Chapter 6

Curcuma oil: nitric oxide system and apoptosis

"Man cannot discover new oceans unless he has the courage to lose sight of the shore."

- Andre Gide -

(1947 Nobel Prize for literature, 1869-1951)
6.1 Introduction

In the previous chapter and in a published report (Rathore et al., 2007), the potent antioxidant effect of C.oil on cerebral ischemia and its outcomes was shown. The present exercise was undertaken to evaluate the mechanism of neuroprotection by curcuma oil (C.oil) in the focal cerebral ischemia model in the rat. In the present study, the occlusion time was reduced and the dose of C.oil was half of the dose reported earlier. The oil was administered before producing ischemia. The reasons for doing so was that at lower doses some of the "benefits" will not show up while other effects would: it was felt that this was necessary to explore the relationship between the "clinical outcome" (function) and the biochemical variables.

In the present study C.oil is given before ischemic insult. As reported earlier treatment strategies under development are aimed at offering neuroprotection acutely soon after focal cerebral ischemic injury, but delayed initiation of therapy may reduce efficacy. Pretreatment i.e. before ischemia begins could offer distinct advantages in patient groups at high risk for ischemic stroke (Fisher et al., 1994). Any form of pretreatment to limit the damage to the susceptible functional brain tissue during neurosurgical procedures may have a significant impact on the patient’s recovery (Jadhav et al., 2007). In another set of experiments the C.oil was given post ischemia after 2hrs. Time window studies confirming efficacy was also carried out as recommended by recommendations of the Stroke Therapy Academic Industry Roundtable (Green and Shuaib, 2006).

6.2 Results

6.2.1 Neurological evaluation

At 24hrs of reflow, pretreatment with C.oil improved the neurological score 1.5 ± 0.09 as compared to the ischemia/reflow group 2.9 ± 0.06 (*P<0.05) (Fig.1). After 2hrs of reflow, post treatment of C.oil, signification improvement of neurological score 1.7 ± 0.07 was observed (Fig.1).
Fig. 1: The bar chart showing the neurological evaluation score of sham, I/R and C.oil pretreatment group. C.oil improved neurological evaluation scores after 24 hrs of I/R. Data are expressed as mean ± S.E.M of five animals per group. (*$P<0.001$) was considered highly significant when comparisons were made with the I/R group by one-way ANOVA followed by Newman Keuls post hoc test.

6.2.2 Infarct volume and brain edema

Pretreatment with C.oil after 24hrs decreased the infarct volume to $92 \pm 5.60 \text{ mm}^3$ (n=5), *$P<0.001$ as compared to the I/R group (n=5) $261.93 \pm 21.89 \text{ mm}^3$ as shown in (Fig.2 & 3). Pretreatment with C.oil decreased the edema volume to $15.08 \pm 3.08 \text{ mm}^3$ (n=5), *$P<0.001$ as compared to the I/R group (n=5) $49.56 \pm 4.08 \text{ mm}^3$ as shown in (Fig.4). In a subsequent study it was found that giving C.oil to rats, 2 hrs after MCAo, resulted in a decrease in infarct ($112.83 \pm 6.63 \text{ mm}^3$) (*$P<0.001$) and edema volume ($23.56 \pm 2.56 \text{ mm}^3$) (*$P<0.001$) (n=5).

Fig. 2: Representative TTC stained brain sections of sham, I/R, C.oil pretreated and C.oil post treated group of rats after 24 hrs of refloow. Unstained areas represent the infarcted brain tissue. (five animals per group)
**Fig. 3:** Bar chart showing the infarct volume of brains of sham, I/R, C.oil pretreatment and C.oil post treated group. C.oil decreased the area of infarction after 24 hrs of reflow both in the pretreatment as well as in post treatment. Data are expressed as mean ± S.E.M of five animals per group. (*P<0.001) was considered highly significant when comparisons were made with the ischemia/reflow group by one-way ANOVA followed by Newman Keuls post hoc test.

**Fig. 4:** Bar chart showing the edema volume of brains of sham, I/R, C.oil pretreatment and C.oil post treated group. C.oil decreased the edema volume after 24 hrs of reflow both in the pretreatment as well as in post treatment. Data are expressed as mean ± S.E.M of five animals per group. (*P<0.001) was considered highly significant when comparisons were made with the ischemia/reflow group by one-way ANOVA followed by Newman Keuls post hoc test.

### 6.2.3 Brain neutrophil infiltration

MPO in the I/R rats after 24hrs was found to be, 169mU/g/min ± 3.56 that is significantly high as compared to the sham-operated group 44mU/g/min ± 1.96. C.oil pretreatment significantly reduced the MPO activity to 54mU/g/min ± 2.68 (*P<0.001) (Fig.5).
Fig. 5: Myeloperoxidase (MPO) activity after 24hrs I/R from sham, I/R and C.oil pretreatment groups of rats. C.oil pretreatment decreased the MPO levels after 24 hrs of ischemia/reflow. Data are expressed as mean fluorescence ± S.E.M of five animals per group. Statistical significance was assessed by one-way ANOVA. (*P<0.001) was considered highly significant when comparisons were made among different groups by Newman Keuls post hoc test.

6.2.4 Neuronal NO and Brain tissue NOx levels

In the flowcytometric estimations, significantly increased levels of NO were observed in the I/R group neuronal rich populations as compared to the sham-operated rats group neuronal rich populations. C.oil pretreatment showed a significant decrease in NO content (*P<0.001) in the neuronal rich populations isolated (ex vivo) (Fig. 6).

Fig. 6: NO estimation: mean fluorescence of DAF-2DA from neurons (10⁶/ml) of sham, I/R and C.oil pretreatment I/R was done. Data are expressed as mean ± S.E.M of five animals per group. (*P<0.001) was considered highly significant when comparisons were made with the ischemia/reflow group by one-way ANOVA followed by Newman Keuls post hoc test.

Increase in nitrite levels was observed in the I/R group of rats 24hrs after reflow 89.30 ± 7.02 μmol/mg as compared with tissue from the sham-operated rats 15.65 ± 3.75 μmol/mg.
Pretreatment with C.oil reduced the nitrite formation significantly 40.61 ± 6.23 μmol/mg (*P<0.05) (Fig.7).

Fig.7: Bar chart showing the tissue nitrite content in sham, I/R and C.oil pretreatment group measured at 24hrs after I/R. Increased tissue nitrite level in I/R was significantly reduced by C.oil pretreatment. Data are expressed as mean ± S.E.M of five animals per group. (*P<0.05) was considered highly significant when comparisons were made with the ischemia/reflow group by one-way ANOVA followed by Newman Keuls post hoc test.

The I/R rats showed an increase in the nitrate levels in the tissue at 24hrs after reflow 45.72 ± 3.2 μmol/mg as compared to that from the tissue from the sham-operated rats, which was 10.23 ± 2.02 μmol/mg. Pretreatment with C.oil 30 min before I/R reduced the nitrate formation significantly 19.20 ± 4.56 μmol/mg (*P<0.05) (Fig.8).

Fig.8: Tissue nitrate content in sham, I/R and C.oil pretreatment I/R group measured at 24hrs after C.oil pretreatment significantly reduced ischemia induced increased tissue nitrate level as compared to I/R. Data are expressed as mean ± S.E.M of five animals per group. (*P<0.05) was considered highly significant when comparisons were made with the ischemia/reflow group by one-way ANOVA followed by Newman Keuls post hoc test.
6.2.5 NOS isoform immunoblotting and immunolabeling in brain cryosections

The immunolabeling patterns (in the brain cryosections) of iNOS (Fig.9), nNOS (Fig.10) and eNOS (Fig.11), expression were examined in brain tissue sampled after 24hrs of reflow. An increase in the expression of iNOS, nNOS and eNOS was observed in the I/R group. C.oil pretreatment decreased the nNOS, iNOS and eNOS immunoreactivity with well-defined cells in the cerebral cortex region. However, co-localization in the section from the C.oil treated group reveled that the immunoreactivity was decreased in comparison to sections from the I/R group. This finding was further confirmed by immunoblotting. A significant increase in the expression of iNOS (Fig.12), nNOS (Fig.13) and eNOS (Fig.14) was observed in I/R group after 24hrs in the immunoblot, which was decreased in the C.oil treated group.

Fig.9: Photomicrographs (representative of 5 photomicrographs) showing inducible nitric oxide synthase (iNOS) immunoreactivity in brain cryosections from sham group (A-C) cerebral tissue subjected to 1hr of transient ischemia followed by 24hrs of reflow in I/R (D-F), and I/R-C.oil (G-I) groups. Sections were immunolabeled with a polyclonal anti-iNOS antibody, which was observed as green fluorescence (B, E & H) and neuron specific marker NeuN shown as red fluorescence (A, D & G). The merger is shown in C, F & I. Magnification: 40X (Bar 20μm)
**Fig. 10:** iNOS expression by immunoblotting. Bar chart shows the optical density of bands in iNOS/β-actin ratio. Lower Panel showing the bands for iNOS and β-Actin as lane 1: sham, lane 2: I/R with C.oil pretreatment, and lane 3: I/R (50 μg/lane). I/R induced iNOS expression was greatly reduced by C.oil pretreatment. Data are expressed as mean ± S.E.M of five animals per group. (*) (P<0.05) was considered highly significant when comparisons were made with the ischemia/reflow group by one-way ANOVA followed by Newman Keuls post hoc test.

**Fig. 11:** Photomicrographs (representative of 5 photomicrographs) showing neuronal nitric oxide synthase (nNOS) immunoreactivity in sham group (A-C) normal cerebral tissue subjected 1hr of transient ischemia followed by 24hrs of reflow in I/R (D-F), and I/R-C.oil (G-I) groups. Sections were immunolabeled with a polyclonal anti-nNOS antibody, which was observed as green fluorescence (B, E & H) and neuron specific
marker NeuN shown as red fluorescence (A, D & G) indicated by arrows. The co-localization is shown in (C, F & I). Intensity of nNOS immunoreactive cells was comparatively greater in I/R sections (E). Magnification: 40X (Bar 20μm).

Fig.12: nNOS expression by immunoblotting. Bar chart shows the optical density of bands in nNOS/β-actin ratio. Lower Panel showing the bands for nNOS and β-Actin as lane 1: sham, lane 2: I/R with C.oil pretreatment, and lane 3: I/R (50 μg/lane). I/R induced nNOS expression was greatly reduced by C.oil pretreatment. Data are expressed as mean ± S.E.M of five animals per group. (*P<0.05) was considered highly significant when comparisons were made with the ischemia/reflow group by one-way ANOVA followed by Newman Keuls post hoc test.

Fig.13: Photomicrographs (representative of 5 photomicrographs) showing endothelial nitric oxide synthase (eNOS) immunoreactivity in brain cryosections from sham group (A-
C) cerebral tissue subjected to 1 hr of transient ischemia followed by 24 hrs of reflow in I/R (D-F), and I/R-C.oil (G-I) groups. Sections were immunolabeled with a polyclonal anti-eNOS antibody, which was observed as green fluorescence (B, E & H) and neuron specific marker NeuN shown as red fluorescence (A, D & G). The colocalization is shown in right hand panel (C, F & I). Magnification: 40X (Bar 20 μm).

**Fig.14:** eNOS expression by immunoblotting. Bar chart shows the optical density of bands in eNOS/β-actin ratio. Lower Panel showing the bands for nNOS and β-Actin as lane 1: sham, lane 2: I/R with C.oil pretreatment, and lane 3: I/R (50 μg/lane). I/R induced eNOS expression was greatly reduced by C.oil pretreatment. Data are expressed as mean ± S.E.M of five animals per group. (*P<0.05) was considered highly significant when comparisons were made with the ischemia/reflow group by one-way ANOVA followed by Newman Keuls post hoc test.

### 6.2.6 Neuronal ROS, calcium, peroxynitrite and mitochondrial membrane potential (ΔΨm) levels

Rats in the I/R group showed an increased level of ROS in the neuronal rich populations as compared to that from the sham-operated rats. C.oil treated I/R group showed significant decrease in ROS content (*P<0.05) (**Fig.15**).
Fig. 15: Mean fluorescence of neuronal rich populations (10^6/ml) from sham, I/R and C.oil pretreatment I/R for quantification of different parameters assessed by acquiring and analyzing in cell quest program of Becton Dickinson Flowcytometer. Data are expressed as mean fluorescence ± S.E.M of five animals per group. (*P<0.001) was considered highly significant when comparisons were made with the I/R group by one-way ANOVA followed by Newman Keuls post hoc test. For ROS estimation the mean fluorescence of DHDCF-DA in isolated neuronal rich populations from sham, I/R and C.oil pretreatment I/R group of rats was measured.

The I/R rats showed significant increase in the levels of calcium in the neuronal rich populations as compared to that from the sham-operated rats. C.oil pretreatment showed a significant decrease in calcium overload (*P<0.001). Results are presented in (Fig. 16).

Fig. 16: Mean fluorescence of neuronal rich populations (10^6/ml) from sham, I/R and C.oil pretreatment I/R for quantification of different parameters assessed by acquiring and analyzing in cell quest program of Becton Dickinson Flowcytometer. Data are expressed as mean fluorescence ± S.E.M of five animals per group. (*P<0.001) was considered highly significant when comparisons were made with the I/R group by one-way ANOVA followed by Newman Keuls post hoc test. For measurements of intracellular calcium, fluorescence of Fluo-3AM was obtained from neuronal rich populations of sham, I/R and C.oil pretreatment I/R groups of rats.
An increase in fluorescence for mitochondrial membrane potential ($\Delta \Psi_m$) was observed in the neuronal rich populations from I/R group. C. oil pretreatment offered significant protection as the fluorescence was decreased ($*P<0.05$) (Fig.17).

**Fig.17:** Mean fluorescence of neuronal rich populations ($10^6$/ml) from sham, I/R and C. oil pretreatment I/R for quantification of different parameters assessed by acquiring and analyzing in cell quest program of Becton Dickinson Flowcytometer. Data are expressed as mean fluorescence ± S.E.M of five animals per group. ($*P<0.05$) was considered highly significant when comparisons were made with the I/R group by one-way ANOVA followed by Newman Keuls post hoc test. For estimation of mitochondrial membrane potential, the mean fluorescence of Rhod 123 obtained from neuronal rich populations of sham, I/R and C. oil pretreatment I/R groups of rats was used.

Significantly increased level of peroxynitrite was observed in the neuronal rich populations from I/R group as compared to sham group. C. oil pretreatment significantly decreases the ischemia induced peroxynitrite content ($*P<0.01$). As a control study, when the cells were treated with superoxide dismutase (SOD) the signals were significantly reduced, while with treatment with Catalase (CAT) there was no significant change in the fluorescence which further confirmed that the signals were coming from peroxynitrite and not due to the ROS or NO (Fig.18).
Fig. 18: Mean fluorescence of neuronal rich populations (10^6/ml) from sham, I/R and C. oil pretreatment I/R for quantification of different parameters assessed by acquiring and analyzing in cell quest program of Becton Dickinson Flowcytometer. Data are expressed as mean fluorescence ± S.E.M of five animals per group. (*P < 0.01) was considered highly significant when comparisons were made with the I/R group by one-way ANOVA followed by Newman Keuls post hoc test. For estimation of peroxynitrite, the mean fluorescence of DHR 123 in neuronal rich populations from sham, I/R and C. oil pretreatment I/R groups of rats was used, and as a control neuronal rich populations of the I/R group were treated with superoxide dismutase (SOD) or catalase (CAT).

6.2.7 Determination of neuronal mode of death

Significantly large numbers of neurons in the in the neuronal rich populations from the rats of the I/R group, were found apoptotic in comparison to other groups. The C. oil pretreatment group showed reduction in cell death. The percentage of viable cells was increased significantly, with reduction in the early apoptotic cells population (Fig. 19 a & b) (*P < 0.001).
Fig. 19 (a): Histogram showing the viable and apoptotic population in neurons were obtained from sham, I/R and C.oil pretreatment I/R groups of rats. Data are expressed as mean ± S.E.M of five animals per group. (*P < 0.001) was considered highly significant when comparisons were made with the ischemia/reflow group by one-way ANOVA followed by Newman Keuls post hoc test. (b) Representative dot plots for quantification of mode of death the mean florescence from neuronal rich populations using FITC- conjugated Annexin V and Propidium iodide (PI). Neuronal rich populations (10^6/ml) were obtained from sham, I/R and C.oil pretreatment I/R groups of rats; lower left quadrant shows viable cells (Annexin V negative and PI negative), lower right quadrant shows early apoptotic cells (Annexin V positive and PI negative), upper right quadrant shows late apoptotic/necrotic cells (Annexin V positive and PI positive) and upper left quadrant shows necrotic cells (Annexin V negative and PI positive).

6.2.8 TUNEL staining

TUNEL-positive nuclei showing chromatin condensation and fragmentation into apoptotic bodies could be detected in the I/R group after 24hrs of reflow. The TUNEL-positive cells seemed to have disintegrated membranes. C.oil pretreatment greatly reduced the TUNEL-positive cells. These cells might have been diffusely stained pointing towards attenuation in damage caused by ischemia/reflow (Fig. 20).
Fig. 20: Representative of Laser confocal images of in situ DNA fragmentation as detected by the TUNEL method in the ischemic cortex (D-F). Comparisons were made among sham control and ischemia with or without C.oil (250 mg/kg body weight), which was injected i.p. within 30 min before MCAo. Cell death was demonstrated by TUNEL staining appears in green fluorescence (B, E & H), indicated by arrowheads and all nuclei are visible with Propidium iodide as red fluorescence (A, D & G) and merger of both is shown in C, F & I. Arrows in the cortical region 24hrs indicate the fluorescence after ischemia. (A, B & C) represents sham; (D, E & F) as ischemia reflow (I/R); (G, H&I) represent I/R-C.oil group rat sections. Magnification: 40X (Bar 20μm)

6.2.9 Caspase-3 activity in brain tissue

Ischemia induced a significant increase in mean fluorescence obtained from the caspase-3 activity (0.875 ± 0.052) in the brain tissue when compared with sham (0.434 ± 0.02). C.oil pretreatment significantly reduced the ischemia induced increase in caspase-3 activity (0.415 ± 0.0517) (*P<0.001) close to the level of the sham group (Fig. 21).
Fig. 21: Mean fluorescence of fluorometric substrate Ac-DEVD-AMC, in the brain tissue from sham, I/R and C.oil pretreatment groups of rats (n=5) was estimated. C.oil pretreatment decreased the Caspase-3 levels after 24hrs of ischemia/reflow. Data are expressed as mean ± S.E.M of five animals per group. (*P<0.001) was considered highly significant when comparisons were made with the ischemia/reflow group by one-way ANOVA followed by Newman Keuls post hoc test.

The increased levels of cleaved caspase-3 (17 kDa) in I/R group of rats were further confirmed by immunoblotting. The expression of cleaved caspase-3 was reduced by C.oil pretreatment as comparable to I/R group. Since the antibody used in the present study labels both of the procaspase-3 (32 kDa) and the cleaved caspase-3 (~17 kDa), the procaspase-3 expression was greater in the sham which was reduced in the I/R group and was more in the C.oil pretreatment group (Fig. 22).
**Fig.22:** Caspase-3 expression by immunoblotting. Bar chart shows the optical density of bands in caspase-3/β-actin ratio. Lower Panel shows the bands for procaspase-3 (32 kDa) and cleaved caspase-3 (~17 kDa) and β-actin. Lane 1: sham, lane 2: I/R with C.oil pretreatment, and lane 3: I/R (65 μg/lane). I/R induced cleaved caspase-3 expression was greatly reduced by C.oil pretreatment. The procaspase-3 levels were similar to sham group in C.oil pretreatment group the level was lowered in I/R group. Data are expressed as mean ± S.E.M of five animals per group. (*P<0.001) was considered highly significant when comparisons were made with the I/R group.

### 6.2.10 Cytochrome c, Bax, Bcl-2, and p53 immunoblotting and immunolabeling in brain cryosections

Expression of cytochrome c in the brain cryosections was increased in the I/R groups after 24 hrs. Double labeling (in the brain cryosections) using neuronal marker NeuN determined that cytochrome c positive cells were neurons (**Fig.23**).

**Fig.23:** Photomicrographs (representative of 5 photomicrographs) represents double immunostaining confocal images of cytochrome c in cortex region. Arrows indicate immunoreactive cells. Double fluorescent immunostaining with cytochrome c and NeuN at 24hrs after reflow was done in sham (A-C), I/R (D-F) and I/R-C.oil (G-I) groups of rats. Sections were immunolabeled with a polyclonal anti-cytochrome c antibody, which was observed as green fluorescence (B, E & H) and neuron specific marker (NeuN) positive cells shown as red fluorescence (A, D & G). The co-localization of both is shown in right hand panel (C, F& I). Magnification: 40X. (Bar 20μm).

C.oil treatment reduced cytochrome c immunoreactivity as compared to that in the ischemia/reflow group. Expression of cytochrome c in the immunoblot from the I/R group
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was increased in the I/R group after 24hrs, which was decreased in the C.oil treatment group (Fig.24).

![Graph showing cytochrome c expression](image)

**Fig.24:** Cytochrome c expression by immunoblotting. Bar chart shows the quantification of bands. Lower Panel showing the representative bands for cytochrome c and β-Actin as lane 1: sham, lane 2: I/R, and lane 3: I/R with C.oil pretreatment (65μg/lane). I/R induced cytochrome c expression was greatly reduced by C.oil pretreatment. Data are expressed as mean ± S.E.M of five animals per group. (*P<0.05) was considered highly significant when comparisons were made with the I/R group by one-way ANOVA followed by Newman Keuls post hoc test.

There was a significant increase in the expression of p53 (Fig.25) in the immunoblot from the I/R group after 24hrs, which was decreased in the C.oil treatment group in an almost similar pattern with the sham group. Immunolabeling of p53 (Fig.26) in the brain cryosections revealed localization of p53 in the cytoplasm in the I/R rats. Weak staining was observed in C.oil treated group.

![Photomicrographs showing p53 expression](image)

**Fig.25:** Panel shows the photomicrographs (representative of 5 photomicrographs) showing expression of p53 in the cerebral cortices of sham, I/R and I/R-C.oil. P53 positive cells are indicated by arrows. Magnifications 40X. (Bar 20μm)
Fig. 26: p53 expression by immunoblotting. Bar chart shows the quantification of bands in p53/β-actin ratio. I/R induced p53 expression was greatly reduced by C.oil pretreatment. Lower Panel showing the representative bands for p53 and β-actin as lane 1: sham, lane 2: I/R, and lane 3: I/R with C.oil pretreatment (65µg/lane). Data are expressed as mean ± S.E.M of five animals per group. (*P<0.01) was considered highly significant when comparisons were made with the ischemia/reflow group by one-way ANOVA followed by Newman Keuls post hoc test.

Bax expression (Fig. 27) was significantly increased in the ischemia/reflow group after 24hrs, that was lowered in the C.oil treatment group but the amount was not equal to that in the sham group.

Fig. 27: Bar chart shows the quantification of bands for Bax expression from immunoblotting, C.oil pretreatment decreased the Bax levels after 24 hrs of ischemia/reflow. Lower Panel showing the representative bands for Bax and β-actin as lane 1: sham, lane 2: I/R, and lane 3: I/R with C.oil pretreatment (65µg/lane). Data are expressed as mean ± S.E.M of five animals per group. (*P<0.01) was considered highly significant when comparisons were made with the ischemia/reflow group by one-way ANOVA followed by Newman Keuls post hoc test.
Bcl-2 expression (Fig.28) was reduced in ischemia/reflow group after 24hrs, which was significantly increased in the C.oil treatment group, but the amount was more than that recorded in the sham group.

![Bar chart showing Bcl-2 expression levels](image)

Fig.28: Bar chart shows the quantification of bands for Bcl-2 expression from immunoblotting, C.oil pretreatment increased the Bcl-2 levels after 24 hrs of ischemia/reflow. Lower Panel shows the representative bands for Bcl-2 and β-actin as lane 1: sham, lane 2: I/R, and lane 3: I/R with C.oil pretreatment (65μg/lane). Data are expressed as mean ± S.E.M of five animals per group. (*P<0.05) was considered highly significant when comparisons were made with the ischemia/reflow group by one-way ANOVA followed by Newman Keuls post hoc test.

6.3 Discussion

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 (Fig.29).
Fig. 29: Diagrammatic representation of putative pathways showing the mechanism of ischemia induced neuronal death. During ischemia, excessively released glutamate activates NMDA receptors, leading to the calcium influx. The intracellular calcium rise then activates the calcium-dependent multiple pathways, promoting neuronal injury. The NO and oxygen radicals generated via activation of the NOS and catabolic enzymes, further leads to the increase in the levels of ROS and thereafter ONOO⁻, which brings about changes in the mitochondria, due to which the translocation of p53 to the outer membrane and release of cytochrome c occurs which further activates the family of caspases which are known to cleave nuclear DNA resulting in apoptosis. The picture depicts the crucial molecular changes involved in the neuronal death.

The therapeutic goal certainly is the protection of neurons, but this cannot be achieved without counteracting secondary phenomena such as edema formation in the conditions of stroke. Cellular edema is the very first visible morphologic feature after ischemic insult. In conditions of stroke, tissue swelling is located mainly in the penumbra region, where destabilization of the blood brain barrier (BBB) may lead to extravasations and progressive cellular edema (Reempts and Borgers, 2000). Cerebral edema volume is an initial and important event in the injury pathway, which was attenuated in the present experiments by C. oil pretreatment. From a functional or clinical point of view, the penumbra can be considered as the area at risk for the patient and is the area of hope for therapy, since changes detected in this region can be reversed. After severe ischemia, such as MCAo, the BBB is damaged, leading to uncontrolled swelling and infarction within a few hours of the onset of
ischemia. The significant attenuation of the area of infarct by C. oil treatment led us to further explore the injury pathway and the modifications thereof. Another important factor - NO probably contributes to BBB breakdown and ONOO− is involved in neuronal cell death. C. oil knocks down the damaging chain of events in an orderly fashion. A significant reduction was observed with C. oil treatment in the levels of calcium, of reactive oxygen species, of NOx and NO and the mitochondrial membrane potential. ROS and myeloperoxidase activity are measures of polymorphonuclear leukocyte infiltration in inflammation. A similar but a graver situation develops during reflow after cerebral ischemia and this process is significantly inhibited by C. oil pretreatment (Fig. 30).

Fig. 30: Picture depicting the main molecular targets of C. oil that leads to different types of cell death. Arrowheads indicate the main biological end points preceding cell death. C. oil significantly reduced the Calcium overload there by decreasing the mitochondrial membrane potential. C. oil significantly reduces the nitrosative stress by inhibiting the iNOS, nNOS & eNOS expression resulting in the reduction of NO and its metabolites, and moreover the oxidative stress by decreasing the levels of MPO and ROS, all these events together leads to the apoptosis in the neurons which is checked by C. oil pretreatment. The significant inhibition of these putative events leads to the increased rate of survival of neurons.

In addition to, being associated with neurodegeneration, NO and ONOO− have also been implicated in BBB breakdown (Tan et al., 2004). In the whole animal, ONOO−, which has a short half-life, might not be able to interact with the BBB (Beckman et al., 1990), but in the cellular milieu, it can damage DNA severely and trigger apoptosis (Estevez et al., 1995).
Despite the increased presence of NO during excitotoxicity (Almeida and Bolanos, 2001), its specific role remains unclear. Depending upon the cellular source from which the NO is produced, it may be protective or a degenerative or may even have no effect on excitotoxicity (Dawson et al., 1991).

NO is produced endogenously by a family of nitric oxide synthases (NOS), with a wide range of physiological and pathophysiological actions (Almeida and Bolanos, 2001). The increase in NO levels starts as early as 5 min after onset of ischemia and fades out within 1 hr, thereafter a secondary rise ensures between 12 and 24 hrs during reflow (Holtz et al., 2001).

C.oil attenuated the rise in the levels of intracellular calcium. The raised Ca$^{2+}$ levels contribute to the destruction of structural and regulatory proteins. Neurons have a special ability to respond to increases in [Ca$^{2+}$]i with increases in NO production via neuronal NO synthase. The time course of rapid expression of the three different types of NOS's has raised lots of controversies; whether such expression is simultaneous or overlapping needs detailed investigation.

The study was planned to monitor the expression of all the three NOS isoforms in the neurons, in the brain cryosections and in the western blot. The eNOS, originally identified in the vascular endothelial tissue, has been shown to be expressed in threatened neurons (Leker et al., 2001). It may be argued that the preservation of eNOS in the ischemic penumbra may limit neurological deficit and attenuate ischemic burden. Whether eNOS expression in neurons is the target, or the source of NO, or is dependent on the severity of injury needs further investigation. Expression of eNOS is reported in cortical neurons of the penumbra at all time points after the onset of stroke (Leker et al., 2001). As per the initial findings, by the co-localization of the eNOS and NeuN immunofluorescent signals, clearly identified these cells as neurons. Defined eNOS expression was observed in the neurons of the ischemic penumbra, while nNOS was particularly abundant in the core region.

As reported earlier (Holtz et al., 2001), an increase in the nNOS expression was observed during ischemia/reflow. C.oil treatment significantly reduces nNOS expression in neurons, as observed in brain cryosections, and has been further confirmed by western blot.

The iNOS is reported to be expressed in macrophages, neutrophils etc (Nathan and Xie, 1994). There is contradictory evidence for the absence or presence of iNOS in neurons. Nathan and Xie (1994) proposed that iNOS could be expressed via both the calcium-
dependent and the calcium-independent pathways. The implications for these dual routes of NO expression remain controversial. Zhu et al (2002) have observed expression of iNOS in glial cells within the ischemic brain, and it is reported to be involved in enlargement of infarct volume. Neurons, threatened by the severe drop in perfusion, may use the robust and sustained production of NO, by eNOS or iNOS, in an effort to restore blood flow or to initiate delayed intracellular signaling cascades that may lead to cellular preservation, or even, to cellular degeneration (Holtz et al., 2001). The present data show that C.oil inhibited iNOS expression, which may be responsible for its protective effects against ischemic stroke. There is a recent claim that in the glial cells, iNOS expression is dependent on NF-κB activation (Togashi et al., 1997). C.oil has a COX-2 inhibitory effect, and a combination of several of the fractions that are contained in the turmeric oil was effective at inhibiting PGE2 (Lantz et al., 2005). It is tempting to infer, that the inhibition of iNOS expression by C.oil may be due to the blockage of NF-κB activation, but that is yet to be proved.

It was observed that cerebral ischemia results in increased oxidative stress and a rise in the intracellular calcium concentration. These have been linked to the opening of mitochondrial permeability transition pores. Bcl-2 acts on mitochondria to prevent the release of cytochrome c and inhibits caspase-3 activation. Caspases, once cleaved, can cleave nuclear DNA repair enzymes, leading to nuclear DNA damage and apoptotic cell death (Niwa et al., 2001). As the mitochondrial membrane potential was held to normal levels by the administration of C.oil, the release of cytochrome c was inhibited. C.oil pretreatment up-regulated the Bcl-2 expression and down-regulated the Bax expression. C.oil salvaged the ischemic tissue by reducing the rise in the level of caspase-3.

Most studies provide evidence that the production of free radicals damages cellular components, including DNA (Watanabe et al., 1999) and acts as the most pervasive activator of p53. Is the effect of C.oil on p53 due to the attenuation of levels of MPO and ROS? In agreement with earlier reports (Watanabe et al., 1999), p53 expression following middle cerebral artery occlusion was also observed. Post-injury increase in p53 is essential for the neuronal apoptosis that ensues, a conclusion perhaps best exemplified by the finding that kainate treatment caused the death of p53+/+, but not of p53−/− neurons both in vivo and in vitro (Xiang et al., 1996). C.oil treatment inhibited the elevation of p53 expression caused by ischemic injury. Recent studies have recognized the important role of mitochondria in neuronal apoptotic processes. The present results clearly demonstrate the ability of C.oil to inhibit the mitochondria-mediated apoptotic pathway.
It has been argued that, if the cellular damage is too extensive and the energy machinery fails apoptotic processes may switch to necrosis. Much work remains to be done in the area of cerebral stroke to explore the potential of antiapoptotic drugs. In the present study, ischemia/reflow induced neuronal apoptosis is demonstrated by labeling neurons by FITC-conjugated Annexin V and PI, Terminal deoxynucleotidyl transferase-mediated dUTP Nick End Labeling (TUNEL) of DNA fragmentation, nuclei condensation. The apoptosis induced was observed to be caspase-dependent, as was indicated by the increase in caspase-3 cleavage.

Concluding the findings and inferences, firstly, C.oil suppressed the rise in the intracellular concentration of Ca\(^{2+}\) - a common component in the signaling pathways. Intracellular calcium induces NO production through the activation of NOS isoforms. The high levels of NO generated by NOS isoforms that are partially responsible for exacerbating the neuronal damage were reduced by Coil. In addition, C.oil prevented post ischemic brain neutrophil infiltration and NO metabolites and thus markedly reduced the production of reactive oxygen species. Secondly, C.oil suppressed the elevated protein level of Bax, and the mitochondrial translocation and activation of Bcl-2 that is triggered by altered mitochondrial membrane potential. It has reduced the cytosolic release of apoptogenic molecules cytochrome c, inhibited the activation of caspase-3, almost completely eliminated p53 expression and ultimately inhibited apoptosis. Lastly, the observations of present studies clearly demonstrate that C.oil exerted its major action in the penumbral region of the infarct; it is this region that is protected by modulation of apoptosis. The therapeutic time window of C.oil was 2hrs and the neuroprotective effect is at least in part due to the reduction of NO-induced formation of peroxynitrite and apoptosis in the transient MCAo model in rat.