupts transmembrane ionic gradients. The products of lipid peroxidation are aldehydes, hydrocarbon gases, and other metabolites that cause cytotoxic and vasogenic oedema, as observed in our electron micrographs. Iron derived from hemoglobin of intraparenchymatous hemorrhages can serve as a catalyst for free radical mediated oxidation leading to enhanced secondary tissue damage (Wilberger, 1996).

Marked alterations of phospholipids structures of different cell membranes have been observed in experimental models during acute or subacute cerebral aggression. Tissue surrounding haematomas, traumatic lesions, infective zones and certain tumors undergo autocatalytic peroxidation, which attacks fatty acid chains that include double bonds (Cohadon, 1984).

Alterations of astrocytic gap junctions have been widely reported in a variety of experimental conditions. According to Hossain et al. (1994), rat astrocytes respond to ischemic insult by reorganizing their gap junction, and increasing the junctional protein connexin 43. Ochalski et al. (1995) reported extensive gap junctions, disruption and gap junction disassembly in kainic lesion sites of rat brain. Theriault et al. (1997) described gap junction remodeling in rat spinal cord after acute compression injury. Li et al. (1998) found astrocytic gap junction internalization in rat brain after cerebral focal ischemia. Soroceanu et al. (2001) reported that high-grade brain

tumors show reduced intercellular communication, and a decrease in connexin-43 protein levels. Aronica et al. (2001) found high expression of connexin proteins in low grade tumors, and in the peritumoral reactive astrocytes. Nakase et al. (2003 a,b) reported a protective role of astrocytic gap junctions in ischemic stroke reducing apoptosis and inflammation following ischemic insult. According to Perez- Velazquez et al. (2006), the actions of gap junctional coupling during injuries may be causally related to oxidative stress.

In the mammalian CNS, excessive release of glutamate and overactivation of glutamate receptors are responsible for the secondary (delayed) neuronal death following neuronal injury, including ischemia, traumatic brain injury (TBI), and epilepsy. The coupling of neurons by gap junctions (electrical synapses) increases during neuronal injury. These Authors reported that the ischemic increase in neuronal gap junction coupling is regulated by glutamate via group II metabotropic glutamate receptors (mGluRs). (Cullen et al., 2012).

Concluding Remarks

In moderate brain edema a continuous plasma membrane is observed in some neurons, but the cytoplasmic membranes, such as smooth and rough endoplasmic reticulum membranes appear damaged. In severe edema, fragmentation of plasma membrane, enlargement and focal necrosis of rough endoplasmic cisterns and nuclear envelope, detachment of membrane-bound ribosomes, and reduction of polysomes are found. Shallow and deep invaginations of plasma membrane, and the formation of endocytic and clathrin-coated vesicles are seen. In astrocyte cells, areas of focal necrosis and fragmented limiting plasma membrane, overdistended rough endoplasmic reticulum cisterns with extense degranulated membrane domains, and vacuoles of smooth endoplasmic reticulum with necrotic limiting membrane are observed. Oligodendroglial

cells show also notably edematous changes featured by lacunar enlargement of rough endoplasmic reticulum and nuclear envelope, detachment of membrane bound ribosomes, and discontinuous plasma membrane. Plastic changes and damage of synaptic membranes are found. Synaptic vesicle exocytosis at the synaptic active zone, and endocytosis at the non specialized regions of presynaptic ending limiting membrane are frequently observed at activated synapses. In severe brain edema, synaptic disassembly occurs featured by wide separation of pre- and post synaptic membranes, and lost of perisynaptic astrocytic glial ensheathment. Disruption, fusion and disassembly of interastrocytary gap junctions have also been observed. The endothelial cell luminal membrane of brain capillaries shows profound activity changes that characterize increased cerebrovascular permeability, such as increased formation of micro- and macropinocytotic vesicles, deep invaginations, clathrin coated vesicles, and emission of pseudopods to form endothelial vacuoles.

The alterations of nerve cell plasma membranes and cytomembranes are related with the anoxic-ischemic conditions of brain parenchyma. The role of free radical and lipid peroxidation, disturbed energy metabolism, altered metabolic cascades, glutamate excitotoxicity, hemoglobin toxicity, protein aggregation, and presence of extracellular edema fluid are discussed in relation with the derangement of nerve cells membranes.

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