

The Thioredoxins Protein System: a Key Target in Perinatal Asphyxia

Francisco Capani^{1,2,3,*}, Tamara Logica Tornatore¹, Eduardo Blanco⁵, Maria Inés Herrera^{1,4}, Rodolfo Kolliker¹ and George Barreto⁶

¹*Instituto de Investigaciones Cardiológicas (ININCA), Facultad de Medicina, UBA-CONICET, Marcelo T. de Alvear 2270, C1122AAJ, Ciudad de Buenos Aires, Argentina*

²*Departamento de Biología, UAJFK, C1197AAR, Ciudad de Buenos Aires, Argentina*

³*Investigador Asociado, Universidad Autónoma de Chile*

⁴*CIPP, Facultad de Psicología, Universidad Católica Argentina*

⁵*Unidad de Gestión Clínica de Salud Mental, Laboratorio de Medicina Regenerativa, Instituto de Investigación Biomédica de Málaga (IBIMA), Hospital Universitario Regional de Málaga, Universidad de Málaga Málaga, Spain; Departament de Pedagogia i Psicologia, Facultat d'Educació, Psicologia i Treball Social, Universitat de Lleida Lleida, Spain*

⁶*Departamento de Nutrición y Bioquímica, Facultad de Ciencias, Pontificia Universidad Javeriana, Bogotá D.C., Colombia*

Abstract: Hypoxia during birth is one of the leading childhood mortality worldwide and results in many neurodisabled survivors. Several lines of evidence demonstrated that reactive oxygen species are the final step in the metabolic disorders produced by a deficit of oxygen. However, any specific therapy has been effective to ameliorate the devastating consequences of perinatal asphyxia. In the last few years, the Thioredoxin Family Protein has emerged as a key player in the regulation of the redox state of the cells in normal and pathological conditions. This review summarizes new insight of the role of Trxs, Prxs and Grxs in physiopathology of perinatal asphyxia.

Keywords: Trxs protein family, Perinatal asphyxia, Neuroprotection, Redox regulation, Central nervous system, Retina.

1. PERINATAL ASPHYXIA

1.1. Overview

Perinatal asphyxia (PA) is characterized by an acute or subacute brain injury due to asphyxia and is considered one of the most frequent causes of morbidity and mortality in term and pre-term neonates, accounting for 23% of neonatal deaths globally [1]. As many as a million deaths worldwide might be caused by PA. Following PA, approximately 45% of newborns die and 25% have permanent neurological deficits including cerebral palsy, mental retardation, developmental disability, learning disabilities, visual impairments, hearing problems, and different issues in school readiness [2].

During early development, brain injury results in significant damage in different areas of the central nervous system (CNS) such as: cortex, hippocampus,

neostriatum, cerebellum and substantia nigra [3]. The type and distribution of human brain lesions differ markedly between premature and term newborns, likely as a consequence of the stage of brain maturation and subsequent regional vulnerability, as described in several previous studies [4-6]. The neonatal brain, with its high concentrations of unsaturated fatty acids, high rate of oxygen consumption, low concentrations of antioxidants, and availability of redox-active iron, is particularly vulnerable to oxidative damage [7].

1.2. Models of Perinatal Asphyxia

Different model of PA has been used for many laboratories in the world. One of them is a murine model of PA. In this model uterus horns are isolated through an abdominal incision and placed in a water bath at 37°C during different period of time. According of the rate of survival, PA is classified in: mild PA (15 minutes, 100 % of survival), moderate (19 minutes of PA, 50 % of survival) and severe (20 minutes of PA, 10 % of survival). Following PA, uterus horns are opened, pups are removed and stimulated to breathe by performing tactile intermittent stimulation with pieces of

*Address correspondence to this author at the Instituto de Investigaciones Cardiológicas (ININCA), UBA, CONICET, Marcelo T. de Alvear 2270, C1122AAJ, Buenos Aires, Argentina; Tel/Fax: +5411 4508 3886/8; E-mail: franciscocapani@hotmail.com; fcapani@fmed.uba.ar

medical wipes until regular breathing was established and then animals were left to recover for 1h under a heating lamp. When their physiological conditions improved, they are given to surrogate mothers that had delivered normally within the last 24h. This PA model has three main advantages: first, it mimics the asphyxia just in the moment of delivery; second, it allows for the studying of both the short- as well as the long-term effects, since it is a non-invasive procedure; and third, it is easily reproducible across laboratories. The most obvious and serious short-term consequence of PA observed within this model is the mortality. Asphyxia period longer than 20 min generates an ATP deficit that leads to the consequent activation of anaerobic glycolysis and the accumulation of extracellular acidosis in the extracellular compartments [8]. Prolonged PA leads to an increase in expression of the hypoxia inducible factor (HIF-1) and a decrease of transcription and translation in general terms [9]. Re-oxygenation is associated with cell death, probably induced by glutamate over-activation and excessive reactive oxygen species (ROS) release [8, 9]. Rats subjected to asphyxia for 20 min at 37°C, and monitored by microdialysis, showed chronic defects in neurotransmitters, such as a decrease in dopamine, aspartate and glutamate release [9]. In addition, an increase in nitric oxide (NO) levels in the neostriatum and neocortex, both at short- and long-term periods was extensively described [10].

Long term consequences after 30 days of PA include modifications in cytoskeletal organization [11, 13]. After 6 months of PA proteins misfolding triggers an increment in protein ubiquitination levels, and synaptic degeneration [9, 11]. Moreover, different behavioral deficits such as exploration of new environments, spatial reference and working memory were observed 3 months after PA [12]. However, this PA model fails in the proper determination of the exact grade of neuronal damage since the PA is induced when rats do not have the brain totally developed and neurons are more resistant to the oxygen deprivation. Cerebral hypoxia-ischemia in 7-day-old rats is another murine model of PA frequently used [14, 15]. Basically consists in the ligation of the right common carotid artery, which remains occluded permanently. Whereas the cerebral blood flow normalizes rapidly, this produces an ischemic condition in the brain, which eventually leads to a focal hypoxia. This focal hypoxia is followed by exposure of rat pup to an anoxic environment for 3 minutes causing a global hypoxia. This model displays some features that do not correlate to the cases of PA suffered during human delivery.

First, at postnatal day 7 the rat is lissencephalic, in contrast to the gyrencephalic human brain [16]. Also, under the model conditions, ligation of the common carotid artery leads to unilateral regional reduction of cerebral blood flow; thus, the distribution of the brain injury caused by the experimental model does not correspond to human cases of severe hypoxic-ischemic encephalopathy [15]. The multi-organ involvement, one of the main characteristic after severe clinical asphyxia, is not seen after exposure to the experimental model. However, this model presents several advantages: (a) low-cost; (b) easily reproducible; (c) high rate of long-term survival (90 %), allowing the evaluation of neurological functional and neuropathological outcomes; (d) postnatal day 7, the rat's brain corresponds histologically to that of a near-term human fetus of 32-34 weeks, meaning that the cerebral cortical neuronal layering is complete, the germinal matrix is involuting, and white matter has undergone some myelination [17, 18]. (Table 1)

Table 1: Comparison Among Main Features of Murine Perinatal Asphyxia Model

	Global Perinatal Asphyxia	Global Hypoxia-Ischemia
Procedure	Non invasive. Place animal in water bath at 37 C. Easily reproducible	Invasive. -Ligation of the right common carotid artery, which remains occluded -Exposure of rat pup to an anoxic environment for 3 minutes causing a global hypoxia. Easily reproducib
Age of pups	New born	7 days old
Rate of survival	Related to the grade of asphyxia (100 % in mild asphyxia 3% in severe asphyxia)	90,00%
Brain damage	Related to the grade of asphyxia	Severe damage
Clinical correlation	Reproduces umbilical circular cordon. Multiorgan involvement	Grade of brain damage
Experimental possibilite	Short and long term studies	Short and long term studies
Brain maturity	Corresponds histologically to a pre term human fetus of 28-30 weeks	Corresponds histologically to that of a near-term human fetus of 32-34 weeks,

1.3. Physiopathology

Different mechanisms of injury have been proposed for the immature brain versus the adult brain. Most obvious difference is that apoptotic mechanisms are several-fold more pronounced in immature animals [19]. Substantial body of evidences, suggest that the developing brain shows a marked susceptibility to both changes in redox regulation and neuronal apoptosis, which may be related with this age-dependent injury vulnerability [20-24].

Mechanisms that cause neurological damage after PA might divided schematically into three metabolic phases [25-27]. Hypoxia leads to primary energy failure (phase 1). Shortly after re-oxygenation, aerobic metabolism is re-established (phase 2). However, as a result of a cascade of cellular mechanisms [28, 29], after 6–24 hours' mitochondrial energy production begins to fail one more time. This secondary energy failure (phase 3) is induced for 24–48 hours after the hypoxic event. Damage that occurs during phase 3 is considerable and leads to deep cellular alterations [30]. Although many modifications induced by PA are related to biological reactions that result in partial recovery, PA also generates over-expression of several metabolic, molecular and cell cascade pathways, extending the energy deficit and redox imbalance, associated with further neuronal damage, to either apoptosis (with several associated genes such as Bcl2, Bax among others) or necrosis. Molecular cascades involved in the removal of damaged cells implying activation of ubiquitination, peroxisome, and caspases pathways.

On the other hand, redox imbalance is strongly connected with re-oxygenation after PA, resulting in both over-activation and inactivation of a number of buffering enzymes; including those modulating mitochondrial activity [31, 32]. Finally, resuscitation may even imply hyperoxemia, leading to a further production of ROS and redox imbalance, worsening brain injury [31, 32].

Finally, neuroimaging studies has shown that brain injury evolves over days, if not weeks [33], which has been substantiated in different animal models [34]. If time permits, various therapeutic interventions may be attempted as the injury evolves [35]. Clinical observation of babies with asphyxia and extensive laboratory research with experimental models led to a heuristic model in which hypoxia-ischemia triggers a delayed series of events that lead to brain cell death [36, 37]. This period of latent interval suggested that

post-insult interventions could be protective if started on time [36]. Being able to complement these findings with those regarding the elucidation of brain injury mechanisms could enable the identification of new therapeutic targets.

2. REDOX REGULATION OF THIOREDOXIN PROTEIN FAMILY DURING PERINATAL ASPHYXIA BRAIN INJURY

2.1. Central Nervous System

Reperfusion after an ischemic event leads to an increased production of ROS [39, 40], that have been proposed as causative agent for the death and degeneration of neurons following the insult [8, 40-42]. As we describe above, we have previously shown that Trxs family (and related) proteins are distributed in a wide region and cell-type specific manner in the rat and mouse CNS [38, 43], implying specific functions and a complex crosstalk between the Trxs family members. In addition, not all areas of the brain are equally vulnerable to hypoxia-ischemia. Among the most vulnerable areas are the superior brainstem, cerebellum, white matter and subcortical structures supplied by the distal branches of deep and superficial penetrating blood vessels, cerebral white matter at the zones between the major cerebral artery territories, CA1 region of the hippocampus, and neocortical layers 3, 5, and 6 [44-46] and retina.

Although no studies on Trxs family proteins in PA have been published, both the release of ROS and RNS have been demonstrated under these conditions [8], potentially leading to the deregulation of redox signaling, which could be responsible for the induction of apoptosis and de-differentiation in the CNS associated with long-term neurological deficits. The disruption of redox control or generally, the generation of ROS [47, 48] by uncoupling of the mitochondrial chain [49] and inflammation [50, 51] were described as the most destructive causes of hypoxia/ischemia and reoxygenation injury.

Grx2-overexpressing HEK293 and HeLa cells exposed to hypoxia and reoxygenation showed less oxidative damage and increased survival and proliferation rates compared to wild-type controls [52]. Besides the anti-apoptotic function of mitochondrial Grx2a [53, 54], cytosolic Grx2c was recently shown to be essential for embryonic brain development. Zebrafishes with silenced expression of the oxidoreductase were characterized by the loss of the all types of neurons due to apoptosis and a developed

axonal scaffold [55]. This data suggests that the isoforms of Grx2 could be crucial for the long-term regeneration of physiological redox signaling and the recovery of the affected tissues.

Previous studies have shown that the Trx family of proteins contributes to mechanisms of brain tolerance in early periods after exposure to stress [56, 87]. Cells have developed response mechanisms to cope with these low oxygen concentrations. HIF-1 and 2 constitute transcription factors that regulate the expression of more than 180 genes under hypoxic conditions [58]. The HIF-1 target ATIA (anti-TNF α -induced-apoptosis) was shown to protect cells against hypoxia-induced apoptosis via Trx2 and the generation of ROS [59]. Moreover, hypoxia-induced mitochondrial ROS are essential for HIF activation [60, 61], regulate inflammatory responses via interleukin (IL-6) [62], and apoptosis via p38 phosphorylation [63]. Other studies suggested that over-expression of Trx1 leads to elevated HIF-1 α levels in cells cultured under both normoxic and hypoxic conditions, whereas inhibition of TrxR1 activity blocked the activation of HIF-1 α [64]. Trx1 leads to an induction of HIF-1 α , possibly by activating Akt [65]. Trx1 might also be involved in depleting HIF-1 α levels upon reoxygenation [66].

Hypoxic insults induce molecular and cellular changes, affecting cell morphology, cell polarity, osmoregulation, protein synthesis and the release of neurotransmitters [67, 68]. The reoxygenation phase causes rapid changes in the redox properties of the affected tissue and an increase in the cytokine and chemokine levels, as well as the infiltration of immune cells [69]. Not surprisingly, members of the Trx family protect against ischemic injuries. Over-expression of

Trx1, Grx1, Grx2, and Prx2 attenuate ischemic damage of neurons [70-73]. Recently our group studied the expression and distribution of Trx family and related protein in the CNS. Trxs, Grxs and Prxs displayed a complex expression pattern following hypoxia/ischemia and reoxygenation event (Figure 1). Trx1 and Grx2 as late as 60 days after hypoxia/ischemia were expressed consistently. Over expression and silencing of these proteins in SH-SY5Y cells subjected to hypoxia-reoxygenation showed that these proteins are required to maintain the normal neuronal phenotype. [74].

2.2. Retina

Retina is a tissue that undergoes high levels of stress due to its exposure to elevated oxygen levels and different light intensities [75]. Ganglion cells are the ones in charge of sending the visual input to the brain (visual cortex). This layer expresses every protein belonging to the Trx family proteins [38] and over expression of Trx1 in retinal ganglion cells increases their survival after glutamate/buthionine sulfoximine treatment. In addition, Trx1 levels decreased after glaucoma induction and retinas that over expressed Trx1 and Trx2 showed a reduced loss of retinal ganglion cells after glaucoma induction [76]. On the other hand, retinal pigment epithelium (RPE) cells are located near the choroidal capillaries, making them particularly susceptible to ischemia or hypoxia [77]. Sugano *et al.* [78] shows that Trx2 has a protective effect in ARPE-19 cells treated with 4-hydroxynonenal (4-HNE) or C2-ceramide. Over expression of Trx2 reduced cell death induced by both agents in low concentrations. Moreover, Trx2 played a key role in cell viability through regulation of mitochondrial metabolic activity and Hsp70 expression

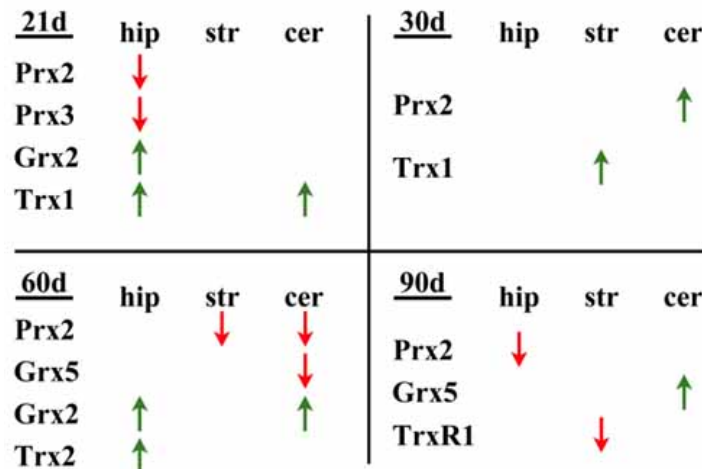


Figure 1: Scheme showing the time course of the expression of the Trx protein family after PA.

On other hand, transgenic mice that express human Trx1 are less susceptible to light induced retinal damage [79]. Consistent with this view, Trx-1 expression is reduced after retinal damage [80], while Prx6 is also diminished in cell cultures submitted to a hypoxia event [81]. Caprioli *et al.* [82] reported a decrease in Trx1 levels more pronounced at 5 weeks after intra ocular pressure (IOP). In addition, Trx1 is also down-regulated after IOP in a rat glaucoma model, whereas its overexpression after IOP leads to an attenuated cell death [83]. The administration of N-methyl-D-aspartate (NMDA) to mammalian eyes stimulates glutamate receptors and induces retinal damage mimicking retinal ischemia and glaucoma. An intravitreal Trx1 injection effectively attenuates retinal cell damage induced by NMDA [84].

2.3. Inflammatory Mediated Regulation During Oxygen Homeostasis Restoration

Studies on the hypoxia signaling pathway have helped the concept that hypoxia can induce inflammation gain general acceptance. In persons with acute mountain sickness, for example, levels of circulating pro-inflammatory cytokines increase and vascular leakage causes pulmonary or cerebral edema. Hypoxia signaling and members of the nuclear factor κ B (NF- κ B) family of transcription factors regulate inflammation and orchestrate immune responses to achieve tissue homeostasis [85]. Members of this

family interact with members of the HIF pathway in ways that link inflammation to hypoxia (Figure 2).

Ischemia reperfusion activates NF- κ B in epithelial cells, which in turn increases the production of tumor necrosis factor α (TNF- α), a pro-inflammatory cytokine, but simultaneously attenuates epithelial apoptotic hypoxic activated pathways. These pathways were related with the Thioredoxin protein family [86, 87]. Choksi *et al.* [57] reported the identification of an NF- κ B-independent ATIA (anti-TNF α -induced apoptosis)-Trx2 (Trx2) axis that inhibits TNF α - and hypoxia-induced apoptosis directly through elimination of excessive ROS. Current paradigm for inhibition of TNF α -induced apoptosis centers on NF- κ B, which inhibits caspases and prevents prolonged JNK activation. In addition, the anti-apoptotic effect of NF- κ B also was suggested to associate with the elimination of excessive ROS. In the same report, Choksi *et al.* [57] has shown that ATIA (anti-TNF α -induced apoptosis) inhibits TNF α -induced apoptosis independently of NF- κ B through Trx2-mediated elimination of excessive ROS. These findings provide a novel hypothesis for inhibition of apoptosis induced by TNF α and other death signals by directly controlling ROS accumulation. The pleiotropic inflammatory cytokine TNF α regulates immune responses, inflammation, proliferation, and cell death (apoptosis and necrosis), and its regulation of apoptosis is mainly mediated by its membrane receptor 1 (TNF-R1). Upon TNF α stimulation, TNF-R1 trimer

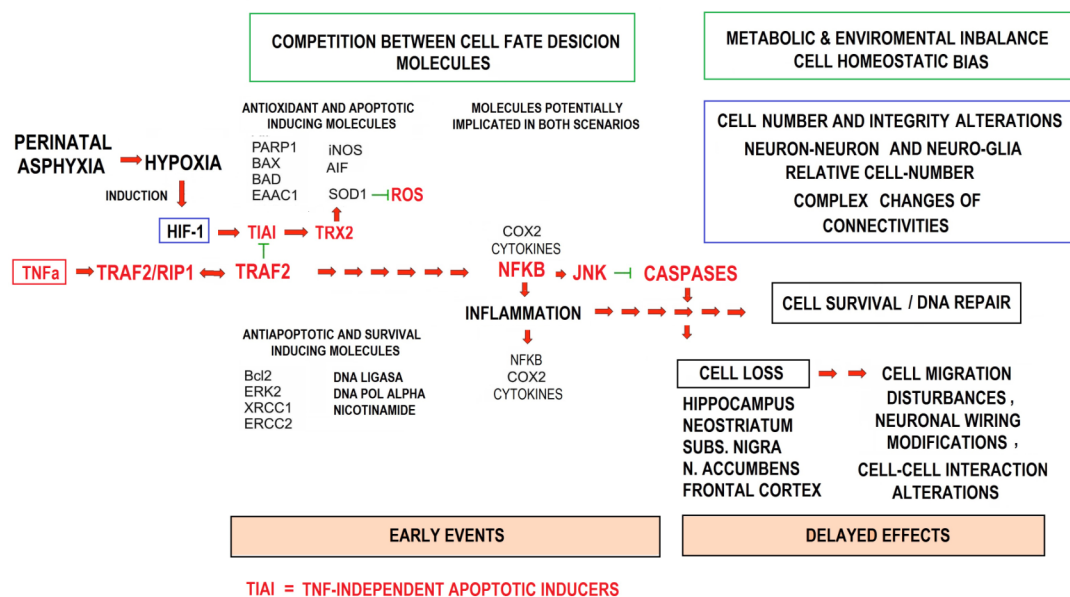


Figure 2: Schematic illustration of ATIA serving as a Link between inflammation and hypoxia: The ATIA-TRX2 axis inhibits apoptosis induced by both TNF α and low O₂ through elimination of excessive ROS on mitochondria. This scenario rescues parenchymal cells from apoptosis. ATIA activity may be a key regulator in carcinogenesis because tumor cells usually take advantage from normal tissue under hypoxic conditions. Blockade of antitumor TNF- α cytotoxicity.

recruits multiple adaptors such as, TRAF2, TRAF5, RIP1, cIAP1 and cIAP2 and other modulators or regulators such as Miz1 and the linear ubiquitin chain assembly complex [88, 89],

2.4. Thioredoxin, Hypoxic-Ischemic Brain Injuries and Cognitive Alterations

The hippocampus is commonly associated with learning and memory processes in mammals [90]. Previous studies have demonstrated that hypoxic-ischemic brain injuries can induce hippocampal neuron loss, which is implicated in spatial learning and memory deficits [91-93]. It has been demonstrated that transient bilateral common carotid arteries occlusion (BCCAO) can destroy more than 50% of hippocampal CA1 neurons [94] inducing learning and memory deficiencies tested in the Morris water maze [95, 96]. Redox regulatory proteins, such as Trxs, play a key role in the cellular response to redox imbalance, against brain damage induced by hypoxic-ischemic injuries [38]. Acute hypoxia leads to long-lasting behavioral consequences (motor, emotional and cognitive dysfunctions) in adult asphyctic rats [12]. However, only few works have studied the role of redox imbalance in the origin of behavioral alterations induced by hypoxic-ischemic injuries.

Regarding to Trx1, endogenous Trx1 production is induced by cerebral ischemia where it is responsible for alleviation of oxidative damage. Exogenously administered recombinant human Trx1 (rhTrx1) can penetrate the blood brain barrier and exert a neuroprotective effect on injured neurons by the middle cerebral artery occlusion (MCAO) [97]. RhTrx1 may also promote neurogenesis and facilitate long-term recovery following bilateral common carotid artery occlusion (BCCAO). Recently, Tian *et al.* [98] have investigated the role of rhTrx1 in neurogenesis and its potential effect on learning and memory deficits measured in the Morris water maze following the BCCAO mouse model. Mice subjected to BCCAO treated with rhTrx1 before reperfusion showed shortened escape latencies in the learning phase and improvements in the spatial memory test. Moreover, rhTrx1 was effective in promoting neurogenesis in the dentate gyrus after cerebral ischemia and facilitating cognitive recovery of treated mice [99]. Previous studies have demonstrated that hippocampal neurogenesis could facilitate long-term potentiation and improve memory and learning [100, 101]. Hence, facilitation of endogenous neurogenesis could be a

promising regenerative strategy for ischemic stroke treatment [100].

In other recent work, Yang *et al.* [101] studied the role of Trxs in neurodegenerative changes of rats subjected to chronic exposure to an intermittent hypoxia (CIH) model. The most notable alterations were increased apoptosis in the CA1 region of the hippocampus and adjacent cortex, as well as substantial neuropsychological impairments of spatial learning in the Morris water maze. CIH-rats showed impaired spatial learning and memory in the water maze (longer latencies to reach the target platform, reduced numbers of passes over the target platform, and smaller percentage of time spent in the target quadrant). Trx mRNA and protein levels were significantly decreased in the CIH-hippocampus; meanwhile, an elevated apoptosis of hippocampal neurons of rats exposed to CIH was found. Rats, which displayed better performance in the spatial memory test, also showed higher levels of the Trx mRNA and protein in the hippocampus and lower number of apoptotic cells in the hippocampus [101]. Hence, Trx deficit may play a significant role in the impaired spatial learning and memory in rats exposed to CIH and this alteration may trigger the apoptosis of hippocampal neurons.

New studies are necessary to understand the relationships between redox system regulations in cognitive alterations after neuronal damage induced by hypoxic-ischemic episodes.

3. CONCLUSIONS

Several lines of evidences propose a key role of Trxs in the physiopathology of the brain ischemia insult. In addition, recently we have demonstrated that Trx-1 and Grx-2 have a key role on the different steps of the nervous cells differentiation. Therefore, Trx-s protein family appears a novel actor in the regulation of the mechanism involved in cell damage during PA.

4. FUTURE DIRECTIONS

Future experiments should be carried out to obtain new insights about Trx-s protein family function in the rescue of the nervous cells subjected to anoxic-hypoxic in order to design new therapeutic tools. These studies might introduce new insights, extremely useful to develop new therapeutic tools for PA and its complications.

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