Chapter 5

Curcuma oil: Oxidative effect

"The larger the island of knowledge,
the longer the shoreline of mystery."
5.1 Introduction

Excess accumulation of free radicals in brain cells can result in detrimental cell dysfunction and death. In particular, neuronal cells have Ca\(^{2+}\)-permeable channels, a high rate of oxygen consumption and low levels of catalase (Nishino et al., 2004; Bengmark, 2006; Maheshwari et al., 2006). In pathological conditions as in cerebral ischemia, the mitochondrial dysfunction and consequent production of reactive oxygen species (ROS) and nitric oxide due to up-regulation of inducible nitric oxide synthase, a cascade of events is initiated leading to death.

Chemical constituents of the curcuma oil used in the present study were patented as an US patent (2006) (Ray et al., 2006) and the detailed chemical composition its stability etc has been recently reported (Jain et al., 2007). This is the first report of effect of C. oil showing neuroprotection. The present study investigates the effects of curcuma oil on the cerebral damages in rats that have been subjected to ischemia by MCAo. The highly lipophilic character of the curcuma oil greatly enhances its access to the brain (Ray et al., 2006)

5.2 Results

5.2.1 Brain lesion and edema volume analysis

The ischemic lesion after focal ischemia was observed as increased signal intensity on the diffusion-weighted imaging scans with a high b value. The lesions were spread within the vascular territory served by the MCA. The hyper intensity of the right hemisphere was compared to that of the contralateral hemisphere and of the sham operated rat. The MCA occluded side of the hemisphere showed hyper intense areas in the parts of the upper and lower frontoparietal cortex as well as the lateral caudate of putamen. The contralateral side of the brain showed weak signal intensity in the corresponding slices of brain. In the C. oil-treated group, the signal intensity between both the hemispheres was not significantly different in the corresponding slice of brain. MRI scan revealed a clear-cut reduction (%) in the hyper intense area (ischemic area) after C. oil treatment resulting in highly significant attenuation of hyper intense area as compared to I/R (**P<0.001) (Fig.1 & 2).
**Fig.1**: Diffusion-weighted imaging scans of brain from (A) sham operated, (B) I/R (2h of reflow) and (C) C.oil pretreated-I/R (2h of reflow) rats. Ischemic lesion was evident as hyper intense signal area in the right cerebral cortex and attenuated by C.oil pretreatment (n=13).

![Diffusion-weighted imaging scans](image)

**Fig.2**: Bar chart showing quantification of lesion areas in rat brain MRI scan from sham, I/R (2h of reflow) and C.oil pretreatment-I/R (2h of reflow). The bars indicate the Mean ± S.E.M. C.oil-I/R vs. I/R ***P<0.001

![Bar chart](image)

**Fig.3**: Effect of C.oil on brain infarct and edema volume in rat brain. Representative coronal brain sections (2-mm thick) stained with 2% triphenyl tetrazolium chloride (TTC). Rat brain sections obtained from sham operated, I/R (24h of reflow) and C.oil pretreated-I/R (24h of reflow) (n=5).

Brain section stained with TTC revealed no lesion in seven slices of the brain in sham-operated rats. A clear infarction (290.6 ± 36.2 mm$^3$) was observed in the ischemia 24h reflow group (2/24). Pretreatment by C.oil to the MCAo group attenuated the infarct area (99.88 ± 10.2mm$^3$) (**Fig.4**).
Fig. 4: Bar chart showing quantification of areas of infarct of brain from sham operated, I/R (24h reflow) and C.oil pretreated-I/R (24h of reflow) in mm$^3$ in rat brains. The area of infarct was significantly attenuated by C.oil pretreatment the bars indicate the Mean ± S.E.M. C.oil I/R vs. I/R rats ** $P<0.01$ (n=5).

An increase in edema volume (98 ± 10mm$^3$) was observed in the MCAo-reflow group at 24h reflow. Pretreatment of C.oil to the MCA occluded group attenuated the edema volume (58 ± 8mm$^3$). C.oil attenuated area of infarct and edema volume significantly (Fig. 5).

Fig. 5: Bar chart showing quantification of brain edema volume from sham operated, I/R (24h of reflow) and C.oil pretreated-I/R (24h of reflow) rats (n=5). The bars indicate the Mean ± S.E.M. *** $P<0.01$ as compared with the I/R group.

5.2.2 Neurological Evaluation

The neurological deficit was evaluated after 24 h of middle cerebral artery occlusion. C.oil improved the neurological score (2.6 ± 0.3) as compared I/R (5.4 ± 0.28). In C.oil treated group the neurological score significantly increased (***$P<0.001$) as compared to I/R group of rats. Mean neurological disability scores paralleled the changes in infarct volume in untreated and treated groups. (Fig. 6)
5.2.3 Measurement of enzymatic activity

The effects of C.oil on brain malondialdehyde (MDA) content, superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GSH-Px) activities in Ischemia/reflow rats are shown as bar charts. The level of malondialdehyde was higher in I/R as compared to the sham group. In the rats pretreated with C.oil, the elevated levels of malondialdehyde seen after middle cerebral artery occlusion were attenuated.
Fig. 8: Bar chart showing change in SOD levels (U mg\(^{-1}\) protein) in the tissues from brains of sham operated, I/R (24h of reflow) and C.oil pretreated-I/R (24h reflow) rats. C.oil pretreatment restored the SOD levels but not significantly. Data is expressed as Mean ± S.E.M. (n=5).

Fig. 9: Bar chart showing change in Catalase levels (U mg\(^{-1}\) protein) in the tissues from brains of sham operated, I/R (24h of reflow) and C.oil pretreated-I/R (24h reflow) rats. C.oil pretreatment restored the Catalase levels but not significantly. Data is expressed as Mean ± S.E.M. (n=5).

These changes were insignificant. The levels of GSH-Px activity was decreased as compared to sham operated. A significant rise in the levels of GSH-Px was observed with sham vs I/R group of rats.
Fig. 10: Bar chart showing change in GSH-Px levels (U mg\(^{-1}\) protein) in the tissues from brains of sham operated, I/R (24h of reflow) and C.oil pretreated-I/R (24h reflow) rats. C.oil pretreatment restored the GSH-Px levels but not significantly. Data is expressed as Mean ± S.E.M. (n=5).

5.2.4 Studies in Neurons

Strong fluorescent signals for reactive oxygen species were detected in brain neurons from I/R group (43.8 ± 2.31) and the sham, (26.1 ± 2.4). Rats with pretreatment with C.oil produced attenuation in fluorescence for ROS estimation (21.2 ± 3.41). A significant (\(*\!*P<0.01\)) decrease in ROS levels was seen after C.oil pretreatment (Fig. 11).

Fig. 11: Bar charts showing change in fluorescence of isolated neurons from sham operated, I/R (24h of reflow) and C.oil pretreated-I/R (24h of reflow) rats. Bar chart showing reactive oxygen species (ROS) measured with the help of fluorochrome DCFH-DA. Neurons were isolated from sham operated, I/R (24h of reflow) and C.oil pretreated - I/R (24h reflow) rats. Data is expressed as Mean ± S.E.M. (n=5). Effects of C.oil I/R vs. I/R rats, \(*\!* P<0.01\).
Strong fluorescent signals of peroxynitrite levels were detected in the neurons from I/R group (63.50 ± 8.8) as compared to sham group (25 ± 0.001). Pretreatment by C.oil produced attenuation in fluorescence signal (36.08 ± 1.79). C.oil treatment significantly reduced (**P<0.01) the ischemia induced peroxynitrite levels (Fig.12).

![Fig.12](image-url)

**Fig.12:** Bar chart showing change in mean fluorescence from neurons isolated from brains of sham operated, I/R (24h of reflow) and C.oil pretreated-I/R (24h reflow) rats. The extent of rhodamine123 fluorescence reflects the amount of peroxynitrite formed due to ischemic insult. C.oil pretreatment reduced the rhodamine123 fluorescence by reducing the formation/scavenging of peroxynitrite. Data is expressed as Mean ± S.E.M. (n=5). Effects of C.oil I/R vs. I/R rats, **P<0.01.

A shift to the right in fluorescence for mitochondrial membrane potential (ΔΨm) was observed in brain neurons from the I/R group of rats, which was restored by the pretreatment of C.oil (Fig.13).

![Fig.13](image-url)

**Fig.13:** Representative Fluorogram showing the mitochondrial membrane potential as shown by Rhd-123 fluorescence in neurons. Neurons were isolated from brains of sham operated, I/R (24h of reflow) and C.oil pretreated-I/R (24h reflow) rats (n =5).
The fluorescence observed from I/R-C.oil was close to that observed in the sham. C.oil pretreatment offered significant protection as the fluorescence decreased and the peak obtained was closer to that of the sham operated group. These observations were confirmed by confocal microscopy where the population of Rhod positive neurons was higher than sham. C.oil treated rats had as few Rhod positive neurons as neurons from sham (Fig.14).

**Fig.14:** Representative confocal microscopic image of brain cryosections stained with Rhodamine 123, the Rhod 123 positive neurons are stained green. Rat brain cryosections were obtained from (A) sham operated, (B) I/R (24h of reflow) and (C) C.oil pretreated-I/R (24h reflow) (n =5).

### 5.2.4.1 Flowcytometric estimation of mode of neuronal death

All most all neurons from sham group of rat were viable (97.7%) whereas neurons from I/R group were apoptotic (70.96%) and late apoptotic (3.79%). C.oil pretreatment significantly attenuated the apoptotic and late apoptotic population of neurons to (23.87%) and (1.07%) respectively and increased the number of viable neurons (69.25%). (Fig.15)

**Fig.15:** Fluorescence was determined from isolated neurons loaded with FITC-labeled Annexin V and Propidium iodide (PI). The dot plot represents percentage of cells; lower left quadrant-viable cells, lower right quadrant-early apoptosis, upper right quadrant-late apoptotic/necrotic and upper left quadrant-necrotic cells. Brain neurons were isolated from sham operated, I/R (24h of reflow) and C.oil pretreated-I/R (24h reflow) rats.
5.2.5 Assay of caspase-3 enzymatic activity

Ischemia induced a significant increase in mean fluorescence obtained from caspase-3 activity (0.935 ± 0.11) in comparison to sham operated rats (0.419 ± 0.02). C.oil pretreatment significantly (**P<0.01) reduced the ischemia induced increase in caspase-3 activity (0.206 ± 0.078) (Fig.16).

Fig.16: The bar chart showing caspase-3 activity in brain tissue from sham operated, I/R (24h of reflow) and C.oil pretreated-I/R (24h reflow) rats. The fluorometric substrate Ac-DEVD-AMC was used for the determination of caspase-3 activity. The bars indicate the Means ± S.E.M. (n =5). Effects of pretreatment of C.oil -I/R vs. I/R group, * P< 0.05.

5.3 Discussion

We believe this is the first detailed report of the protective effect of C.oil in cerebral stroke. The protection offered by C.oil is evidenced by the reduction in infarct size and neurological deficit. The significant reduction in tissue levels of NO, ROS, ONOO−, ΔΨm and its anti-oxidant effect greatly attenuated the delayed neuronal death and further salvage the penumbra. C.oil appears to exert its neuroprotective effects by acting at multiple targets in the signaling pathways that are activated in ischemic and neurodegenerative brain diseases. Cerebral ischemia leads to excess production of free radicals upon reflow, this becomes explosive and failing the tissue antioxidant activity results in oxidative stress induced neuronal loss. At this point of time, levels of NO and ROS increase in the brain to further worsen tissue injury to generate peroxynitrite (ONOO−). In the present study, a significant attenuation has been observed in ONOO− and ROS levels by the administration of C.oil.

The brain appears to be particularly vulnerable to oxidative stress and the brain generates many more free radicals than the other tissues (Dringen, 2000). During reperfusion,
the sudden supply of molecular oxygen, which serves as a substrate for xanthine oxidase for nucleotide metabolism, results in increased generation of hydrogen peroxide and superoxide as byproducts (Kong et al., 2000). Upon reflow, this explosive production of free radical production causes damages and the peroxide and superoxides further increase the damage to the tissue. MDA is indicative of lipid peroxidation and is a measure of the oxidative stress. The present study shows that elevation of MDA and depletion of protective enzymes (SOD, CAT & GSH-Px) in ischemic reperfused brain are in agreement with earlier reports (Shah et al., 2005). Pretreatment with C.oil reverses the post-ischemic enhanced MDA level as compared to the I/R group and up-regulation of SOD, CAT and GSH-Px activity, suggesting that it attenuates the excessive formation of ROS secondary to reperfusion injury. This is in agreement with the previous report that Curcuma oil possesses significant anti-inflammatory and antioxidant activity (Jayaprakasha et al., 2002). Although in the present report, C.oil per se did not show any effect on endogenous SOD, Catalase and GSH-Px levels (Data not shown).

The present results clearly indicate that the neuroprotective role of C.oil was mediated via its antioxidant effect through the suppression of lipid peroxidation and oxidative DNA damage which is indicative of lipid peroxidation, a measure of oxidative stress suggesting the involvement of free radicals in neuronal injury. The antioxidant effectiveness of C.oil, is possibly relate to its ability to enter cells and to its orientation in biomembranes (Thomas et al., 1992; Kaneko et al., 1994).

Free radical reactions may be particularly pronounced in the microcirculation and one of the beneficial effects of substances that antagonize free-radicals activity may be an improvement in tissue reperfusion and prevention of increased micro-vascular permeability leading to edema formation. We have shown that there was suppression in brain edema formation following C.oil treatment in our study. This strongly supports the above proposition.

Diffusion-weighted imaging is sensitive to early ischemic lesions as regions of increased signal intensity, i.e. decreased water diffusitivity showing up as early changes (Lo et al., 1997). The hyper intense lesion areas observed by diffusion-weighted imaging eventually become infarcted without timely therapeutic intervention (Li et al., 2000). Due to MCA occlusion the influx of water in the brain cell results in restricted diffusion of water protons. This is pickup as hyperintense signal by MRI in the area affected (Lo et al., 1997). Reperfusion injury is reversible when reperfusion is established as early as 30min and not
later than 2h after ischemia. It is well documented that there is a significant correlation between area of infarct assessed by 2, 3, 5-triphenyltetrazolium and the diffusion-weighted imaging-derived ischemic lesion volume after reperfusion. It has been suggested that early in vivo estimation of injury by diffusion-weighted imaging could be well correlated and could be used for evaluation of the therapeutic efficacy of neuroprotective agents (Minematsu et al., 1992; Tatlisumak et al., 1998). C.oil significantly attenuated these ill effects of ischemia reflow as observed as early as 2h reflow (MRI observation) and at 24h of reflow (TTC evaluation).

Oxidative stress markers such as levels of SOD, CAT, GSH-Px and MDA were estimated at 24 hrs to look for correlation of neurological deficit with infarct volumes. In the present study, the decrease in functional capacity of the neuronal mitochondria produced by ischemia was ascertained by probing the mitochondrial membrane potential (ΔΨm) with Rh123. Under the conditions of the present study, the increase in total fluorescence of Rh123 in ischemic animals indicated the probable depolarization or collapse of the mitochondria within the neurons. Pretreatment with C.oil prevents the increase in the fluorescence indicating a protection of the membrane potential or no depolarization of the mitochondria within the neurons.

The damage to the mitochondrial respiratory chain may cause increase in mitochondrial generation of ROS (Dykens, 1994; Votyakova and Reynolds, 2001). Substantial rise in cytosolic calcium promotes the mitochondrial calcium uptake causing the collapse of (ΔΨm). In the present study a significant reduction in (ΔΨm) depolarization and ROS generation was observed in the C.oil-treated group when compared against the ischemic reperfusion group. ROS profile obtained in this study appears to be in agreement with previous reports that neurons undergo a progressive ROS accumulation following focal ischaemia (Cao et al., 1988; Chan, 2001). This might be part of the "positive feed back loop" that leads to ultimate cell death. ROS, including O2-, ONOO-, OH- and H2O2 are all potential reactants capable of initiating damage to proteins, membrane lipids and nucleic acids.

Damage to neurons may occur through oxidative stress and/or mitochondrial impairment and culminate in activation of an apoptotic stage. Apoptosis or related phenomena are possibly involved in secondary cell death in cerebral ischemia. In pathological conditions, as in stroke, neuronal death exhibits features of both apoptosis and necrosis, or apoptosis and autophagic degeneration. It has been argued that apoptotic processes may switch to necrosis, if damage is too extensive and the energy machinery fails.
Caspase-3 activity is one of the key executioners of apoptosis, being responsible either partially or totally for the proteolytic cleavage of many key proteins. In the present study, C.oil significantly attenuated Caspase-3. Much remains to be done in the area of cerebral stroke to explore the potential of antiapoptotic drugs. It was thought worthwhile exploring the apoptogenic effect of C.oil. It was observed that C.oil exerted its major action on apoptotic activity. Apoptosis is an important mode of cell death occurring in the penumbral region of infarct and it is the region protected by modulation of apoptosis. It is concluded on varied biochemical/ enzymatic alterations, produced subsequent to the occlusion of middle cerebral artery (MCAo) followed by reperfusion, the MDA levels and the activities of CAT, GSH-Px and SOD, were markedly reversed and restored to near normal levels in the groups pre- treated with C.oil. Apart from its direct free radical-scavenging activity, C.oil also has the capability of reducing levels of ONOO\textsuperscript{--}, which is a highly toxic free radical. It is well documented that attenuating oxidative stress is important to evolve neuroprotective strategies so as to enhance neuronal survival after cerebral ischemia.

Summing up the effects of C.oil, the present data demonstrated that C.oil prevents the oxidative stress, attenuates the behavioral deficits and infarction. The present study provides experimental evidence for C.oil as a neuroprotective agent and the next part of the study was focused to understand more in detail the action and its mechanism.