CHAPTER V

DISCUSSION AND CONCLUSION

Oxidative stress refers to the cytotoxic consequences of oxygen radicals like superoxide anion, hydroxyl radical, and hydrogen peroxide, which are generated as byproducts of normal aberrant metabolic processes during ageing and other neurodegenerative diseases and act on polyunsaturated fatty acids (PUFA) in brain, thereby propagating the lipid peroxidation (Coyle and Puttfarcken, 1993). Major antioxidant and oxidative free radical scavenging enzymes are glutathione, superoxide dismutase (SOD) and catalase.

Nitric oxide (NO), first identified as endothelium-derived relaxing factor, is produced by different types of cells in multicellular organisms where it acts as a diffusible messenger in many forms of intercellular communication as well as of intracellular signaling (Moncada, Palmer, and Higgs, 1989; Nathan, 1992; Kerwin, Lancaster, and Feldman, 1995; Contestabile et al., 2003). Its role as a brain messenger molecule, acting as an unconventional neurotransmitter/ neuromodulator, was first recognized about 15 years ago (Garthwaite, Charles, and Chess-Williams, 1988). Since then, an impressive number of studies have addressed the multifarious actions of NO in brain physiology, with particular focus on mechanisms related to synaptic function and neural plasticity. An even larger number of studies have, on the other hand, explored the "dark side" of brain NO, i.e. its implication in several types of neural damage occurring when its production and release escape the control mechanisms and contribute to neurodegenerative processes which are linked to the development of neurological diseases such as vascular disease, Parkinson's disease, and Alzheimer's disease.

Search for natural antioxidants, especially of plant origin, has notably increased in recent years (Loliger, 1991). Antioxidative compounds obtained from natural sources, such as grains, oilseeds, beans, leaf waxes, bark, roots, spices, fruits and vegetables, have been investigated (Chen et al., 1996). As evidenced by previous unpublished observations that *Centella asiatica* extract and asiaticoside might be useful in slowing down neurodegenerative disease and prevention by their antioxidant properties or reducing free radical activity.

In the present research, we demonstrate that in neuroblastmoma cell cultures, SNAP, a NO donor, induces neuronal injury and death in a concentration-dependent manner. We also demonstrate that asiaticoside cannot protect SNAP-induced neurotoxicity whereas *Centella asiatica* extract possesses a certain degree of neuroprotective effect. However, the mechanism by which this beneficial effect may mediate is not fully understood.

In this culture system, exposure of cultured N1E-115 cells to 0.5-3 mM of SNAP for 12-24 hr induced gradual decreases in cellular MTT reduction in correlation with concentrations and time of exposure. A condition of exposure with 1 mM of SNAP for 24 hr was adopted as a standard cytotoxic condition which decreased mitochondrial metabolic activity of the culture to nearly 50% of that of control cultures (Fig.6). This toxic exposure condition resulted in an oxidative stress which was implied by a marked increase in NO generation (as considered from nitrite accumulation), a reduction of mitochondrial metabolic activity, and a reduction of glutathione content.

Asiaticoside and *Centella asiatica* extract have been reported to posses various pharmacological activities. This study primarily investigated effects of asiaticoside and *Centella asiatica* extract in N1E-115 neuroblastoma cell line in term of neuroprotective properties. Preliminary results revealed that the incubation for 24-48 hr, with concentration ranges of 1–100 μM for asiaticoside and 1 –100 μg/ml for *Centella asiatica* extract, had no cytotoxic effects on cultured neuroblastmoma cells as considered from the mitochondrial metabolic activity (Fig. 7 and Fig. 8). However, exposure to a high concentration range (200-500 μM) of asiaticoside revealed cytotoxic effects to cultured cells (Fig. 7). Similar experiments with a high concentration range of *Centella asiatica* extract were not done due to a limited supply of this test agent.

Because of cytotoxic effects of asiaticoside, and possibly *Centella asiatica* extract, at high concentrations, a low concentration range of asiaticoside (1-100 μM) and *Centella asiatica* extract (1-100 μg/ml) was used to investigate the possible neuroprotective effect of these test agents on NO-induced neuronal injuries in neuronal cell line cultures. The results indicated that the inhibition of metabolic activity by NO donor (SNAP) could be attenuated only by co-treatment with 25-100 μg/ml of *Centella asiatica* extract (Fig. 12). In addition, the cytoplasmic LDH release,

an indication of cell death, also significantly decreased under the same condition of co-treatment with 50-100 μg/ml of *Centella asiatica* extract (Fig. 13). Other conditions tested, including pre-treatment with 1-100 μM of asiaticoside or 1-100 μg/ml of *Centella asiatica* extract for 24 and 48 hr before exposure to 1 mM SNAP (Fig. 9 and Fig. 10) and co-treatment with 1-100 μM of asiaticoside and 1 mM of SNAP (Fig. 11), did not reveal any beneficial effects to cultured neurons.

These experimental findings suggest that *Centella asiatica* extract (triterpene rich fraction of the plant extracts) possess a certain degree of cytoprotective activity to cultured neurons which may mediate through its triterpinoid contents. Asiaticoside is clearly one of the active triterpenoids, and is found in the plant in the largest amount, but there may be other active principles including madecassoside. It was notable that the beneficial activity observed in *Centella asiatica* extract at 25-100 μg/ml concentrations on metabolic activity of neuroblastoma cells is unlikely due the asiaticoside content (38.56% in this study). It might be due to other active contents, e.g., madecassoside (37.20% in this study) and other unidentified substances, and these should be investigated as well. In addition, it has often been noted that combinations of antioxidants or cytoprotective agents are more effective than would be expected if they were acting independently of one another (Larson, 1995). Some synergy between them and the whole plant activity may be important.

Several experiments indicated that one mechanism of compound that can modulate oxidative stress and subsequent inflammation process is by scavenging free radicals such as nitric oxide. In this study, no alteration of SNAP-induced nitrite accumulation was observed after co-treatment with *Centella asiatica* extract (Fig. 13). The results suggest that reduction of mitochondrial damage and cell death by *Centella asiatica* extract does not appear to mediate through inhibition of NO generation or facilitation of NO scavenging.

Lipid peroxidation is an auto-catalytic, free-radical-mediated, destructive process, where by polyunsaturated fatty acids in cell membranes undergo degradation to form lipid hydroperoxides (Sevanian and Hochstein, 1985). These latter compounds then decompose to form a wide variety products, including low molecular mass hydrocarbons, hydroxyl aldehydes, fatty acids, ketones, alkenals and alkanals, in particular malonaldehyde (MDA). MDA content was evaluated as an end point, indicative of the extent of lipid peroxidation, since MDA is known to be the one of the

most abundant aldehydes formed as a byproduct of lipid peroxidation. Thus, reduction of MDA production would indicate inhibition of lipid peroxidation. It was rather surprising that exposure to 1 mM SNAP alone markedly reduced lipid peroxidation in cultured N1E-115 cells as compared to that of controls (Fig. 16). Along with this line, Rauhala, Sziraki and Chiueh (1996) found that NO released from SNAP did not stimulate, but rather inhibited brain lipid peroxidation in rat cortical homogenates stimulated by submicromolar concentrations of Fe^{2+}. These results suggest that less reactive NO radicals, by itself, do not mediate oxidative stress such as lipid peroxidation and may have antioxidant properties.

The reduced MDA production caused by SNAP was attenuated in the presence of 100 μg/ml *Centella asiatica* extract. The interpretation and significance of this finding are complicated. An obvious interpretation is that *Centella asiatica* extract did not possess lowering effect on lipid peroxidation but SNAP did. The decreased lipid peroxidation in SNAP-exposed group (compared with control group) in conjunction with suppressed mitochondrial metabolic activity and successive cell death may imply that cytotoxic effects of SNAP may be due to other mechanisms than the generation of lipid peroxidation.

Alternatively, there are several possible pathways associated with NO-induced neurotoxicity. Sustained elevation of extracellular Ca^{2+} produces NO persistently, which increases the chance of exposure to oxidative stress (Kroncke, Fehsel, and Kolb-Bachofen, 1997). Beckman and his group found that a short lived peroxynitrite ($ONOO^{-}$), an oxidized form of NO produced by the reaction with superoxide anions (O_2^{-}), is the central molecule to cause NO-induced cell death in 1990. The main target organelles of $ONOO^{-}$ are the nucleus and mitochondria. $ONOO^{-}$ breaks single strands of DNA (Wink et al., 1991), which activates poly(ADP-ribose) polymerase (PARP) in the nucleus. Activated PARP excessively transfers ADP-ribose from NAD^{+} to histone or PARP itself. This causes insufficient ATP production and energy failure (Zhang et al., 1994), resulting in ATP depletion and cell death. P53, a tumor suppressor protein in the nucleus, is upregulated in reaction to DNA damage by NO (Messmer et al., 1994), resulting in apoptosis (Levine, 1997).

Since NO can easily permeate into mitochondria, and O_2^{-} is constantly generated by the electron transport system, mitochondria have a high risk of exposure to $ONOO^{-}$. $ONOO^{-}$ inactivates succinate dehydrogenase and nicotinamide adenine dinucleotide phosphate (NADH) dehydrogenase, and also mitochondrial ATPase,

such that mitochondrial respiration is irreversibly inhibited (Radi et al., 1994). In addition, $ONOO^-$ inactivates Mn-SOD, which is exclusively located in the mitochondria and promotes the production of $ONOO^-$(Macmillan-Crow et al.,1996). $ONOO^-$ oxidizes thiols and NADPH to induce mitochondrial permeability transition pore (PTP), leading to mitochondrial Ca^{2+} efflux (Packer and Murphy, 1994; Packer et al., 1997). The PTP permits the free movement of a large number of molecules in and out of mitochondria, leading to mitochondrial collapse and cell death (White and Reynolds, 1996). It has been reported that cytochrome c, an important molecule which activates the apoptotic cascade by the polymerization of apoptotic protease activating factor 1 (Apaf 1), followed by the activation of caspase-9 and caspase-3, is released from mitochondria (Eskes et al.,1998). In neuronal cells, $ONOO^-$ nitrates the tyrosine residues in neurofilaments, which results in damage to axonal flow and neuronal death.

In cultured neurons, neuronal cell death produced by a low level of NO donor is characterized by delayed neuronal death, and this is apoptosis rather than necrosis (Bonfoco et al., 1995). One study revealed that in a serum-free medium, SNAP induced apoptosis from concentrations of 500 μM to 1 mM and at a concentration of 250 μM induced caspase-3 activation in cortical neuron. Treatments were performed for 24 hr (Figueroa et al., 2005). It is quite possible that protection by co-exposure with *Centella asiatica* extract against NO-induced cell death may be marginal, not directly involved its antioxidant property but likely to mediate via inhibition of apoptotic mechanisms and may induce certain cellular protective mechanisms by increasing of mitochondrial function and increasing of glutathione levels.

Despite our present knowledge of some of the cellular pathways that modulate CNS injury, complete therapeutic prevention or reversal of acute or chronic neuronal injury has not been achieved. As a result, identification of novel therapeutic targets for the treatment of neuronal injury would be extremely beneficial to reduce or eliminate disability from CNS disorders. Current studies have begun to focus on pathways of oxidative stress that involve a variety of cellular pathways.

The potential antioxidant therapy includes natural free radicals scavenging enzymes and agent which are capable of augmenting the endogenous antioxidant defenses included glutathione. Glutathione is an endogenous antioxidant present mainly in the reduced form within the cells. It reacts with the free radicals and prevents the generation of hydroxyl radicals, the most toxic form of free radicals.

During this defensive process, reduced glutathione gets converted to its oxidized form with the help of the enzyme glutathione peroxidase. The decreased level of reduced glutathione in SNAP-exposed cultures seen in our study indicates that there was an increased generation of free radicals and the reduced glutathione was depleted during the process of combating oxidative stress. Co-treatment of 100 μg/ml of *Centella asiatica* extract with 1 mM SNAP did not significantly attenuate SNAP-induced glutathione diminution in N1E-115 cells in spite of increasing mitochondrial metabolic activity and reduced cell death.

In conclusion, experimental results from the present study suggested that *Centella asiatica* extract, but not asiaticoside, may possess the marginal *in vitro* cytoprotective property against NO-induced neuronal damages. Antioxidant activity of *Centella asiatica* extract may not be directly responsible for this beneficial effect. However, the detailed mechanisms are not fully understood and remain to be further elucidated.