

# Cell Culture Models of Oxidative Stress and Injury in the Central Nervous System

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**Abstract:** Constantly growing body of evidence suggests that hallmarks of oxidative stress are present in various central nervous system (CNS) disorders. Technological advantages in cell culturing made it possible to use neural cell/tissue cultures as experimental models for investigation of molecular mechanisms which underlie the development of oxidative stress condition, damage and adaptive responses to oxidative insults. This review is focused on the application of cell culture methodology for studies of oxidative stress condition in the brain. The review describes studies of biomarkers of oxidative stress-dependent cell damage and adaptive responses in various kinds of brain cell culture models. It discusses the use of cell/tissue culture models for elucidation of the role and pathogenesis of oxidative stress in neurodegenerative brain disorders, AIDS-associated brain pathology, drug abuse, and aging. The review underscores the importance of cell/tissue-based studies for testing of new antioxidants and development of therapeutic strategies for amelioration of oxidative damage in the CNS. The impact of new advances in gene and protein expression analysis on the cell/tissue culture-based research of oxidative stress in the CNS is also discussed.

**Key Words:** Cell/tissue culture, *in vitro*, Alzheimer's disease, Parkinson's disease, Huntington disease, HIV-associated dementia, Prion diseases, oxidative damage, brain.

## INTRODUCTION

The role of free radical-mediated reactions in human neuropathology continues to attract significant interest. In general, it has been shown that different disorders caused by different genetic or environmental insults may have one common molecular basis, namely, oxidative stress. Evidence has been accumulating that free radicals and reactive oxygen species (ROS) have been associated with the etiology and progression of different neurodegenerative diseases. Free radicals play an important role in the development of neurodegenerative disorders such as Alzheimer's disease (AD), Huntington's disease (HD), Pick's disease, amyotrophic lateral sclerosis, epilepsy, schizophrenia, cerebral ischemia. Free radical generation leads to oxidative damage of lipids, nucleic acids and proteins, abnormal aggregation of cytoskeletal proteins, mitochondrial dysfunction, antioxidant enzymes upregulation, reactive nitrogen species formation, advanced glycation endproducts formation, inactivation of key enzymes (Aksenov *et al.*, 2001a; Butterfield *et al.*, 1997; 2001a; Markesbery, 1997; Rao *et al.*, 2002).

During past decade free radical generation was established to be an early biochemical event subsequent to spinal cord injury and head trauma (Azbill *et al.*, 1997; Barut *et al.*, 1993; Hall, 1989; Hall *et al.*, 1986). Recent studies have indicated that many other disorders such as HIV infection are associated with oxidative stress. HIV increases the level of ROS both within the infected cells and the plasma of

serum levels of lipid peroxidation products, malondialdehyde, and hydroperoxide (Revillard *et al.*, 1992) indicated by ongoing oxidative stress in HIV-infected patients. Although various markers of oxidative stress noted above can be detected all over the body, for example in HIV-infected T-cells and peripheral monocytes, the brain is considered the most vulnerable site of oxidative damage mainly because the brain is rich in polyunsaturated fatty acids, accumulates redox metal ions, consumes a large amount of inspired oxygen, is relatively low in antioxidants, and is composed largely of non-mitotic cells. Accumulation of oxidative damage in neurons accounts for the increased incidence of neurodegeneration in AD, ALS, Parkinson's disease etc., and also in HIV-1 infection where neuronal cell loss and AIDS-associated dementia has been observed (Perl *et al.*, 2000). While the commonality of oxidative stress in many of the above-mentioned disorders is well-studied, there is a gap in understanding of the relationships among oxidative stress, neurotoxicity, and cell death.

Model systems such as experimental animals and cell cultures are often used to understand how oxidative stress can produce neurotoxic effects, which later lead to neuronal dysfunction, degeneration, and cell death. This review focuses on the use of neural cell cultures for studies of the role of free radical species in pathogenesis of neurodegeneration associated with aging, viral infections, and drug abuse. It attempts to summarize an up-to-date methodology of the investigation of oxidative stress condition and its consequences for different types of CNS cells in culture and discusses current approaches to model oxidative stress-related neuropathology using different types of brain cells or tissue cultures.

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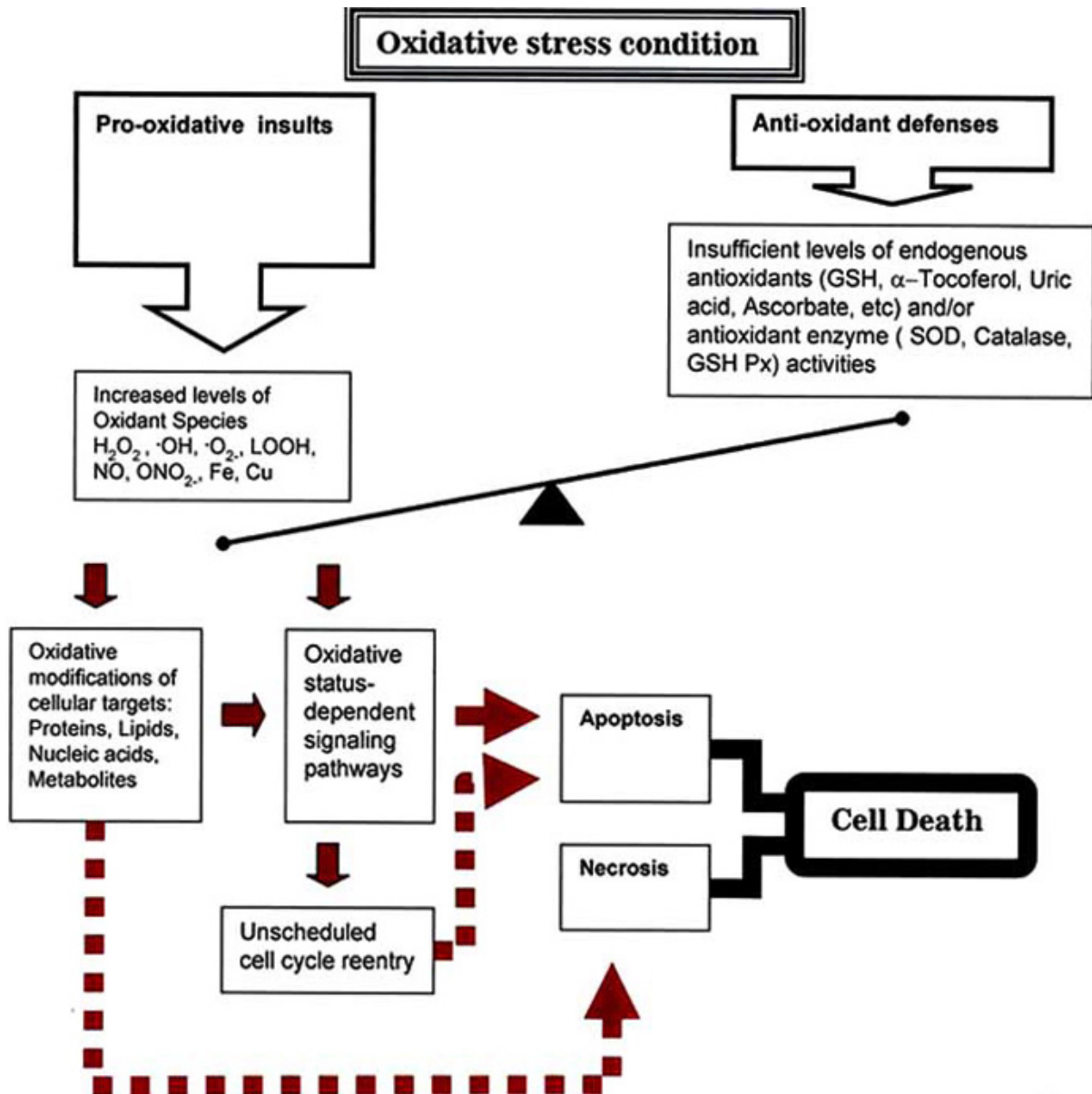
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### Oxidative Stress and CNS Detection of Biological Markers of Oxidative Damage

Oxidative stress reflects the disruption of an intricate balance between the formation and clearance of highly reactive free radical species in living organisms (Fig. 1). Among different organs and tissues, the CNS is one of the most sensitive to changes in the redox status and possible oxidative damage because of the high level of oxidative metabolism and the abundance of postmitotic highly differentiated cell elements that are hard to replace if damaged. Aging and diseases can increase the probability of

oxidative insults or decrease the ability of CNS structures to absorb their damaging consequences (Bowling *et al.*, 1995; Butterfield *et al.*, 1997; Beal, 2002).

As it follows from the general definition of oxidative stress, the direct marker of that condition must be increased levels of free radical species. However, due to a huge variety in chemical nature of potential prooxidants, short life span of highly reactive free radical substances, and obstacles of *in vivo* measurements of their concentrations, this task is very complicated and is rarely possible to complete. Therefore, studies of oxidative stress are limited to the detection of

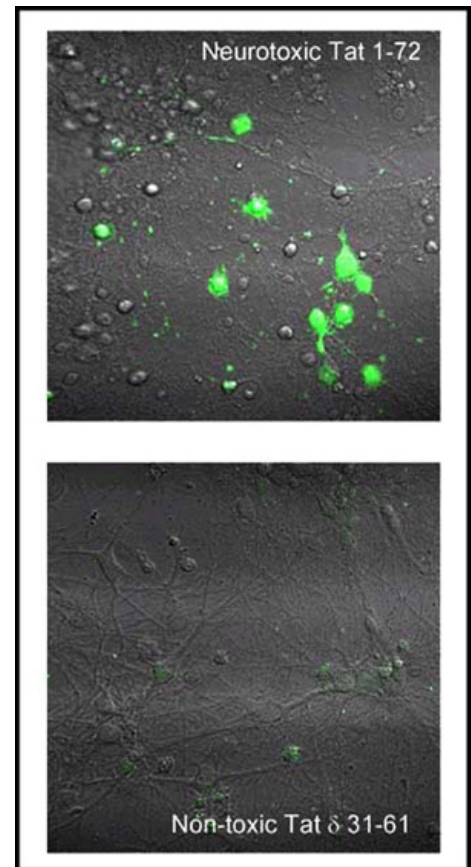


**Fig. (1).** Oxidative stress condition reflects a shift of a delicate balance between pro-oxidative and anti-oxidative events in the cell which leads to oxidative modifications of macromolecular cell components. Oxidative modifications change the functioning of proteins, lipids, nucleic acids, and allow formation of highly reactive products which can further modify these cellular targets. Changes in oxidative status may cause necrosis and/or trigger cascades of ROS-dependent signaling that activates mechanisms of programmed cell death.

indirect markers such as an increase of oxidative stress-related gene expression, a decrease of antioxidant protection, or to the detection of the consequences of oxidative stress using biomarkers of oxidative damage- relatively stable chemical modifications of macromolecular cell components caused by free radicals (Floyd *et al.*, 2002). Among different experimental models, cell or tissue cultures provide a unique possibility to measure directly the changes of oxidative status of neural cells, making tissue/cell culture the model of choice regarding the involvement of oxidative stress in a pathological process (Halliwell, 2003; Lesuisse *et al.*, 2002). One of the widely used approaches to the study, an increased free radical production, is the use of oxidation-sensitive fluorescent molecular probes, such as dichlorodihydrofluorescein (DCFDA or H<sub>2</sub>DCF), which allows detecting of intracellular production of ROS. The example of ROS detection in primary culture of rat hippocampal neurons is shown in Fig. (2). Mitochondria are believed to be a major source of ROS in a cell (Delgi Esposti, 2002). Fluorescent probes for the detection of changes in the mitochondrial transmembrane potential, such as rhodamine (123, 3,3'-dihexiloxadiazolcarbocyanine) and aggregate dye 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolcarbocyanine iodide (JC-1), provide a possibility to monitor changes in mitochondrial function, which may lead to increased ROS production in a living cell. All these techniques work to their greatest potential in tissue/cell culture studies of oxidative stress and related cell pathology.

Since deteriorating effects of oxidative stress on the cell function are the major concerns, detection of biomarkers of oxidative damage is a viable way to reveal a possible role of the unbalanced oxidation/reduction of macromolecular cell components like proteins, lipids and nucleic acids in the pathogenesis of neurodegeneration associated with the disease, trauma, drug abuse, or normal ageing (Facheris *et al.*, 2004; Cui *et al.*, 2004; Moskovitz *et al.*, 2002). The detection of lipid peroxidation products is one of the oldest experimental approaches used to study oxidative stress in the postmortal brain tissue or in the samples obtained from different experimental models of brain pathology. Due to the high concentration of polysaturated fatty acids in the brain compared to other tissues, lipid peroxidation is a major outcome of free radical-mediated injury to brain tissue. Although lipid peroxidation products undoubtedly contribute to oxidative damage in the brain, their usefulness as biomarkers of oxidative modification of lipids *in vivo* is limited because of their chemical instability and rapid metabolism. However, several chemically stable products, such as neuroprostanes (derivatives of docosahexaenoic acid), isoprostanes and isofurans (derivatives of arachidonic acid), are useful markers of lipid peroxidation in neurodegenerative brain pathology (Montine *et al.*, 2004). In cell/tissue culture models of oxidative stress, detection of highly reactive products of lipid peroxidation is less problematic due to higher grade of the investigator's control over the conditions of the experiments. Thus, measuring of TBA-reactive substances (TBARS) that are formed as a result of interaction between various lipid peroxidation products and thiobarbituric acid (TBA), a gas chromatography-mass spectrometric (GC-MS) assay measuring malondialdehyde (MDA) (Liu *et al.*, 1997), and detection of

4-hydroxynoneal and its adducts (Keller *et al.*, 1997; Kruman *et al.*, 1997) are effectively used to detect oxidation of lipids in studies of oxidative stress using *in vitro* CNS cell/tissue culture models.



**Fig. (2).** Green dichlorofluorescein (DCF, the product of the reaction between ROS and DCFDA) fluorescence indicating increased intracellular ROS production in cultured fetal rat hippocampal neurons exposed to neurotoxic recombinant HIV-1 transactivating protein Tat (Tat 1-72, top image). Control cell culture was treated with the same dose of biologically inactive, non-toxic recombinant analog of Tat with scrambled sequence of the region responsible for neurotoxicity (Tat 31-61, bottom image). Tat protein is believed to play a major role in pathogenesis of HIV-associated dementia. Neurotoxicity of Tat is associated with oxidative stress (Kruman *et al.*, 1998; Perez *et al.*, 2001).

Another wide variety of biochemical and molecular biology methods were developed to detect oxidative stress-induced damage in DNA and RNA. In general, the techniques in that field are aimed at the search for oxidatively modified bases or strand breaks that may result from interactions of nucleic acids with oxidants. Formation of 8-hydroxy-2'-deoxyguanosine (8-OHdG) is the most common modification of DNA caused by oxidative stress. Levels of 8-hydroxy-2'-deoxyguanosine, measured using immunochemical techniques or by HPLC, serve as an indicator of oxidative DNA damage in neurological diseases

and in normal brain ageing (Markesbery *et al.*, 1999; Floyd *et al.*, 2002). Oxidative damage of brain RNA has been assayed by immunodetection of oxidized nucleosides (Shan *et al.*, 2003) and reverse transcription (RT) or RT-PCR-based methods (Rhee *et al.*, 1995).

In a few past decades methods were developed that allow reliable detection of oxidative modifications in proteins. Coupled with the constantly increasing knowledge of the importance of redox status of proteins for the course of biochemical processes they are involved in, the development of methods for the detection of protein oxidative modification spurred the research in that area. Oxidation of proteins can lead to hydroxylation of aromatic groups and aliphatic amino acid side chains, nitration of aromatic amino acid residues, nitrosylation of sulfhydryl groups, sulfoxidation of methionine residues, chlorination of aromatic groups and primary amino groups, and to conversion of some amino acid residues to carbonyl derivatives. Oxidation can lead also to cleavage of the polypeptide chain and to formation of cross-linked protein aggregates. Furthermore, functional groups of proteins can react with oxidation products of polyunsaturated fatty acids and with carbohydrate derivatives (glycation/glycooxidation) to produce inactive derivatives (Stadtman *et al.*, 2003). Levels of protein oxidation in the brain and other tissues reflect complex relationships between production of substances which can cause oxidative modifications, antioxidant levels, and proteolytic elimination of oxidized forms of proteins. The formation of protein carbonyls by various oxidative stress-related biochemical reactions is widely used indicator of protein oxidative damage. The most popular current methods of measuring protein carbonyl formation are based on the detection of protein-bound carbonyl derivatives with specific antibodies (Aksenova *et al.*, 1999a,b; Aksenov *et al.*, 2001a). Variants of this technique have been successfully used in cell culture models of neural cell aging and neurodegenerative pathology (Fig. 3). Immunochemical techniques have been developed for the detection of nitrated tyrosine residues, the indicator of peroxynitrate-induced protein damage. Oxidation of protein-bound thiols also can be detected with highly specific probes that covalently bind to SH groups in proteins. Unlike other kinds of protein oxidative modifications, the oxidation of sulfur-containing amino acid residues is reversible. Cyclic oxidation and reduction of sulfur-containing amino acid residues may be an important antioxidant mechanism and plays an important role in regulation of some protein biological activities. Thus, measuring of free SH-groups in cellular proteins is also a useful tool in the investigation of oxidative stress-mediated cell pathology (Aksenov *et al.*, 2001b).

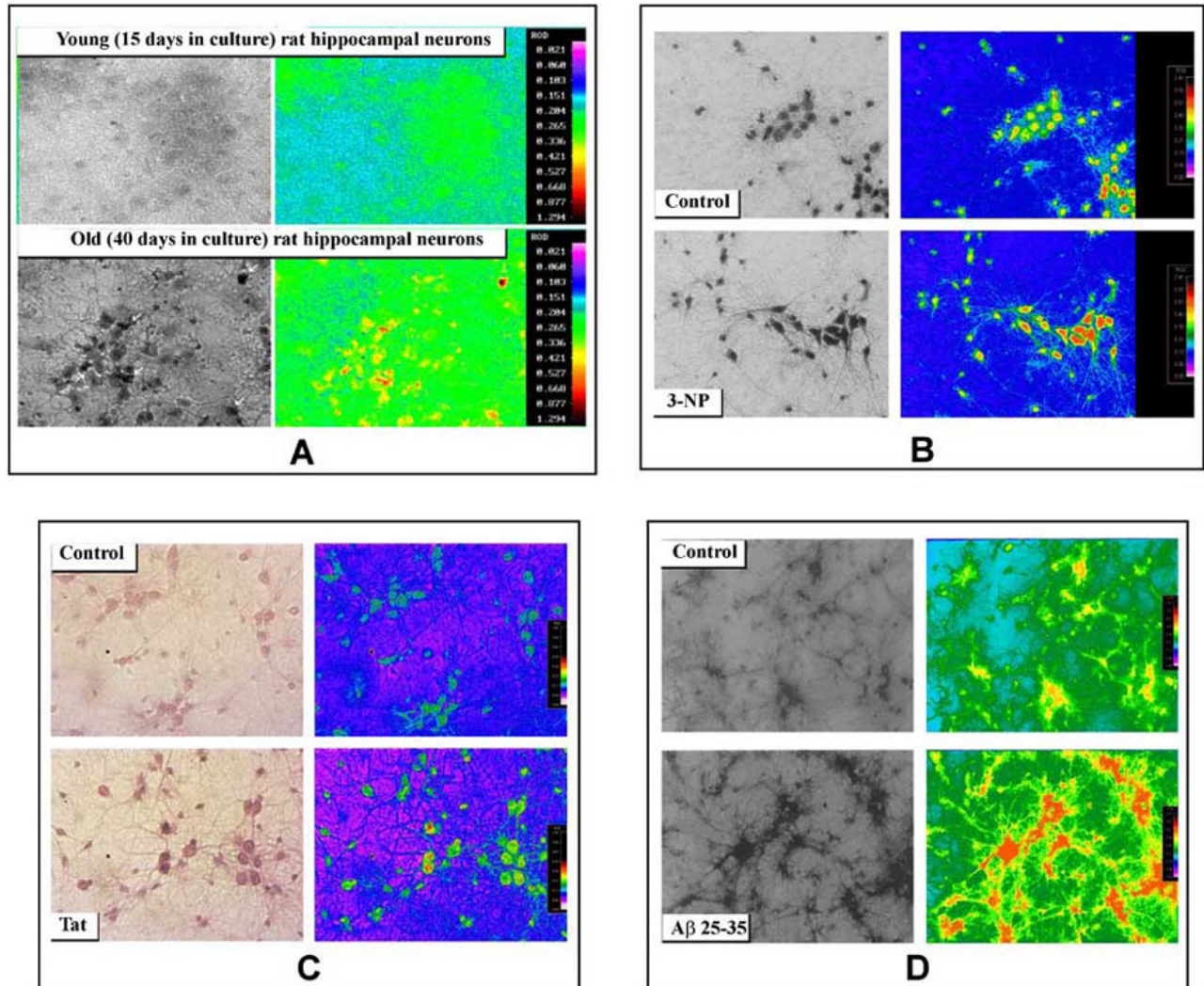
The detection of indirect indicators of oxidative stress such as changes in gene expression that marks a specific cell response to oxidative stress or changes in levels of antioxidant compounds are also of a great value for the investigation of oxidative stress-related pathology. The usage of neural cell/tissue cultures provides an advantage of quick analyses of the oxidative stress-dependent mechanisms of cell death following the interaction of CNS cells with neurotoxins or conditions that are able to trigger oxidative stress.

### **Oxidative Stress “*In Vitro*”: Modeling of Oxidative Stress Conditions in the CNS Using Cell/Tissue Cultures**

Constantly growing evidence of the involvement of oxidative stress in various pathological processes that impair CNS functioning stimulates the investigation of basic mechanisms of oxidative damage and protection against it. Brain cell/tissue cultures are extremely useful to study the effects of oxidative stress, in terms of both toxicity and adaptive cell responses. The most common approaches to modeling of CNS oxidative stress include the exposure of primary neural cell cultures, brain tissue explants, and/or neural cell lines to pro-oxidative conditions such as hyper- or hypoxia, deficit of energy sources (glucose), and addition of external prooxidants. Methodological and practical aspects of cell culture-based models of oxidative stress were discussed in several research reports and reviews (Gille *et al.*, 1992; Halliwell, 2003; Ricart *et al.*, 2001; Hirrlinger *et al.*, 1999) during the past decade.

Different brain cell types are used to study effects of oxidative stress in culture. Subjection of different types of CNS cell in culture to prooxidative agents allows to investigate possible differences in the sensitivity of different brain cells to oxidative insults that take part in the pathogenesis of the particular brain disorder. Comparison of abilities to dispose exogenous hydrogen peroxide by oligodendroglial, astroglial, microglial cells and neurons demonstrated prominent differences in prooxidant detoxication capacities of these CNS cells and showed that oligodendroglial cells in culture have a prominent machinery for ROS disposal, which is likely to support the protection of oligodendrocytes *in vivo* in the brain under oxidative stress condition (Hirrlinger *et al.*, 2002). It has been shown that oxygen and glucose deprivation induces mitochondrial dysfunction in neurons but not in astrocytes in primary culture (Almeida *et al.*, 2002). Cell culture studies generated data that suggest a protective role of astrocytes and microglia against neuronal cell death induced by reactive oxygen and nitrogen species (Hirrlinger *et al.*, 2000; Pentreath *et al.*, 2000; Tanaka *et al.*, 1999; Wang *et al.*, 2000; Wilson, 1997), but at the same time oxidative stress-triggering role of astrocytic/microglial activation has been shown (Manning *et al.*, 2001; Moss *et al.*, 2001; Wang *et al.*, 2002). Cell culture studies have made a significant contribution to our understanding of an important role of glial and microglial cells in monitoring of the microenvironment in the CNS and respond to CNS injuries.

It is still debated whether these reactions are beneficial or detrimental (Tacconi, 1998; Von Bernhardi *et al.*, 2001). Comparison of prooxidative effects of suspected pathogens both in pure neuronal and in mixed neuronal/astrocytic or neuronal/microglial cell cultures can help to determine if direct interactions with neurons or glial/microglial cells are responsible for oxidative damage and neurodegeneration (Bonavia *et al.*, 2001; Le *et al.*, 2001; Piani *et al.*, 1992; Qui *et al.*, 2002; Viviani *et al.*, 2001). Results of the investigation of HIV-1 gp 120 toxicity in sandwich co-culture of primary rat hippocampal neurons and glia indicated that ROS produced by gp 120 – activated glia caused oxidative damage and degeneration of neurons (Viviani *et al.*, 2001). Study of another HIV-1 neurotoxic protein, Tat, in mostly



**Fig. (3).** This figure illustrates the use of immunochemical detection of protein carbonyl derivatives in: (A) long-term primary rat hippocampal cell culture (in vitro model of neural cell ageing); in (B) rat hippocampal neurons exposed to 3-nitropropionic acid (one of the suspected pathogens involved in the development of oxidative stress condition in Huntington disease); in (C) rat hippocampal neurons treated with HIV-1 Tat (virotoxin which plays major role in pathogenesis of HIV-associated dementia); in (D) rat hippocampal neurons subjected to amyloid beta peptide (A<sub>25-35</sub>), neurotoxic self-aggregating polypeptide which plays a key role in pathogenesis of Alzheimer's disease. In all four panels, images on the right are computer-generated false color (red-green-blue or RGB) maps of protein carbonyl immunoreactivity in cultured neural cells. Scales represent the relationship between color code and relative optical density of the protein carbonyl-specific immunostaining. Imaging analysis of protein carbonyl levels in all these studies was performed using MCID 4 or MCID 7 imaging systems (Imaging Research Inc, Ontario, Canada).

neuronal and neuronal/astrocytic cell cultures suggested that Tat-induced neuronal apoptotic death was not influenced by astrocyte co-culture (Bonavia *et al.*, 2001). Oxidative stress-related toxicity of amyloid beta peptides was shown to result from direct interactions with cultured neurons (Harris *et al.*, 1996). However, enhancement of beta-peptide toxicity to cortical and mesencephalic neurons by ROS-producing microglia was recently reported (Qin *et al.*, 2002). Thus, the use of cell culture models allows initial screening of hypotheses about mechanisms of oxidative stress – related neuronal cell death associated with neurological disorder of interest.

#### Cell/tissue Culture-Based Experimental Models for Studying the Role of Oxidative Stress in the Pathogenesis of Neurodegenerative Brain Disorders and Degeneration Associated with Neuroinfections and Drug Abuse

Numerous studies performed over the last decade demonstrated the presence of oxidative stress hallmarks in many different types of neuropathology. The possible role of oxidative stress in neurodegenerative disorders has become a subject of several scientific reviews (Bowling *et al.*, 1995; Butterfield *et al.*, 2001a; Moskovitz *et al.*, 2002; Rao *et al.*, 2002; Shor-Posner *et al.*, 2002) and cell culture models have

been widely used in the studies. In the investigation of a complicated pathogenesis of neurodegenerative diseases neural cell/tissue cultures help to model *in vitro* neurotoxic effects of substances that are suspected to play a role in neuronal cell death associated with a particular disorder (Table 1). This variant of “*in vitro* toxicology” is one of the most popular applications of CNS cell cultures for studies of oxidative stress in neurodegenerative disorders. The use of cell culture models provides powerful tools for checking whether neurotoxic effects of a substance suspected to play a role in the disease pathogenesis may account for oxidative stress and damage associated with that kind of neurodegenerative disorder.

Experimental reports have been published that used cell/tissue cultures to study neurotoxicity of beta amyloid peptides in Alzheimer’s disease, prion proteins in prion disorders, MPTP in Parkinson’s disease, 3-NP in Huntington’s diseases, HIV-1 proteins in HIV-associated dementia. Since the support for the idea of an important role of oxidative stress in many disorders caused by different genetic or environmental insults and affecting different CNS regions is growing (Moosmann *et al.*, 2002; Rao *et al.*, 2002), many other disorders that affect the brain are studied with an aim to test prooxidant abilities of the specific pathogens towards neural cells in culture.

#### **Alzheimer’s Disease: Cell/tissue Culture Studies of Beta-Amyloid (A $\beta$ ) Neurotoxicity**

Oxidative stress in the AD brain is manifested by lipid oxidation, increased levels of oxidatively modified proteins, oxidative damage of nucleic acids, and altered antioxidant enzyme activity and expression (Aksenov *et al.*, 1998;

2001a; Aksenova *et al.*, 1999b; Arlt *et al.*, 2002; Beal 2002; Markesbery *et al.*, 1999). It is believed that 42- or 40-amino acid amyloid beta peptides that are the major constituents of senile plaques in the AD brain are central to the pathogenesis of the AD neuropathology (Butterfield *et al.*, 2001b; Selkoe 1989; 1994). The toxicity of beta amyloid (A $\beta$ ) was demonstrated using neuronal cell cultures and this experimental model helped to advance progress in understanding of mechanisms of neurodegeneration caused by A $\beta$ . Studies of primary neuronal cultures, neural cell lines and organotypic brain cultures, which were subjected to different A $\beta$  peptides, provided significant amounts of data that supported the hypothesis of possible role of beta amyloid in the development of oxidative stress condition in Alzheimer’s disease (Butterfield 1996; 1999). The ability of beta peptides to cause increased production of ROS, oxidative damage to lipids and proteins, changes of antioxidant defenses, and oxidative stress-related changes of gene expression have been documented in numerous research papers (Aksenov *et al.*, 1998; Butterfield *et al.*, 1994; Harris *et al.*, 1996; Varadarajan *et al.*, 2000; Yatin *et al.*, 1998), (Fig. 3D).

#### **Prion Diseases: Neurotoxicity and Oxidative Stress Induced by Prion Peptide (PrP) in CNS Cell Cultures**

The exact pathogenic mechanism of transmissible spongiform encephalopathies (prion diseases, TSEs) remains uncertain, but it is believed that oxidative stress plays a central role (Milhavet *et al.*, 2002). Prion diseases are characterized by the production of self-aggregating isoform of the prion protein that forms protease-resistant amyloid-like fibers and causes neuronal cell death in the brain. Increased levels of oxidative damage markers (lipid peroxidation products, oxidized and/or nitrated proteins)

**Table 1. The Table Presents Several Examples of the Use of In Vitro Cell/Tissue Culture Models for Studies of Specific Aspects of Pathogenesis of Different Neuropathological Processes which May Involve the Development of Oxidative Stress Condition**

Neuropathological process (disorder)	Pathogen*	Cell/tissue culture model	Reference**
Alzheimer’s disease	Amyloid beta-peptide, 4-hydroxynonenal (HNE)	Primary rat hippocampal cell cultures PC 12 cells Organotypic slice cultures	Butterfield <i>et al.</i> , 1999; Keller <i>et al.</i> , 1999 Bozner <i>et al.</i> , 1997 Bruce <i>et al.</i> , 1996
Prion disease	102-126 PrP peptide	Mixed neuronal/microglial cell culture	Brown <i>et al.</i> , 1996
Huntington disease	3-nitropropionic acid (3-NP, a potent inhibitor of mitochondrial complex II)	Primary cultures of striatal neurons	Garcia <i>et al.</i> , 2002
Parkinson’s disease	6-hydroxydopamine, MPP+	Primary cultures of dopamine (DA) neurons and dopaminergic cell lines	Collier <i>et al.</i> , 2003
HIV-associated dementia	HIV-1 neurotoxic proteins: Tat, gp 120	Primary rat hippocampal cell cultures Primary rat cortical cell cultures Human fetal neuronal cell cultures	Kruman <i>et al.</i> , 1998 Bonavia <i>et al.</i> , 2001 Turchan <i>et al.</i> , 2001
“Normal” aging of CNS cells	NA	Long-term primary rat hippocampal cell cultures	Aksenova <i>et al.</i> , 1999

\* Neurotoxic substance, which is believed to play a prominent role in the pathogenesis of the disorder and may cause oxidative injury to affected neural cells.

\*\* Representative reference picked up from a number of related articles.

have suggested the involvement of oxidative stress in those neurodegenerative disorders. The toxicity of synthetic peptide corresponding to 106-126 amino acids of PrP was used by different groups to mimic neurotoxicity of prions. A peptide corresponding to 102-126 of human prion protein was found to be toxic to cultured neurons (De Gioia *et al.*, 1994). Testing of this peptide in neuronal and astroglial cell cultures suggested that it triggers changes in expression of the inducible form of antioxidant enzyme heme oxygenase, which is considered to be one of the genetic markers of oxidative stress, in cultured astrocytes but not in neurons (Milhavel *et al.*, 2002). As it was demonstrated using a mixed neuronal/microglial cell culture, neurotoxic effect of 102-126 PrP peptide requires the presence of microglia which responds to the presence of the peptide by increasing the oxygen radical production (Brown *et al.*, 1996). Thus, recent findings from cell culture studies suggested that mechanism of PrP peptide toxicity involves activation of microglia, oxidative stress, and direct interaction with PrP-synthesizing neurons that reduce their ability to cope with oxidative stress (Giese *et al.*, 2001). However, it is not clear if the use of synthetic prion peptide to model TSEs pathogenesis is really pertinent since the peptide does not present *in vivo* in courses of the disease (Milhavel *et al.*, 2002).

#### **Huntington Disease: Cell/Tissue Culture Studies of "Huntingtin"-Induced Neurodegeneration**

An autosomal mutation in chromosome 4 that results in expression a mutant "huntingtin" protein with abnormally expanded polyglutamine region is a characteristic genetic feature of. The role of mutant huntingtin in striatal neurodegeneration associated with HD remains unclear. One of the hypotheses of the pathogenesis of Huntington's disease suggests the role for a mitochondrial metabolic defect that leads to the development of oxidative stress in this neurological disease (Gutekunst *et al.*, 2000). Several laboratories provided evidence supporting this view. Decreased activity of mitochondrial complex II has been reported in the postmortal brain tissue obtained from patients who suffered from Huntington's disease. Increased levels of lipid peroxidation products such as isoprostanes and 3-nitropropionic acid (3-NP) were also found in HD CSF, cortex and striatal tissue samples. Since 3-NP is known to be a potent irreversible inhibitor of complex II, this substance is used widely in modeling of Huntington-associated oxidative stress. Studies of 3-NP neurotoxicity in cultures have demonstrated that oxidative damage is involved in the mechanism of neuronal cell death induced by that substance (Fig. 3B) and may account for hallmarks of oxidative stress associated with HD (Garcia *et al.*, 2002; Teunissen *et al.*, 2002).

#### **Parkinson's Disease: Cell Culture Studies of 6-Hydroxydopamine (6-OHDA) and N-Methyl-4-Phenyl-1,2,3,6-Tetrahydropyridine (MPTP) Neurotoxicity**

The major symptoms of Parkinson's disease are related to the loss of the midbrain dopamine neurons within the substantia nigra which normally project to the caudate nuclei and putamen (Bernheimer *et al.*, 1973; Fearnley *et al.*, 1991). Genetic transmission only represents a small percentage, mostly early-onset cases and involving mutations of

synuclein and parkin genes. Environmental factors, especially pesticides, have been implicated. Although there are a number of hypotheses attempting to explain the biochemistry of Parkinson's disease (PD), the one on oxidative stress has attracted major interest (Koutsilieris *et al.*, 2002; Olanow, 1990; Youdim *et al.*, 1993). Biomarkers of oxidative damage in PD are primarily located in the nigrostriatal system. Combined with the selective loss of dopaminergic neurons, this illuminated the hypothesis on the role of disruptions in dopamine metabolism as a possible reason for oxidative stress in the PD brain, and the oxidative stress-based toxicity of 6-hydroxydopamine (6-OHDA) was demonstrated in cell culture models (Carrasco *et al.*, 2002; Grunblatt *et al.*, 2000a). About two decades ago N-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) was identified as a causative agent of parkinsonism in the young drug abusing population (Speciale, 2002). After the causative role of MPTP in human clinical syndrome was proven, numerous studies were undertaken to create *in vivo* and *in vitro* models of MPTP neurotoxicity (Leonardi *et al.*, 1998). Cell cultures and animal models helped to further the understanding that oxidative stress is a key aspect of MPTP neurotoxicity (Grunblatt *et al.*, 2000b).

#### **HIV-Associated Dementia: Role of Viral Proteins in Pathogenesis of Neurodegeneration Associated with HIV Infection**

HIV-associated dementia complex, a serious disabling condition that develops in approximately 20% of patients with HIV-1 infection, is now among other human dementive pathologies where oxidative stress is hypothesized to play its role (Shor-Posner *et al.*, 2002). Neurotoxic properties of some HIV-1 proteins and compounds released by HIV infected cells in the CNS have been reported (Nath, 2002). Viral proteins Tat, Nef, Vpr, protease, and gp120, have been implicated in initiation and/or intensification of oxidative stress (Perl *et al.*, 2000). Significant amounts of the results in this field were generated using neural cell cultures as an experimental model. Neurotoxicity of Tat and gp120 was studied in cultures of rat hippocampal (Kruman *et al.*, 1998), cortical neurons (Bonavia *et al.*, 2001), and in human fetal neuronal cell cultures (Turchan *et al.*, 2001). Cell culture studies made a significant contribution to characterization of neurotoxic properties of HIV-1 proteins and provided substantial evidence for the role of oxidative stress in the pathogenesis of AIDS-associated brain pathology. From the *in vitro* studies that use cell cultures it becomes obvious that direct interactions of viral products with susceptible neurons and their ability to activate astrocytes and microglia may trigger oxidative stress-based forms of neuronal apoptosis (Bruce-Keller *et al.*, 2001; Haughey *et al.*, 2001; Howard *et al.*, 1999; Kruman *et al.*, 1998; Perez *et al.*, 2001). Our own studies of Tat neurotoxicity in rat hippocampal cell cultures show that direct interactions of HIV-1 Tat protein with neurons induce significant increase in intracellular ROS production followed by increased protein oxidation (Fig. 3C). Cell culture studies also provided evidence that antioxidant compounds offer protection against neurotoxicity of Tat and gp120, thereby confirming that oxidative stress plays a key role in neuronal cell damage mediated by these virotoxins.

### **Cell Culture Models of Drug Abuse and Synergistic Toxicity of Drugs and Infectious Pathogens**

Cell culture studies have also been performed to investigate oxidative stress and neuropathology associated with alcoholism and drug abuse. Human brain microvascular endothelial cells (HBMEC) have been used to test the hypothesis if cellular and molecular mechanisms of methamphetamine (METH)-induced neurotoxicity may involve alterations of cellular redox status and induction of inflammatory genes. Results indicated that METH-induced disturbances in cellular redox status and activation of AP-1 and NF-kappaB can play critical roles in the signaling pathways leading to upregulation of inflammatory genes in HBMEC (Lee *et al.*, 2001a). Another report from the same team of the researchers through the link between cerebrovascular pathology associated with cocaine usage and the ability of this drug to trigger activation of redox-sensitive transcription factors and induce the inflammatory gene responses in cultured endothelial cells (Lee *et al.*, 2001b). The possible role of changes in ROS production has been investigated in astroglial cell cultures subjected to chronic treatment with ethanol. Along with the decrease of cell viability, an increased formation of malondialdehyde (a marker of lipid peroxidation) and the decrease of activity of oxidative-sensitive enzyme, glutamine synthetase has been demonstrated (Muscoli *et al.*, 2002). It was also shown that exposure of cloned hippocampal cell line HT22 to different doses of ethanol causes dose-dependent increase in protein oxidation (Pirlich *et al.*, 2002).

During recent years cell culture-based studies became a source of valuable information in the quickly emerging field of the biomedical research – molecular mechanisms of synergistic effects infective pathogens and drugs of abuse. These studies help to establish and confirm the ability of drugs of abuse to enhance neurotoxic effects of infective pathogens, such as HIV-1 proteins Tat and gp120 (Turchan *et al.*, 2001; Maragos *et al.*, 2002). In course of this research, evidence starts to accumulate suggesting that cocaine and several other drugs of abuse can influence specific molecular processes (possibly oxidative stress-dependent signaling pathways) that further contribute to toxic effects of HIV-1 transactivating protein Tat and virus' envelope protein gp120 (Gurwell *et al.*, 2001; Maragos *et al.*, 2002; Nath *et al.*, 2002; Turchan *et al.*, 2001).

### **“Aging in A Dish”: Investigation of Oxidative Stress Associated with Normal Aging and the Use of Cell/Tissue Culture Models**

Cell cultures are widely used as models to study the molecular mechanisms of aging. Following the discovery of a finite proliferation capacity of normal dividing cells *in vitro*, a phenomenon interpreted as aging at the cellular level and known as “replicative senescence” (Hayflick, 1998), more than 30 years of intensive research provided deep insights into the division counting mechanism in dividing mammalian cells. However, questions remain whether investigation of replicative senescence will shed insight into the senescence of postmitotic differentiated cells (Cristofalo, 1996; Rubin, 1997). Several investigators have suggested that stationary phase cell cultures (Khokhlov, 1992; Rubin,

1997) might be a more advisable model to study mechanisms of aging of postmitotic differentiated cells. During the last decade due to a significant progress in the technology of culturing of highly differentiated cells it became possible to consider the application of long-term neuronal primary cultures in neurobiology of aging. In general, brain aging studies can benefit from using one of the following cell culture-based experimental approaches: the use of long-term primary cultures of fetal neurons; the use of primary CNS cell cultures obtained from young and senescent donors for comparative brain aging studies; the use of primary neuronal cultures prepared from animals with genetically modified pace of aging process.

The overall evidence indicates differentiated cells *in vivo* accumulate damage over lifetime that results in gradual loss of function and increased probability of degeneration (Drachman, 1997; Rubin, 1997). A significant body of evidence has been accumulated saying that increased oxidative damage plays a role in the normal aging process and the aging brain exhibits markers of oxidative stress (Bowling *et al.*, 1995; Butterfield, 1996; Markesbery, 1997). It was demonstrated that neuronal survival in long-term primary culture changes according to the Gompertz law that mathematically describes dynamic changes of death probability in aging populations and non-neuronal stationary cell cultures (Aksenova *et al.*, 1999a). “Aging in a dish” of rat hippocampal neurons (Aksenova *et al.*, 1999a) was associated with increased oxidative damage of cell proteins (Fig. 3A). During aging in long-term primary culture, rat cortical neurons exhibited a complex profile of protein nitration and age-related variations in the biochemistry of neuronal apoptosis (Lesuisse *et al.*, 2002). Investigation of neuronal ageing in long-term cultures demonstrated age-related changes of Ca<sup>2+</sup> homeostasis similar to those obtained in aged brain slices (Toescu *et al.*, 2000).

Novel techniques that allow isolation and culturing of living neurons from rapidly autopsied human elderly brains (Konishi *et al.*, 2002; Picaud *et al.*, 1998) provide a well-suited *in vitro* model to study age-related biochemical changes in the brain at cellular levels. Recent studies report successful development of methods for dissociation, purification and culturing of neurons from adult and aged rat CNS tissue (Brewer, 1997; Orike *et al.*, 2001). However, the use of dissociated cell cultures in neurobiology of aging remains limited. Organotypic slice cultures from adult and elderly brain are easier to prepare and maintain. Tissue cultures from adult human postmortem brains may enable researchers to study age and disease-related processes directly in human brain tissue (Verwer *et al.*, 2002). Brain slices from laboratory animals of different ages can be used to evaluate the oxidative-stress related parameters and order of their appearance in the brain aging process (Sasaki *et al.*, 2001). Age-related changes of ROS production in the brain were found in rat brain slice cultures obtained from 12-, 24-, and 60-day-old animals (Schreiber *et al.*, 1995). Several studies of brain slices obtained from animals of different ages have indicated that brain aging is associated with increased inability to cope with oxidative damage (Joseph *et al.*, 1996a,b). The higher resistance of immature brains to hypoxia compared to adult brain was found to be attributable

to a lower involvement of free radicals due to a lower aerobic glucose metabolic rate (Maruoka *et al.*, 2001).

Transgenic animals with altered rates of the aging process are represented by the SAM (senescence accelerated mice) strain of mice, which is actually a group of related inbred strains consisting of series of SAMP (accelerated senescence-prone, short-lived) and SAMR (accelerated senescence-resistant, long-lived) strains. Comparative studies of SAMP and SAMR mice (including cell/tissue culture studies) showed that the higher oxidative stress observed in SAMR mice is partly caused by mitochondrial dysfunction, and may be one cause of the senescence acceleration and age-dependent alterations in cell structure and function, including neuronal degeneration (Hosokawa, 2002).

### **Neuroprotection Against Oxidative Stress: The Role for Tissue/Cell Culture Models in the Development of New Therapeutic Approaches for Prevention of Oxidative Damage and Neurodegeneration in the Brain**

Antioxidants that prevent detrimental consequences of ROS are considered to be a promising approach to neuroprotection (Behl, 1999; 2002a,b,c; Fang *et al.*, 2002; Floyd, 1999; Moosmann *et al.*, 2002; Prasad *et al.*, 2000; Shor-Posner *et al.*, 2002). For the most part, experimental evidence demonstrating neuroprotective activities of antioxidants has been obtained *in vitro*, in tissue/cell culture models. Rationale for the possible clinical effectiveness of antioxidants in several neurodegenerative conditions has arisen out of years of basic research (Floyd, 1999) involving extensive use of cell culture-based experimental approaches. Due to the high sensitivity of CNS cells to oxidative damage, inclusions of antioxidants were found essential for optimization of cell viability, maintaining the long-term neuronal survival, and prevention of neurodegeneration *in vitro* (Brewer, 1997; Ricart *et al.*, 2001). Using different experimental strategies of induction of oxidative stress condition in culture (Gille *et al.*, 1992), various antioxidant compounds were shown to provide effective neuroprotection against ROS (Floyd *et al.*, 2002; Khaspekov *et al.*, 1995; Lee *et al.*, 2000; Lezoualc'h *et al.*, 1996; Ricart *et al.*, 2001; Shea *et al.*, 2002; Uhr *et al.*, 1998; Wei *et al.*, 2000). Three major experimental approaches for modeling of oxidative stress in cell cultures: extracellular sources of ROS, normobaric/hyperbaric hyperoxia, and hypoxia/reoxygenation are usually used in neuroprotective antioxidant activities testing. Each of these approaches has some specific methodological and practical aspects that must be taken into account for proper analysis of the results of antioxidant neuroprotection studies. Exposure of tissue/cell cultures to extracellular sources of ROS presents a group of experimental paradigms that involves subjection of a culture to enzymatic or non-enzymatic free radical generating system for certain amount of time. For protection studies, antioxidant compounds are added before or together with free radical generators. Under these experimental conditions compounds that are able to scavenge the particular type of free radical generated usually demonstrate various degrees of protection. Antioxidant compounds can be tested for neuroprotection *in vitro* against the system known to generate the particular kind of free radical in order to get insight into the specificity of their free radical scavenging abilities. The protective effect of Ginkgo

biloba extract against oxidative damage of cerebellar granule cells exposed to Fenton reagent (hydrogen peroxide/ferrous sulfate, hydroxyl radical generator) suggested a potential effectiveness of this drug in brain pathology that is associated with hydroxyl radical production (Guidetti *et al.*, 2001; Wei *et al.*, 2002). Antioxidant protection against hydroxyl and peroxy radical by ferulic acid in neuronal cell culture has been recently demonstrated by Kanski and co-authors (Kanski *et al.*, 2002). Because of implications of peroxynitrite (neurotoxic product of chemical interactions between nitric oxide and ROS) in various types of human brain pathology, there is considerable interest in *in vitro* testing of potential peroxynitrite scavengers (Kanski *et al.*, 2001; Rodriguez-Martin *et al.*, 2002; Wei *et al.*, 2002; Whiteman *et al.*, 2002). Protective effects of red wine-derived flavonoids were investigated using cultured hippocampal neurons subjected to the nitric oxide free radical donors nitroprusside and 3-morpholinopyridone (Bastianetto *et al.*, 2000a). Testing in neuronal cell culture models subjected to different types of exogenous free radicals revealed that efficacy in reducing oxidative damage in CNS of many natural antioxidants, such as melatonin (Reiter *et al.*, 1999), plant flavonoids and polyphenols (Scapagnini *et al.*, 2004; Bastianetto *et al.*, 2000a, b) derives from their abilities to directly scavenge a number of free radicals.

Subjection of cultured cells to high concentration of oxygen (95-100%) at normal atmospheric pressure causes non-apoptotic oxidative stress-related cell death (Kazzaz *et al.*, 1996). Neuronal cell death produced by hyperoxia may be related to lipid peroxidation induced by ROS when cellular antioxidant defenses are overwhelmed (Ahdab-Barmada *et al.*, 1986). It has been shown that hyperoxia causes its damage due to generation of free radicals and antioxidants can afford protection. However, cell culture models of hyperoxia-induced oxidative stress are rarely used in studies of CNS antioxidant protection. Due to its ability to scavenge oxygen-derived free radicals, melatonin was shown to be protective against hyperoxia-induced death of bovine cerebellar endothelial cells in culture (Shaikh *et al.*, 1997).

Hypoxia/reoxygenation-induced oxidative stress is considered to play a significant role in human brain pathology. Therefore, this kind of cell culture models of oxidative stress is used for investigation of mechanisms of antioxidant neuroprotection and testing of potential neuroprotectants. Hypoxia/reoxygenation procedure induces oxidative form of apoptosis in cultured neurons (Chen *et al.*, 2002; Lievre *et al.*, 2000). Neuronal injury resulting from hypoxia/reoxygenation is mediated by activation of NMDA receptors, the production of NO, and the generation of free radicals (OH<sup>-</sup> and O<sub>2</sub><sup>-</sup>). Although the mechanisms of such an injury are rather complex, increased intracellular ROS production that occurs in CNS tissue/cell cultures submitted to hypoxia/reoxygenation is believed to play a key role in pathogenesis of degeneration. The results of studies in dissociated primary cultures (Wang *et al.*, 2002) and in tissue slice cultures (Vlkolinsky *et al.*, 1999) demonstrated that compounds with antioxidant activity may effectively protect neurons against hypoxia/reoxygenation oxidative injury. As far as antioxidant neuroprotection is concerned, this model helps to test the ability of potential antioxidants to quell intracellular

increase in free radical production. It has been employed in testing of cell permeable ROS scavengers (Yamada *et al.*, 2003), phenolic antioxidant compounds (Khaspekov *et al.*, 1992) and lipophilic antioxidants (Horakova *et al.*, 1997; Tagami *et al.*, 1998). Study of neuronal primary cultures subjected to hypoxia/reoxygenation demonstrated that natural antioxidants, such as melatonin (Cazeville *et al.*, 1997) and vitamin E (Tagami *et al.*, 1998), can decrease intraneuronal ROS accumulation and protect neurons from degeneration. Using this cell culture model it was found that neuroprotective effects of estrogens, 17-beta and 17-alpha estradiols, against hypoxia/reoxygenation-induced neurodegeneration are independent from the ability of the steroid to bind the estrogen receptor (Zaulyanov *et al.*, 1999).

CNS tissue/cell cultures subjected to specific neurotoxic substances which may play a role in pathogenesis of neurological diseases associated with oxidative stress are employed in numerous studies that investigate mechanisms of antioxidant protection or try to find, develop and test new antioxidant compounds. Many of such studies emerge as soon as the association of neurotoxic effects of the particular substance with oxidative stress is revealed and before the mechanism of oxidative injury associated with the particular disease is well understood. There is no doubt that studying cell culture models of oxidative stress caused by disease-specific neurotoxic substances can provide valuable information supporting the rationale of antioxidant strategies in treatment of the particular neurological disorder and shed light on the molecular basis of oxidative damage and antioxidant protection against it. However, due to obvious limitations of the cell toxicology approach to modeling of neurodegenerative disorders with complex and multifactorial pathogenesis, significant parts of promising results may not pass *in vivo* testing or clinical trials and, therefore, will not evolve into viable antioxidant therapeutic strategies.

In Alzheimer's disease (AD) the free radicals that have been incriminated as causing neuronal degeneration are believed to be generated by beta-amyloid (Butterfield *et al.*, 1999) and/or activated microglia (Anderson *et al.*, 2001; Colton *et al.*, 2000). Neuronal damage may be attributed to oxidative processes initiated by amyloid-derived free radicals species. Evidence from electron paramagnetic resonance (EPR) spin labeling techniques and spectrophotometric assays suggested that synthetic  $\beta$ -amyloid exhibits hydrogen peroxide-like reactivity toward Fe<sup>2+</sup>, nitroxide spin probes (Butterfield, 1996). Studies that showed effective blockade of  $\beta$ -amyloid toxicity by free radical scavengers added to CNS cell cultures support this hypothesis (Yatin *et al.*, 2000; Bruce *et al.*, 1996; Harris *et al.*, 1995; Zhou *et al.*, 1996). Evidence of the effective protection against amyloid beta peptide toxicity in cultures by lipophilic chain-breaking antioxidants supported the role of increased lipid peroxidation in the mechanism of  $\beta$ -amyloid-induced neurodegeneration (Behl, 1999; Varadarajan *et al.*, 2000). The ability of different antioxidants to protect neurons in culture against amyloid beta-peptide toxicity has been intensively investigated (Butterfield *et al.*, 1999b; Yatin *et al.*, 2000; Sultana *et al.*, 2004; Behl *et al.*, 1997; Goodman *et al.*, 1994; 1996; Pappolla *et al.*, 1997). Those studies added significantly to the understanding of the basic mechanism of  $\beta$ -amyloid toxicity but had limited impact on the development of novel antioxidant strategies for treatment of

Alzheimer's disease.  $\beta$ -amyloid toxicity has its key role in Alzheimer's disease, but it is only a part of a very complex pathogenesis of this devastating age-related neurodegenerative disorder. It is unlikely to expect *in vitro* testing of antioxidant compounds against  $\beta$ -amyloid peptides to provide more than a rationale of their use and initial recommendations for intensive investigation of their efficacy in other experimental models of the disease.

Evidence from postmortem analysis implicates the involvement of microglia in the neurodegenerative process of several degenerative neurological diseases, including Alzheimer's disease (McGeer *et al.*, 2002). It remains to be determined, however, whether microglial activation plays a role in the initiation stage of disease progression or occurs merely as a response to neuronal death (Liu *et al.*, 2003). Nevertheless, neuroinflammation may be an important part of the pathogenesis of oxidative stress in AD. Therefore, possible ways to quell negative effects of microglial activation are under investigation and cultures of microglial cells are employed in these studies (Liu *et al.*, 2003; Lue *et al.*, 2001). Aggregated amyloid peptides, neurofilaments, oxidized and glycosylated proteins that present in increased quantities in the AD brain, can activate microglia and trigger production of prooxidants by activated microglial cells. Quenching of neurotoxic free radical products by various natural and synthetic compounds can be studied effectively *in vitro* and may be a source of useful information for the development of neuroprotective agents that may be beneficial not only for treatment of AD, but also for treatment of other neurodegenerative diseases where the involvement of inflammation is evident (Durany *et al.*, 1999; Floyd, 1999; Wong *et al.*, 2001).

Since MPTP/MPP<sup>+</sup> toxicity is believed to model some basic aspects of neuronal degeneration in Parkinson's disease, MPTP-treated tissue/cell culture models are used in studies of possible neuroprotective drug strategies in parkinsonism. Another type of Parkinson-specific *in vitro* model, cultured CNS cells submitted to 6-hydroxydopamine (6-OHDA), is also employed in studies with the intent to develop drugs able to protect neurons against oxidative stress in this neurologic disease. Furthermore, recent work from human and animal studies has provided evidence for inflammatory component of Parkinson's disease pathogenesis (Grunblatt *et al.*, 2000a,b). Therefore, results of antioxidant protection studies that involve activated microglia cell culture models may be useful. *In vitro* findings of effective protection of dopaminergic neurons against MPP<sup>+</sup>-mediated oxidative damage by melatonin (reviewed in Reiter, 1998), 1-deprenyl (selegiline) (Wu *et al.*, 1994), epigallocatechin gallate, lizaroids U74389G and U83836E, resveratrol, MnTBAP, MCI 186, and Trolox (Stull *et al.*, 2002) indicated that clinical trial of some of those antioxidants should be considered. Mechanism of antioxidant protection of dopaminergic neurons against MPTP-induced injury is associated with scavenging of hydroxyl radical (Li *et al.*, 2002). Autooxidative formation of H<sub>2</sub>O<sub>2</sub> plays a key role in 6-hydroxydopamine neurotoxicity, and the compounds able to promote the removal of the excess of H<sub>2</sub>O<sub>2</sub>, prevent its decomposition into hydroxyl radical and superoxide, or able to quench those ROS may offer neuroprotection and may be efficient therapeutics in treatment of

Parkinson's disease. Cell culture models of MPTP/MPP+ and 6-OHDA toxicity are useful tools in studies intended to find appropriate drugs to protect dopaminergic neurons from oxidative injury. Studies in PC12 cell cultures treated with 6-OHDA demonstrated a potential ability of green tea and black tea polyphenols to protect dopaminergic neurons in the Parkinson's disease brain from oxidative damage (Levites *et al.*, 2002; Nie *et al.*, 2002). Protective effects of pyruvic acid, a neuronal metabolic fuel that undergo decarboxylation to diffuse hydroxyperoxide into water, against 6-OHDA toxicity in murine neuroblastoma cell cultures have been recently reported (Mazzio *et al.*, 2003). Potent antioxidant protection of PC12 cells by radical scavenger and iron chelator apomorphine against 6-OHDA and MPTP has also been reported (Grunblatt *et al.*, 1999).

Copper-mediated oxidative damage is proposed to play a critical role in the pathogenesis of Cu/Zn superoxide dismutase (SOD1)-linked familial amyotrophic lateral sclerosis (FALS) (Beckman *et al.*, 2001; Bush, 2002; Hand *et al.*, 2002; Okado-Matsumoto *et al.*, 2002). Mutations in Cu/Zn superoxide dismutase (SOD1) are believed to cause neuronal degeneration, paralysis, and death in FALS. These mutations structurally weaken SOD, which indirectly decreases its affinity for Zn. Zn-deficient SOD induces apoptosis in motoneurons through a mechanism involving peroxy-nitrite. Importantly, Zn-deficient wild-type SOD is just as toxic as Zn-deficient ALS mutant SOD, suggesting that the loss of Zn from wild-type SOD could be involved in the other 98% of cases of ALS (Beckman *et al.*, 2001). While the key role of mutant SOD1 isoforms in the development of oxidative stress condition in ALS is still debated (Lino *et al.*, 2002), cultured neuronal cells stably transfected with mutant forms of SOD1 and mixed spinal cord cultures from the Cu/Zn-SOD mutant mice are used as *in vitro* models of ALS-associated oxidative stress (Cookson *et al.*, 2002; Kruman *et al.*, 1999; Menzies *et al.*, 2002). Evidence supporting possible effectiveness of antioxidant therapy in ALS has been accumulated from *in vitro* studies using those cell culture models. It was shown that pretreatment of cultures with spin trapping molecule, 5',5'-dimethylpyrroline-N-oxide (DMPO), prevented mutant SOD1-mediated mitochondrial dysfunction and cell death (Liu *et al.*, 2002). Spinal cord cultures from mice expressing mutant Cu/Zn-SOD exhibited increased vulnerability to glutamate toxicity and vitamin E, nitric oxide-suppressing agents, peroxynitrite scavengers, and estrogen protected the cells from free radical-mediated injury (Kruman *et al.*, 1999).

Oxidative stress has been implicated in neuronal damage associated with drug abuse and xenobiotic neurotoxicity. Since antioxidant therapy has been considered to prevent propagation of brain tissue damage and improve neuronal survival in those types of neuropathological conditions (Gilgun-Sherki *et al.*, 2002; Pushpendran *et al.*, 1998), cell culture models have been developed to investigate protective abilities of antioxidant compounds. Neurodevelopmental damage can occur as a result of *in utero* exposure to alcohol. Oxidative stress is one of many proposed mechanisms thought to contribute to nervous system dysfunction characterized in fetal alcohol syndrome (FAS). The possibility to ameliorate combined toxic effect of acute (2 hr) ischemia, chronic (16 hr) hypoglycemia, and ethanol (200, 400, 800 or

1600 mg/dl) exposure on cultured rat fetal hippocampal neurons by antioxidant (beta-carotene and vitamin E) supplementation was reported by Mitchell and co-authors (Mitchell *et al.*, 1999). Epidemiological studies on arsenic contamination in drinking water indicated presence of arsenic in fetal tissues. Experiments on human fetal brain explants on exposure to arsenic in culture showed disturbance in lipid peroxidation, generation of nitric oxide (NO), reactive oxygen species (ROS) and apoptosis. The administration of vitamins C, E, and DMSA showed partial reversal of the effects indicating possible protection from arsenic toxicity (Chattopadhyay *et al.*, 2002). Vitamin E (alpha-tocopherol), a lipophilic free radical scavenger, was shown to prevent haloperidol-induced free radical-mediated necrosis of neuronal cells *in vitro* (Behl *et al.*, 1995). The effective blockade of methylmercury neurotoxicity by oxygen radical scavengers, such as glutathione, catalase, selenium, and cysteine *in vitro* has been reported by Park and co-authors (Park *et al.*, 1996).

Cell culture models of oxidative stress are widely used for testing of antioxidant neuroprotective properties of a variety of synthetic compounds. Nitron spin-traps have been used for more than 30 years in analytical chemistry and biochemistry for the purpose of identification and characterization of free radicals until alpha-phenyl-tert-butyl nitron (PBN), one of the more widely used nitrones for that purpose, has been shown to have potent neuroprotective activities against oxidative damage (Floyd *et al.*, 2002). The nitrones alpha-phenyl-N-tert-butyl nitron (PBN), sodium 2-sulfophenyl-N-tert-butyl nitron (S-PBN) and disodium 2,4-disulfophenyl-N-tert-butyl nitron (NXY-059) have been shown to be neuroprotective in a variety of experimental models, including cell cultures (Barth *et al.*, 1996; Floyd *et al.*, 2002; Yue *et al.*, 1992). Nitrones oppose oxidative challenges by virtue of their ability to trap very rapidly oxygen or carbon centered radicals thus generating nitroxide radical species, which are more stable, and biochemically less harmful than the original radical (Goldstein *et al.*, 2000). Screening of novel variants of nitrones can be significantly facilitated by the use of cell culture models. Studies on carboxyfullerenes, a class of water-soluble antioxidant compounds, indicated that they are capable of eliminating both superoxide anion and H<sub>2</sub>O<sub>2</sub>. Carboxyfullerenes demonstrated robust protection against excitotoxic, apoptotic and metabolic oxidative stress-mediated insults in cortical cell cultures (Dugan *et al.*, 2001).

Cell culture studies provide valuable information about the mechanisms of antioxidant effects and may open the door toward the rational design of neuroprotective antioxidants with optimized properties. Among the family of steroidal molecules, estrogens have the capability of preventing neuronal cell death caused by increased oxidative burden. Employing neuronal cell lines it has been demonstrated that the neuroprotective antioxidant effects of estrogens may depend on their basic chemical properties as hydrophobic phenolic molecules. This finding opened the way to construction of different synthetic analogs of estrogens that exhibit antioxidant activity but lack of sex hormone properties (Behl, 2000). Testing of neuroprotective abilities of synthetic analogs of natural antioxidants against ROS toxicity in cell culture models helped to understand chemical

basis of antioxidant activities (Moosmann *et al.*, 1999). Starting with melatonin, making step by step changes in the chemical structure and testing various aromatic alcohols as well as phenol derivatives for their potential antioxidant activity, Uhr and co-authors (Uhr *et al.*, 1998) demonstrated that aromatic alcohols with intact phenolic groups protect neurons effectively against oxidative damage and cell death. The tested compounds directly inhibited peroxidation reactions suggesting that the neuroprotection is mediated by radical scavenging properties.

Cell culture models of general and disease-specific forms of oxidative stress allow fast screening and comparison of antioxidant activities. Testing of five catechins [(-)-epigallocatechins gallate (EGCG), (-)-epicatechin gallate (ECG), (-)-epigallocatechin (EGC), (-)-epicatechin (EC), and (+)-catechin (C)] with regard to their effect on 6-OHDA-induced oxidative apoptosis in PC12 cells indicated significant difference in the degree of antioxidant protection by those phenolic antioxidants. The data suggested that two of those five related compounds might be potent neuroprotective agents for Parkinson's disease (Jin *et al.*, 2001). The comparison of the effects of indoleamines (serotonin, melatonin, and tryptophan) on the iron-mediated neuronal oxidative damage in cell culture (PC12 cells) suggested that serotonin shows protective effect comparable to melatonin and may be considered as a possible antioxidant agent in oxidative stress-related brain pathology (Park *et al.*, 2002). In cell culture model, N1-acetyl-N2-formyl-5-methoxykynuramine (AFMK), a naturally occurring biogenic amine with potent free radical scavenging capacity, efficiently reduced hippocampal neuronal death induced by either hydrogen peroxide, glutamate, or amyloid beta (25-35) peptide and thus was suggested to be a valuable new antioxidant for preventing and treating free radical-related disorders (Tan *et al.*, 2001).

Together with other experimental approaches, studies using cell culture models may help to get insight into intricate details of the molecular mechanisms of antioxidant neuroprotection and help to understand how those mechanisms can stretch beyond direct scavenging of ROS. Study of Yoshioka and co-authors on antioxidant activity of the dopamine agonist cabergoline indicated multiple antioxidant mechanisms for that compound, such as activator of the glutathione (GSH) system and direct free radical scavenging (Yoshioka *et al.*, 2002).

### **New Research Advances in Gene/Protein Expression Analysis: An Impact on CNS Culture Modeling of Oxidative Stress Condition**

The use of genomic and proteomic research tools for cell culture-based studies of oxidative stress in the brain enable to carry out global surveys of the alterations at mRNA and protein levels and to unveil their regulation. Combination of CNS cell culture models of oxidative stress with powerful methods of gene expression analysis significantly increases our research potential for the investigation of the molecular basis of oxidative stress and cell responses to oxidative insults.

Cytoskeletal lesions and abnormal proliferation of neurites are among the major dementia-associated

abnormalities in AD. The possible link between oxygen free radical injury and some sprouting abnormalities that occur in the AD brain has been demonstrated using mapping of gene expression changes in cultured human neuronal cells treated with hydroxyl peroxide (De La Monte *et al.*, 2000). It was observed that in addition to the changes in pro-apoptotic and survival gene expression, H<sub>2</sub>O<sub>2</sub>-treated cells also manifested increased expression of growth and sprouting molecules, including GAP-43, nitric oxide synthase 3, neuronal thread protein (NTP; ~17 kD and ~21 kD forms), proliferating cell nuclear antigen, and phospho-Erk MAPK. In addition, the H<sub>2</sub>O<sub>2</sub>-treated cells had increased levels of p25, the catalytically active and stable cleavage product of p35, which regulates cdk-5 activity. Previous studies demonstrated p25 accumulation in AD brains and p25-induced hyperphosphorylation of tau and neuronal apoptosis.

Toxicogenomics, a rapidly developing genomics-derived discipline, is specifically focused on the investigation of the impact of chemicals and drugs on gene expression in biological systems. Enormous amount of data can be provided from the use of DNA microarray techniques for the simultaneous analysis of the expression pattern of multiple genes. The high-throughput requirement of these approaches necessitates *in vitro* cell culture systems (Corvi, 2002). Therefore, the combination of expression profiling tools with cell culture models is used now in neurotoxicological studies of potential prooxidant agents. Recently, the use of high-throughput approaches to studying gene expression have been used to identify functional changes occurring in cultured neural cells chronically exposed to ethanol (Rahman *et al.*, 2001). The use of the cDNA microarray approach in nigral dopaminergic cell line subjected to MPP<sup>+</sup> and several neurotoxins which were identified as epidemiological risk factors for PD, helped to test the hypothesis that these agents might induce cell death through a common molecular mechanism involving increase of ROS and following activation of apoptotic gene expression cascades (Chun *et al.*, 2001).

The mechanisms by which alterations to SOD1 elicit neuronal death remain uncertain despite intensive research effort. Analysis of differentially expressed individual proteins in the presence of mutant SOD1 represents a novel approach to further investigate this toxic gain-of-function. Effects of SOD1 mutations on protein expression were investigated in stably transfected motor neuron-like cell line NSC34 using proteomic approach and provided further evidence for involvement of alteration in antioxidant defenses (downregulated GST isoenzymes), proteasome function (downregulated proteasome 5i subunit) and nitric oxide metabolism in the development of FALS (Allen *et al.*, 2003).

Identification of individual proteins modified by ROS using proteomic techniques is a novel powerful tool that may significantly boost the research of the molecular basis of oxidative stress-mediated neurodegeneration in cell culture models. It provides the possibility to search for specific proteins most affected by the particular type of oxidative stress-mediated brain pathology and search for effective ways to protect those targets from oxidative damage. Proteomic identification of oxidatively modified proteins has

been recently used to investigate pattern of protein oxidation in postmortal AD brain (Aksenov *et al.*, 2001; Butterfield, 2004; Castegna 2002; Korolainen *et al.*, 2002). It has been successfully used in non-CNS cell culture models and clearly will be employed soon in cell culture models of brain oxidative stress as well.

## CONCLUSIONS

CNS cell/tissue cultures models are essential parts of the contemporary research tool set which is used for studies of oxidative stress in the brain. With constant developments in cell culture technology, gene engineering, and new methods of analysis, the research potential of *in vitro* modeling of general and disease-specific oxidative stress conditions in CNS cultures continue to grow. This approach offers definite advantages for investigation of cell type-specific vulnerabilities of neural cells to oxidative insults, oxidative stress-related cell responses and interactions of different CNS cell types with each other in oxidatively challenged brain. CNS culture models of oxidative stress are the method of choice in a variety of neurotoxicological studies and in studies of antioxidant neuroprotection since it makes possible to carry out wide-scale, high-throughput screenings and surveys of the parameters of cellular function under well-controlled experimental conditions. However, this is and always will be an *in vitro* experimental modeling of complicated and multifactorial *in vivo* pathological condition. Therefore, clear understanding of the limitations of these experimental models is required in order to take advantage of using CNS cell/tissue culture models in studies of oxidative stress in the brain.

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