CHAPTER II

LITERATURE REVIEW

***Centella asiatica* (Linn.) Urban**

Synonyms: *Hydrocotyle asiatica*, Gotu kola, Indian pennyworth, Brahmi, Indian water, Navelwort, Mandukaparni

Thai name: Bua Bok, Pak Nhok, Pak Van, Jumpakrue

Family: Apiaceae/Umbelliferae

Habitat: Asia, South Africa

Description of plant: weedy, creeping, rooting at the nodes, low-growing with round-lobed leaves and tiny pink flowers.

Part Used: Whole plant

Chemical Components:

Centella asiatica leaves and above-ground parts are rich in terpenoids including asiatic acid, madecassic acid and asiaticoside. Other terpenoids include: centelloside, centellic acid; centoic acid, asiaticentoic acid, madecassoside, brahmoside, brahminoside (saponin glycosides). Aglycones are referred to as hydrocotylegenin A-E; compounds A-D are reported to be esters of the triterpene alcohol R1-barrigenol. The herb also contains: several amino acids; flavonoids including quercetin, kaempferol and various glycosides; volatile oils including beta-caryophyllene, farnesene, germacrene D (sesquiterpenes) as major components; alpha and beta pinene. The major terpenoid is stated to be unidentified. Other constituents include an alkaloid called hydrocotylin, a bitter principle called vallerine, fatty acids including oleic, linoleic, linolenic, palmitic, stearic acids and lignocene; phytosterols including campesterol, sitosterol and stigmasterol; resin and tannin. The root region contains 14 different polyacetylenes.

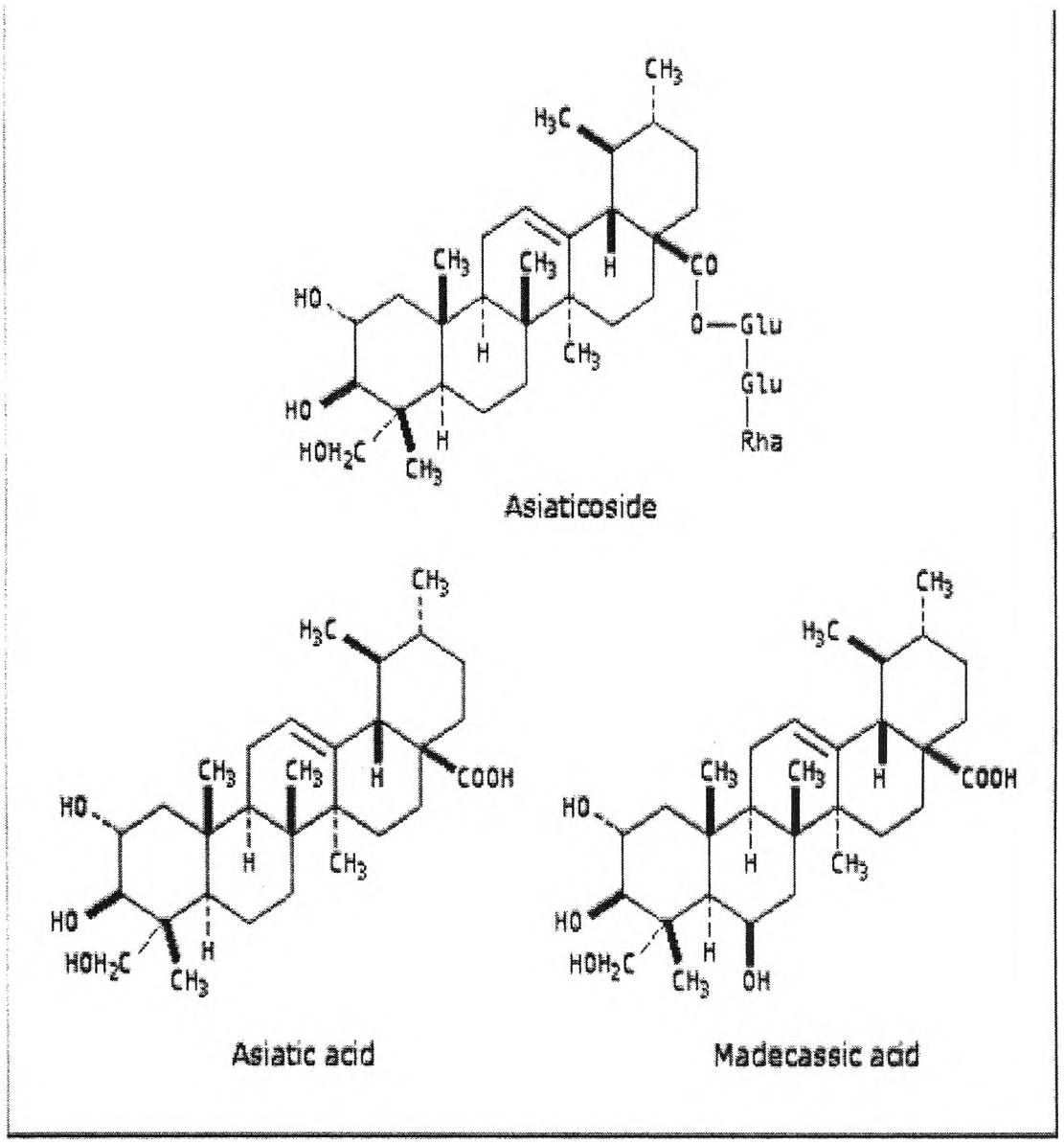

Fig.1 Chemical structures of active components in *Centella asiatica*
(Source: http://bodd.cf.ac.uk/BotDermFolder/BotDermU/UMBE-3.html)

Traditional Uses

Centella asiatica has been widely used in man for several decades. *Centella asiatica* is used as a nourishing food and a valuable medicine in many cultures. It was used as anxiolytic, memory and brain tonic, nervine, antispasmodic, vulnerary, sedative, antibacterial and anti-inflammatory.

Fresh extracts of this plant have been used by the people of Java and the Malay Peninsula for many years, as both topical and internal agents, for healing of wounds (Kartnig, 1988). In India (the plant is commonly known as 'Mandukaparni') and Madagascar, this plant was used to treat leprosy (Sahu et al., 1989), while In Malaysia, although this herb is commonly eaten fresh as a vegetable (salad), especially among the Malay communities, it is also said to have beneficial effects in improving memory and in treating mental fatigue, anxiety, and eczema (Goh, Chuah Mok, and Soepadmo, 1995).

In Srilanka and Indonesia it is given the name 'Thankuni Sak'. In classical Indian Ayurveda medicine, it is considered to be one of 'Rasayana' (rejuvenator) drugs which was used as a calming and rejuvenating herb, especially for nerve and brain cells. It is used to increase intelligence, longevity, and memory, retarding aging and senility. Also used to reduce anxiety, treat petit mal epilepsy, for rheumatic pain, as a diuretic, and for varicose veins. Often used as a wash topically for skin infections, leprosy, and burns. It is said to improve texture of skin. It is also commonly used as a porridge for feeding pre-school children in Sri Lanka in combating nutritional deficiencies (Cox et al., 1993).

In Chinese medicine, was often used interchangeably with several other species of low-growing, round-leaved plants under the name of Zhi xue cao. Used for treating dermatitis, wounds, sores, dysentery, tuberculosis, jaundice, hematuria, and hemoptysis and as a nerve tonic. The Chinese prescribed the leaves in curing leukorrhea and toxic fever (Kan, 1986).

Pharmacological Properties

Extracts of *Centella asiatica* exhibit various pharmacological actions including:

1) Antipsoriasis

Natarajan and Paily (1973) reported that a cream formulation of *Centella asiatica* was found to be successful in treatment of psoriasis in seven patients to whom it was applied.

2) Anxiolytic properties

Gotu kola (*Centella asiatica*) is a psychoactive medicinal plant that has been used for centuries in Ayurvedic medicine to alleviate symptoms of anxiety and to promote a deep state of relaxation and mental calmness during meditation practices. Recent investigations using human and animal models anxiety have confirmed that Gotu kola does indeed possess anxiolytic activity. Bradwejn et al. (2000) reported that a single 12 g dose of Gotu kola administered orally was more effective than placebo in decreasing acoustic startle response in healthy humans. This effect was most pronounced 60 min after treatment. In animals, Gotu kola increases pentobarbitone-induced sleeping time and decreases immobility in the forced swim test (Sakina and Dandiya, 1990). Gotu kola also elicits anti-anxiety effects in the elevated plus maze (Lucia et al., 1997) and an aqueous extract of Gotu kola was reported to have cognitive-enhancing as well as antioxidant effects in rats (Veeranda Kumar and Gupta, 2002).

3) Antioxidative properties

Oral treatment with 50 mg kg^{-1} day^{-1} of crude methanol extract of *Centella asiatica* for 14 days significantly increased the anti-oxidant enzymes, like superoxide dismutase (SOD), catalase and glutathione peroxidase (GSHPx), and anti-oxidants like glutathione (GSH) and ascorbic acid decreased in lymphoma-bearing mice (Jayashree et al., 2003). Recent study has revealed that *C. asiatica* extract and powder may ameliorate H_2O_2-induced oxidative stress by decreasing lipid peroxidation via alteration of the antioxidant defence system of the rats (Abdul-Hamid et al.,2005). The antioxidative activity of the ethanol extract, of both roots and leaves of *C. asiatica*, was found to be as good as α-tocopherol (Abdul-Hamid et al., 2002). It is also found

that different parts of *Centella asiatica* (leaf, root, and petiole) contain high phenolic contents, which exhibit strong antioxidative activities (Abdul-Hamid et al., 2003). Besides, it was found to have a challenging role in quenching free radical induced lipid peroxidation, protein carbonyls and also useful against age-related decline in antioxidant status in aged rat brain regions (Subathra et al., 2005).

4) Anticancer activity

Oral administration of the Centella extracts and its partiallly purified fractions retarded the development of solid and ascites tumours and increased the life span of these tumour bearing mice (Babu et al., 1995).

5) Radiation ulcer

Extracts of *Centella asiatica* has shown its ability of reducing acute radiation reactions by its anti-inflammatory activity (Chen et al.,1999), and it offers good behavioural radioprotection against conditioned taste aversion in rats during clinical radiotherapy (Shobi and Goel, 2001).

6) Gastric ulcer

Studies in rats have documented (Ravokatra, Nigeon-Dureuil, and Ratsimamanga, 1974a; Ravokatra et al., 1974b) asiaticoside exhibits a protective action against stress-induced gastric ulcers, following subcutaneous administration, and accelerates the healing of chemical-induced duodenal ulcers, after oral administration. In another study, Oral administration of *Centella* extract (0.05g/kg, 0.25g/kg and 0.50g/kg) prevented ethanol induced gastric mucosal lesions in rats by strengthening the mucosal barrier and reducing the damaging effects of free radicals (Cheng and Koo, 2000).

7) Wound healing, scar healing, and skin diseases

Centella asiatica extract was found to be useful in preventing and treating keloids and hypertrophic scars (Bosse et al., 1979). A Centella extract containing asiaticoside (40%), asiatic acid (29-30%), madecassic acid (29-30%), and madasiatic acid (1%), was documented to be successful as both a preventive and curative treatment, when 227 patients were given with keloids or hypertrophic scar.

The effective dose in adults was reported to be between 60 and 90 mg. Its activity appears to be on the connective tissue by stimulates synthesis of hyaluronidase and chondroitin sulfate. It is also believed to have an effect on keratinization in areas of infection and to stimulate the reticuloendothelial system. Its topical form is claimed to improve tissue healing, particularly in skin, connective tissue, lymph, and mucous membranes. It also may stabilize connective tissue growth in scleroderma. These properties have been ascribed to the active principles: asiatic acid , asiaticoside, madecassic acid, and madecassoside, which are pantacyclic triterpenes, found to display chronic venous insufficiency (Pointel et al.,1987), varicose vein and wound healing properties. Madacassol, a formulation based on *Centella asiatica* plant extract, when applied locally on wounds in rats prompted the proliferation of granulation and increased tensile strength (Vogel, De Souza, and D'sa, 1990). It decreased the wounds area of the skin necrosis induced by burn (Tsurumi et al., 1973). Asiaticoside in its leaves has yielded encouraging results in treatment of leprosy (Bailey, 1945; Boiteau, 1949; Chopra, Nayar, and Chopra, 1956; Viala et al., 1977). In addition, it has been shown to enhanced the rate of wound healing by promoting fibroblast proliferation and stimulating collagen synthesis (Maquart et al., 1990). Rosen, Blumenthal, and McCallum (1967) have also reported wound healing activity of the plant. Titrated extract of *Centella asiatica* (TECA), contains three principal components such as asiaticoside, asiatic acid and madecassic acid (Nakajima and Ajiyoshi, 1972), have been reported to be effective in treating systemic scleroderma, abnormal scar formation and keloids (Kiesswetter, 1964; Tallat and Abbas, 1971) by strongly inhibiting the biosynthesis of acid mucopolysaccharides and collagens in carrageenin granulomas (Sasaki et al., 1972). Brahmic acid, a biologically active triterpenoid (Singh and Rastogi, 1968) have therapeutic value in ulcerations, extensive wounds, eczemas etc. (Yoshinori, Reiko, and Tsunematsu, 1982), and inhibitory effect on the biosynthetic activity of fibroblast cells (Veechai et al., 1984). Its wound healing effects may be due to its up-regulation of human collagen I expression (Bonte et al., 1994) and increase in tensile strength of wound (Suguna et al., 1996). In guinea pig punch wounds topical applications of 0.2% solution of asiaticoside produced 56% increase in hydroxyproline, 57% increase in tensile strength, increased collagen content and better epithelisation. In streptozotocin diabetic rats, where healing is delayed, topical application of 0.4% solution of asiaticoside over punch wounds increased hydroxyproline content, tensile strength, collagen content and epithelisation

thereby facilitating the healing. Asiaticoside was active by the oral route also at 1 mg/kg dose in the guinea pig punch wound model. It promoted angiogenesis in the chick chorioallantoic membrane model at 40 μg/disk concentration (Shukla et al., 1999b).

Gotu kola can be applied as a topical ointment or compress to the skin, or it can be ingested as dried leaves or an infusion three times a day. (Each dose should be between 0.33 and 0.68 grams).

Extracts from the plant have been formulated into several commercial products including Collaven™, Emdecassol™, Madecassol™, Centelase™, Marticassol™, Blastoestimulina™, and Trofolastin™. These are topical agents applied to the skin.

Since antioxidants have been reported to play a significant role in the wound healing process, the possible involvement of such a mechanism in wound healing promotion by asiaticoside was explored. The result showed that asiaticoside enhanced induction of antioxidant levels at initial stage of healing (Shukla, Rasix, and Dhawan, 1999a). Topical application of 0.2% asiaticoside twice daily for 7 days to excision-type cutaneous wounds in rats led to increased enzymatic and non-enzymatic antioxidants, namely SOD (35%), CAT (67%), GPx (49%), vitamin E (77%) and ascorbic acid (36%) in newly formed tissues. It also induced a several fold decrease of lipid peroxide levels (69%).

8) Immunomodulatory properties

An ethanol extract of *Centella asiatica* dramatically inhibited NO production through the suppression of TNF-α production (Punturee et al., 2005).

9) Mental and Miscellaneous

Gupta, Kurma, and Srivastava (2003) concluded that *Centella asiatica* significantly prevented the cognitive impairment and attenuated the oxidative stress induced by PTZ kindling. Veerandra Kurma and Gupta (2002) have shown that the aqueous extract of whole plant (200 mg/kg for 14 days) showed an improvement in learning and memory in both shuttle box and step through paradigms. The whole plant of *C. asiatica* has been shown to be beneficial in improving memory (Mukerji, 1953; Vaidyaratnam, 1994) and it is reported to improve general mental ability of mentally retarded children (Apparao , 1973; Kakkar, 1990). Nalini et al. (1992) have shown that fresh leaf juice improves passive avoidance task in rats. Two glycosides,

brahmoside and brahminoside, have been shown to exert sedative and hypoglycemic effects in experimental rats.

Pharmacokinetic Properties

A recent study in twelve healthy human volunteers investigated the effects of single or repeated administrations of *Centella asiatica* 30 or 60 mg was administered orally to humans on a single occasion or once daily for 7 consecutive days. The assay method was only able to investigate levels of asiatic acid. The elimination half life was 2 to 3 hours irrespective of the dose used. The peak plasma concentration, area under curve (0 to 24 hours) and the plasma half life were significantly increased following repeated administration of *Centella asiatica*. These increases may in part be explained by the fact that asiaticoside is metabolized in vivo to asiatic acid (Grimaldi et al., 1990). The active ingredients of *Centella asiatica* extract are reported to be excreted primarily in the feces over a 24 to 76 hour peroid, with a smaller percentage being eliminated via the kidneys.

Toxicological Properties

In mice, a dose of 1 g/kg body weight of an extract of *Centella asiatica* (in 50% ethanol) did not lead to any toxic effects. No mortalities were recorded.

The American Herbal Products Association lists *Centella asiatica* as an herb that may be safely used consumed with appropriate use (McGuffin et al., 1997) No signs of toxicity have been found from *Centella asiatica* in animal studies (Sakina and Dandiya, 1990; Babu et al., 1995), mutagenicity (Yen, Chen, and Peng, 2001), and in human studies (compared to placebo) (Brinkhaus et al., 2000). In rats, the acute oral LD50 of a 70% ethanolic extract of the leaves was greater than 675 mg/kg and no toxicity was found from chronic administration (150 mg/kg p.o.) (Lucia et al., 1997). Allergic reaction skin reactions from topical use of preparations containing *Centella asiatica* or triterpene-rich extracts are rare (Danese, Carnevali, and Bertazzoni, 1994; Gonzalo, Revenga, and Bobadilla, 1996). Asiaticoside is only a weak sensitizer (Hausen, 1993).

Adverse Effects

Gotu kola seems to be well tolerated when taken by mouth. Stomach discomfort and nausea have been reported in studies. In theory, gotu kola may cause drowsiness and may raise blood cholesterol and blood sugar levels. Therefore, people with diabetes should avoid gotu kola. Animal studies show that gotu kola may lower the chances of becoming pregnant, but it is not clear if this effect occurs in humans (Ramswamy et al., 1970). Topical administration may cause contact dermatitis in sensitive individuals (Danese et al., 1994).

Interactions

Interactions with drugs, supplements and other herbs have not been thoroughly studied. The interactions listed below have been reported in scientific publications.

Interactions With Drugs

Gotu kola may increase the amount of drowsiness caused by some drugs. Examples include benzodiazepines, such as lorazepam; barbiturates, such as phenobarbital; narcotics, such as codeine; and alcohol. Caution is advised while driving or operating machinery. In theory, gotu kola may increase cholesterol levels and may work against cholesterol-lowering drugs.

Gotu kola may raise blood sugar levels and may work against drugs that lower blood sugar levels. Patients taking oral drugs for diabetes or using insulin should be monitored closely by their health care provider while using gotu kola. Dosing adjustments may be necessary. In theory, dexamethasone and phenylbutazone may decrease any wound-healing abilities of gotu kola.

Nitric Oxide

Since its initial discovery, nitric oxide (NO) has become one of the most highly studies and important bioactive molecules. Along with the many essential roles of nitric oxide *in vivo*, such as neurotransmitter, vasodilation, and immune defense, it can also be involved in reactions that may result in cell damage and death. NO is a relatively unstable molecule that is potentially toxic owing, in part, to the high reactivity of its unpaired electron. It has long been studied as an environmental pollutant because it contributes to the formation of smog, and acid rain and is also involved in the destruction of the ozone layer (Feldman, Griffith, and Stuehr,1993), so it was surprising when NO was found to play an important role in mammalian physiology. NO is biologically relevant because of its production by numerous cell types and because of its role in many physiological processes. In fact, virtually every type of mammalian cell is under the influence of NO (Schmidt and Walter, 1994). NO is synthesized by one of the three distinct NO synthase (NOS) isoforms. Depending on the site of production, the amount of NO produced, and the targets within the local environment, NO can exert many diverse functions. In the nervous system, NO has a dual role as a physiological messenger and a mediator of lethal processes in a variety of neurodegenerative disorders and toxic insults of the nervous system.

There are two distinct categories in the chemical biology of NO: direct and indirect effects. Direct effects are those chemical reactions where NO reacts directly with its biological target. Conversely, the indirect are mediated by reactive nitrogen oxide species (RNOS) which are derived from NO metabolism. The direct effects are very rapid reactions that occur at a low NO concentrations (< 1 μM) and generally involve heme proteins such as guanylate cyclase, cytochrome P450, and hemoglobin. Conversely, the indirect effects require that NO is first activated by superoxide (O_2^-) or oxygen to form RNOS which then undergo further reactions with the respective biological target. These RNOS are highly reactive with biological macromolecules such as protein, lipids, and DNA and are thought to be responsilble for NO-mediated cell death. These reactions are relevant only when the concentrations of NO are high locally (> 1 μM) for prolonged periods of time, such as in the vicinity of activated leukocytes or other cells sensitive to stimulation by cytokines or pathogenic products.(Wink et al.,2001)

The enzymatic combination of a single atom of nitrogen with a single oxygen atom forms the smallest synthetic product of mammalian cells. It also forms a

molecule with an unpaired electron in a frantic search for another molecule to accept or share this odd electron (Nathan,1992). Target molecules include oxygen, other free radicals, thiol groups, and metals. In an environment rich in these targets, NO has a short half-life, in the range of a few seconds or less. Whereas NO can be inactivated through its interaction with oxygen to form nitrite and nitrate, the interaction of NO with other free radicals is a mechanism by which NO exerts many of its effects. The combination of NO with superoxide (O_2^-) forms peroxynitrite ($ONOO^-$) with the capacity to injure target cells. When NO interacts with prosthetic iron groups or thiol groups on proteins, it can form complexes that activate or inactivate target enzymes. It is through this mechanism that NO activates one of its main target enzymes, soluble guanylate cyclase, which increases cellular cGMP concentrations.

Nitric Oxide and Neurodegenerative diseases

Although NO is an important and unique messenger molecule, there is evidence that NO excess is involved in the pathogenesis of several neurodegenerative diseases, e.g., Alzheimer's disease (Stamler, Singel, and Loscalzo, 1992), Parkinson's disease (Good et al., 1998), and progressive supranuclear palsy (Komori et al., 1998). Models of potential diffusion of NO indicate that it can diffuse as far as 300 μm from its site of origin, which could include as many as 2 million synapses (Wood and Garthwaite, 1994).

An early event resulting from oxidative injury to membrane phospholipids is the formation of fatty acyl hydroperoxides. The disruptive effects of these hydroperoxides or derived products on membrane structures apparently triggers a lipid hydrolytic activity, which could be considered the onset of the repair sequence consisting on removal of the LOOH (Van Kuijk et al.,1987). This sequence entails following steps: first, hydrolysis of the phospholipid hydroperoxide by phospholipase A_2 to yield a free fatty acid hydroperoxide and lysophospholipid. Second, the fatty acid hydroperoxide is reduced in the cytosol by a primary antioxidant enzyme; GPOX. Third, the lysophospholipid in the membrane is reacylated through the normal acyl-Co A / acyltransferase reactions which participate in phospholipid synthesis and turnover, a process which is stimulated during membrane oxidant stress and, as such, can be viewed as a repair reaction (Cadenas, 1995)

While O_2^- and H_2O_2 are toxic to cells, the extremely high reactivity of OH and O_2 renders these activated forms most cytotoxic due to deleterious peroxidation

reactions with lipid, proteins and DNA. Briefly, this occurs as exemplified for lipid peroxidation. The OH radical initiates a lipid peroxidation chain reaction with a variety of lipids such as a polyunsaturated fatty acid (PUFA; shown as LH) to form a lipid radical (L) and a lipid hydroperoxide (LOOH). Singlet O_2 attack results in a direct insertion of O_2 in a LH molecule with the formation of LOOH or a cyclic endoperoxide. The L radical can self-react to form LL (a chain termination process), but unless LOOH's are removed L will continue the lipid peroxidation chain reaction by reacting with O_2 and regenerating the peroxidizing lipid peroxylradical, $LO_2^{\cdot}$.

$$LH + {}^{\cdot}OH \rightarrow L^{\cdot} + H_2O$$

$$L^{\cdot} + O_2 \rightarrow LO_2^{\cdot}$$

$$LO_2^{\cdot} + LH \rightarrow L^{\cdot} + LOOH$$

Oxidative stress is exerted by all peroxides which directly damage cells and tissues, or from their more reactive breakdown products such as malondialdehyde and hydroxynonenals (Mannervik, 1985). Moreover, LOOH's are catalytically decomposed by metals such as iron or copper to free radicals such as $LO_2^{\cdot}$ which will contribute to the propagation of the lipid peroxidation chain reaction (Borg and Schaich, 1988). It is evident then that "oxidative stress is a chain-event, and a single initiating event caused by a prooxidant may cascade into a wide spread chain reaction that produces many deleterious products in concentrations many magnitudes greater than the initiator" (Ahmed, 1992)

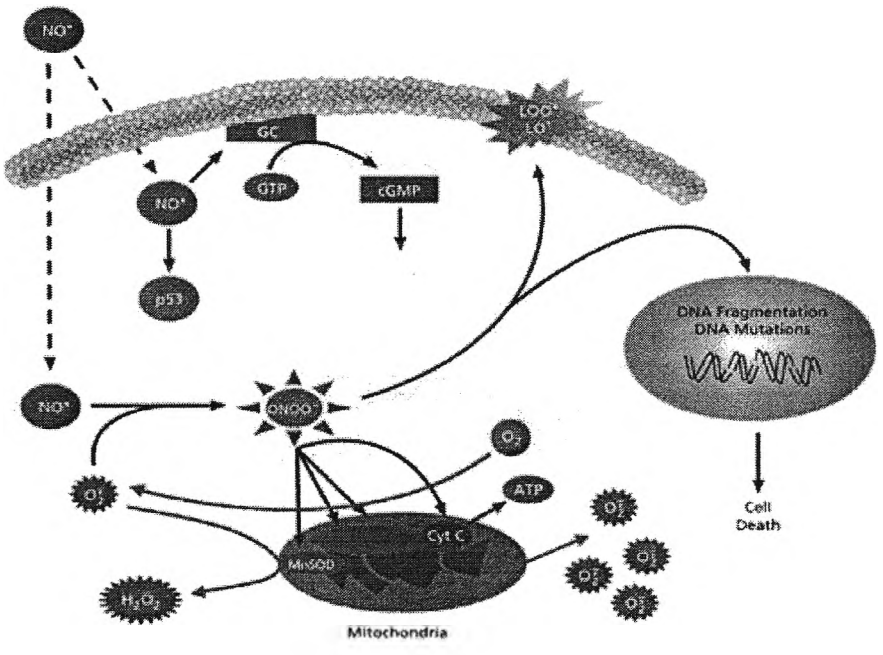

Fig. 2 Nitric Oxide Metabolism and lipid peroxidation.

Source:

(http://www.sigmaaldrich.com/Area_of_Interest/Life_Science/Cell_Signaling/Scientific_ Resources/Pathway_Slides Charts/Nitric_Oxide_Metabolism.html)

Nitric oxide (NO) is as a major signaling molecule in neurons and in the immune system, either acting within the cell in which it is produced or by penetrating cell membranes to affect adjacent cells. Nitric oxide is generated from arginine by the action of nitric oxide synthase (NOS). NO has a half-life of only a few seconds *in vivo*. However, since it is soluble in both aqueous and lipid media, it readily diffuses through the cytoplasm and plasma membranes. NO has effects on neuronal transmission as well as on synaptic plasticity in the central nervous system. In the vasculature, NO reacts with iron in the active site of the enzyme guanylyl cyclase (GC), stimulating it to produce the intracellular mediator cyclic GMP (cGMP), that in turn enhances the release of neurotransmitters resulting in smooth muscle relaxation and vasodilation. NO may also be involved in the regulation of protein activity through S-nitrosylation. In the extracellular milieu, NO reacts with oxygen and water to form nitrates and nitrites. NO toxicity is linked to its ability to combine with superoxide anions (O^{2-}) to form peroxynitrite ($ONOO^-$), an oxidizing free radical that can cause DNA fragmentation and lipid oxidation. In the mitochondria, $ONOO^-$ acts on the respiratory chain (I-IV) complex and manganese superoxide dismutase (MnSOD), to generate superoxide anions and hydrogen peroxide (H_2O_2), respectively.

Neurodegenerative diseases and oxidative stress

The adult brain contains about 10^{11}-10^{12} neurons, which are supported and protected by at least twice as many neuroglial cells (Kruman et al., 1998). The endothelium of the small blood vessels in the brain is much less permeable to molecules than other vascular endothelia, although essential molecules such as glucose, and most lipid soluble molecules can still penetrate. Many other molecules are excluded from the brain by this so-called blood-brain barrier (BBB). BBB also excludes circulating phagocytes from the healthy brain.

The central nervous system (CNS) is said to be especially sensitive to oxidative stress (Emerit et al., 2004). One reason is its high O_2 consumption: in humans, the brain accounts for only a few percent of the body weight but it processes 20% of basal O_2 consumption. A neuron uses much of O_2 it takes up to make, via mitochondria, ATP needed to maintain low gradients (high intracellular K^+, low Na^+, very low and "free" Ca^{2+}). The brain uses glucose for energy production and needs about 4×10^{21} molecules every minute. As the mitochondria in aerobes, are the fount of ATP synthesis, this provides an explanation as to why deep hypoglycemia and

inhibitors of ATP synthesis such as rotenone or cyanide can cause neuronal cell death (Beal, 1992).

The respiratory chain of mitochondria is responsible for most of the reactive oxygen species (ROS) and notably the first produced, superoxide anion ($O_2^{\cdot-}$) in human tissues. Under physiological O_2 level 1–2% of the O_2 consumed is converted to ROS. Another reason is nitric oxide (NO·), a reactive nitrogen species (RNS). This gaseous free radical is an important biological messenger, highly diffusible, that plays a prominent role in the physiology of the CNS (Yun et al., 1996). Three isoforms account for NO· production and include neuronal NO synthase (nNOS; type I), inducible NO synthase (iNOS; typeII) which is produced in very large amounts by activated microglia (macrophages), and endothelial NO synthase (eNOS; type III), In the CNS, nNOS, whose expression is regulated by both physiological and patho-physiological stimuli accounts for most NO· activity. NO· reacts rapidly with $O_2^{\cdot-}$ to form peroxynitrite ($ONOO^-$) which is the most reactive RNS.

ROS and RNS are the cause of oxidative stress in nervous system. They are produced in large amounts in pathologic conditions, especially NO· coming from activated microglia (iNOS) or and from endothelial cells (eNOS). The main sources of ROS in inflammatory process are both damaged mitochondria and activated microglia.

Classically oxidative stress is described as an imbalance between generation and elimination of ROS and RNS. These reactive species were originally considered to be exclusively detrimental to cells (Scandalios, 2002). It is now recognized that redox regulation involving ROS, is key to the modulation of critical cellular functions, notably for neurons astrocytes and microglia, such as mitogene-activated protein (MAP) kinase cascade activation, ion transport, calcium mobilization, and apoptosis program activation. Neurons are post-mitotic cells. Their general inability to divide explains some aging and neurodegenerative disease related loss of function, as neurons die, without chance to be replaced. Fortunately, many parts of the brain have considerable neuronal redundancy.

The biology of oxidative stress

Oxidative stress occurs when oxygen free radicals are generated in excess through the reduction of oxygen. Reactive oxygen species (ROS) consist of oxygen free radicals and associated entities that include superoxide free radicals, hydrogen peroxide, singlet oxygen, nitric oxide (NO), and peroxynitrite. Several of these species are produced at low levels during normal physiological conditions and are scavenged by endogenous antioxidant systems that include superoxide dismutase (SOD), glutathione peroxidase, catalase, and small molecule substances such as Vitamins C and E. Superoxide radical is the most commonly occurring oxygen free radical that produces hydrogen peroxide by dismutation. Hydroxyl radical is the most active oxygen free radical and is generated from hydrogen peroxide through the Haber–Weiss reaction in the presence of ferrous iron. Hydroxyl radical alternatively may be formed through an interaction between superoxide radical and NO (Fubini and Hubbard, 2003). NO interacts with superoxide radical to form peroxynitrite that can further lead to the generation of peroxynitrous acid. Hydroxyl radical is produced from the spontaneous decomposition of peroxynitrous acid. NO itself and peroxynitrite are also recognized as active oxygen free radicals. In addition to directly altering cellular function, NO may work through peroxynitrite that is potentially considered a more potent radical than NO itself (Pfeiffer et al., 2001).

Oxidative stress in the brain occurs when the generation of ROS overrides the ability of the endogenous antioxidant system to remove excess ROS subsequently leading to cellular damage. Several cellular features of the brain suggest that it is highly sensitive to oxidative stress. For example, the brain is known to possess the highest oxygen metabolic rate of any organ in the body (Maiese, 2002). The brain consumes approximately twenty percent of the total amount of oxygen in the body. This enhanced metabolic rate leads to an increased probability that excessive levels of ROS will be produced. In addition, the brain tissues contain increased amounts of unsaturated fatty acids that can be metabolized by oxygen free radicals. Finally, the brain contains high levels of iron which have been associated with free radical injury (Herbert et al., 1994). Yet, given the increased risk factors for the generation of elevated levels of ROS in the brain, it is interesting to note that the brain also may suffer from an inadequate defense system against oxidative stress. Catalase activity in the brain is significantly below other body organs. If one compares the catalase activity of the brain to the catalase activity in the liver, the brain has been shown to

contain only 10% of the catalase activity present in the liver (Floyd and Carney, 1992).

Oxidative stress represents a significant pathway that leads to the destruction of both neuronal and vascular cells in the CNS. The production of ROS can lead to cell injury through cell membrane lipid destruction and cleavage of DNA (Vincent and Maiese, 1999; Wang et al., 2003). ROS result in the peroxidation of cellular membrane lipids (Siu and To, 2002), peroxidation of docosahexaenoic acid, a precursor of neuroprotective docosanoids (Mukherjee et al., 2004), the cleavage of DNA during the hydroxylation of guanine and methylation of cytosine (Lee, O'Connor, and Pfeifer, 2002), and the oxidation of proteins that yield protein carbonyl derivatives and nitrotyrosine (Adams et al., 2001). In addition to the detrimental effects to cellular integrity, ROS can inhibit complex enzymes in the electron transport chain of the mitochondria resulting in the blockade of mitochondrial respiration (Brown and Borutaite, 2004). In cerebral vascular system, the cellular effects of ROS may lead to the destruction of endothelial cell (EC) membranes and an increase in endothelial cell permeability (Sakamaki, 2004).

Lipid Peroxidation

Malondialhyde is one of the products of peroxidative cleavage of polyunsaturated fatty acids containing at least two methylene-interrupts double bonds. Pryor and Stanley (1975) have proposed a mechanism of formation of malon-dialdehyde that involves an intermediate bicycloendoperoxide. But Esterbauer and his group (1991)still propose that the production of malondialdehyde from poly-unsaturated fatty acids with more than two double bonds involves formation of hydroperoxides, β clevage to yield hydroperoxyaldehydes, and finally, via a second β scission, a malondialdehyde or acrolein radical which combines with $OH^{\cdot}$ to form the enol.

The most popular method of lipid peroxidation is the colorimetric or fluorometric determination of malondialdehyde or malondialdehydelike materials by thiobarbituric assay.

Because of an unpaired electron, NO is a free radical, and its effects are largely mediated through other molecules that accept or share this odd electron (Dawson et al., 1998). Target biological molecules include oxygen, other free radicals, thiol groups, and metals. The key reaction of NO *in vivo* is its interaction with

superoxide (O_2^-) to form peroxynitrite ($ONOO^-$), which occurs with a second-order rate constant of 6.7 x 10^9/M/s. A key consideration in the fate of reactive oxygen species is the presence of biologically relevant levels of antioxidant defense.

Glutathione (Anderson, 1997)

Glutathione (γ-glutamylcysteinylglycine; GSH) is the major cellular antioxidant and is found in high concentrations in most mammalian cells (1 to 10 mM). Intracelllular GSH is maintained in its thiol form by glutathione disulfide (GSSG) reductase, which requires NADPH. GSH has several functions (Fig.1), including roles in metabolism, transport, catalysis (coenzyme), and maintenance of the thiol moieties of proteins and the reduced form of other molecules such as cysteine, coenzyme A, and antioxidants such as ascorbic acid ; it is also used in the formation of deoxyribonucleic acids. GSH participates nonenzymatically and enzymatically (GSH S-transferase) in the protection against toxic compounds. Perhaps one of its most important functions is in the protection against oxidative damage caused by reactive oxygen species (ROS), many of which are generated during normal metabolism. GSH can react nonenzymatically with ROS, and GSH peroxidase (and non-Se peroxidase) catalyzes the destruction of hydrogen peroxide and hydroperoxides.

GSH is synthesized intracellularly by the consecutive actions of (γ-glutamylcysteine (1) and GSH (2) synthases:

(1) L-Glutamate + L-cysteine + ATP ⇔ L-γ-glutamyl-L-cysteine + ADP + P_i

(2) L-γ-glutamyl-L-cysteine + glycine + ATP ⇔ GSH + ADP + P_i

The synthesis of GSH is limited by the availability of substrates; cysteine is usually the limiting substrate. γ-glutamylcysteine synthetase is nonallosterically feedback inhibited by GSH (K_i about 1.5 mM)(Richman and Meister,1973). Thus, under physiological conditions, γ-glutamylcysteine synthetase is probably not operating at its maximal rate.

The degradation of the γ-glutamyl moiety of GSH (or GSH S-conjugates) is catalyzed by γ-glutamyl transpeptidase, a membrane-bound enzyme whose active site is on the external surface of certain cells. GSH is normally transported out of cells where transpeptidation occurs in the presence of amino acid is formed. This can then be transported into cells where it, but not GSH, is a substrate for γ-glutamyl

cyclotransferase and forms amino acid and 5-oxoproline. 5-oxoproloine is ring opened by 5-oxoprolinase to form glutamate. Most L-amino acids participate in transpeptidation, but cystine is one of the best acceptor amino acids. When cystine participates, γ-glutamylcystine is formed, transported, and reduced to cysteine and γ-glutamylcystine. Cysteine can be used for the two-step pathway of GSH biosynthesis (1 and 2), and γ-glutamylcystine can be used directly by GSH synthetase (2) to form GSH. This alternative pathway of GSH synthesis serves to conserve cysteine moieties; however, the extent to which it functions physiologically is not yet known.

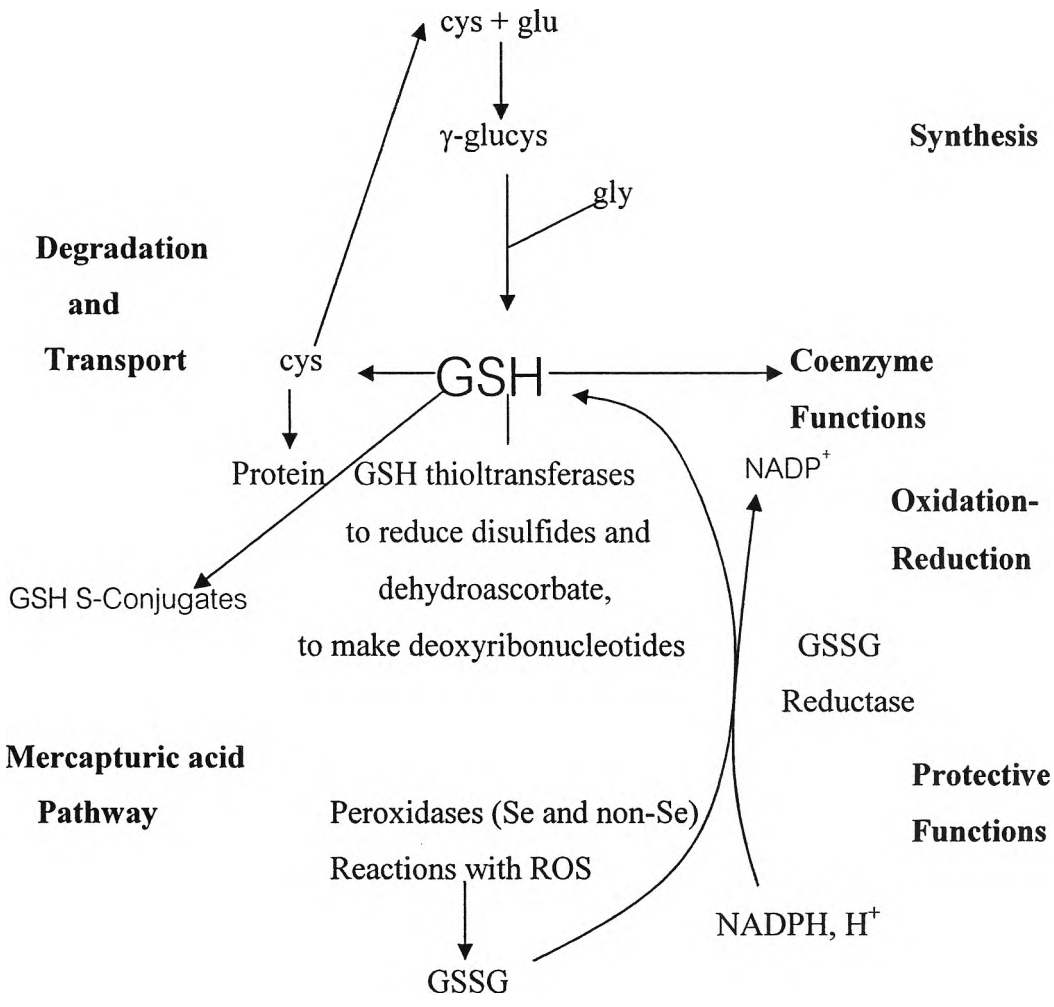

Fig. 3 Overview of GSH function and metabolism (Anderson, 1997)

Much of the work investigating the biological consequences of NO excess has employed "NO donors", of which there are several different major chemical classes (Feelisch, 1998). S-nitroso-N-acetyl-DL-penicillamine (SNAP) is commonly used "NO donor", it is a stable N-nitrosothiols, and spontaneously release nitric oxide under specific physiological condition. It is reported to release NO immediately (Holm et al., 1998).

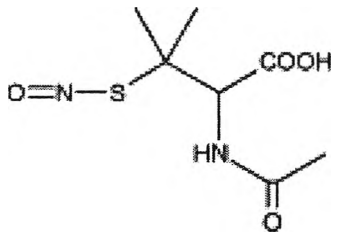

Fig. 4 Chemical structure of S-nitroso-N-acetyl-DL-penicillamine (Singh et al., 1996)

The nervous system comprises two components: the central nervous system (CNS), which is composed of the brain and the spinal cord, and the peripheral nervous system (PNS), which comprises the ganglia and the peripheral nerves lying outside of the brain and spinal cord inclusive of the autonomic nervous system. Two general types of cells are predominant in the nervous system: neurons and neuroglial cells (oligodendrocytes, astrocytes, microglia; and in the PNS, the Schwann cells). Neurons are similar to all other cells of the body in general structure, but they have additional anatomical features of axons and dendrites that allow conduction of nerve impulses for communication with other neural cells and between neuronal populations.

Cell culture (Harry et al., 1998)

Neurons are highly specialized cells and are responsible for the reception, integration, transmission, and storage of information. Afferent neuronal pathways carry information into the nervous system for processing; efferent pathways carry commands to the periphery. In addition, there are interneurons that process local information and transfer data within the nervous system. Within the CNS, neurons are segregated into functionally related nuclei that form interconnecting bundles of axonal fibers. Higher organizational levels consisting of several functionally related neurons are frequently called systems, e.g., motor, visual, associative, and neuroendocrine

systems. The CNS consists of a number of systems responsible for the coordination of receiving and processing information from the environment, maintaining the balance of all other organ systems, and responding to changes in the environment.

The *in vivo* reactions of neurons to injury vary dramatically. Degeneration can be induced by a direct action on the perikaryon or loss of synaptic target site influences and deprivation of trophic factors. A number of chemicals appear to have distinct cellular specificity for neuronal populations. Although specificity can exist and the pattern of degeneration has been used in diagnostic neuropathology, degeneration of any particular neuronal type cannot necessarily identify the damaging agent. Often this pattern reflects the severity and duration of the injury and the acuteness or chronic nature of exposure. The degenerative process of the nerve cell can be either relatively quick or a slow, prolonged process, depending on the underlying mechanism.

In the field of neurobiology, *in vitro* cell culture techniques have been successfully developed and employed to address specific questions of cell biology and nervous system functioning and provide a means to systematically study complex nervous systems (Arenander and de Vellis, 1983; Saneto and de Vellis, 1983; Shahar et al, 1989). Cell lines are cultures that have been serially transplanted or subcultured through a number of generations and can be propagated for an extended period of time. In neurobiology, *in vitro* methods are not ordinarily considered as alternatives to *in vivo* procedures. Instead, *in vitro* methods are selected to address specific hypotheses. Therefore, *in vitro* models are used in an attempt to study biological processes in a more isolated context or in the direct investigation of specific biological processes.

Continuous cell lines are transformed cells derived from neuroblastomas, gliomas, and pheochromocytomas with a useful life span of approximately 50 divisions. Cell lines of limited life span often undergo crisis after which their growth potential changes and their life span becomes unlimited. These cell lines are termed immortalized cell lines. The major attributes of continuous clonal cell lines are homogeneity and the ease with which a large quantity of cells can be grown (Diamond and Baird, 1977).