Chapter 2

Curcumin: nitric oxide system and apoptosis

Basic research is like shooting an arrow into the air and, where it lands, painting a target.

— Homer Burton Adkins (1892-1949) —
2.1 Introduction

Since 2000, many memory and brain benefits of curcumin are being slowly uncovered by scientists, clinicians and gerontologists (researchers who study the aging process). Curcumin has been shown to exhibit anti-inflammatory, antimitagenic, and anticarcinogenic activities. In the previous chapter the dose dependent neuroprotective effect of curcumin, a polyphenolic antioxidant was studied in vivo, in the brain. Curcumin acts as a potent anticarcinogenic, and is known to act in three ways in cancer cells - as an anti-inflammatory agent, as an agent that causes death of cancer cells and more importantly, as an agent that prevents the formation of new blood vessels in the cancer cells, that chokes the life out of cancer cells. These events finally cause apoptosis of cells by impairing the ubiquitin–proteasome system through the mitochondrial pathway (Jana et al., 2004). Curcumin causes rapid decrease in mitochondrial membrane potential and release of cytochrome c to activate caspase 9 and caspase 3 for apoptotic cell death (Jana et al., 2004). Recently, an interesting observation was made in SW480 cells (colon cancer), Curcumin was found to be ineffective to cause apoptosis. What happens in ischemic conditions is that curcumin acts in a diametrically opposite manner, promoting the formation of blood vessels and inhibits apoptosis of neurons.

Nitric oxide (NO) has been found to inflict damage on important biomolecules, and the overproduction of NO in diseases may be implicated in cerebral stroke. However, the modulatory effect of curcumin on the neuronal damage largely remains unknown. The current study examined whether curcumin influenced nitric oxide in neuroprotection against nitrosative stress.

2.2 Results

2.2.1 Brain neutrophil infiltration

MPO activity was evaluated at two-time point at 7 and 24hrs of MCAo. Interestingly no significant difference at 7 hrs in all the groups under study was observed. MPO activity in sham-operated was (3.667 ± 0.9821 mU/g/min), I/R (9.920 ± 0.64 mU/g/min) and curcumin treatment (5.48 ± 2.68 mU/g/min). Whereas in the I/R rats after 24hrs MPO activity was found to be, 74 ± 7.6 mU/g/min that is significantly high as compared to the sham-operated...
group. Curcumin post treatment significantly reduced the MPO activity after 24 hrs of reflow to $32.57 \pm 0.38$ mU/g/min (*$P<0.05$) (Fig.9).

![Fig.9: Myeloperoxidase (MPO) activity after 7 hrs and 24hrs I/R from sham, I/R and Curcumin post treatment groups of rats. Curcumin post treatment decreased the MPO levels after 7hrs and 24 hrs of ischemia/reflow Data are expressed as mean fluorescence $\pm$ S.E.M of five animals per group. Statistical significance was assessed by one way ANOVA. (*$P<0.05$) was considered highly significant when comparisons were made among different groups by Newman Keuls post hoc test.](image)

2.2.2 Brain tissue NOx levels

Increase in nitrite levels was observed in the I/R group of rats. After 7hrs of reflow a significant rise was observed ($32.59 \pm 1.174 \mu$mol/mg) as compared to sham-operated rats $15.65 \pm 3.75 \mu$mol/mg. Post treatment with Curcumin reduced the nitrite formation significantly $8.163 \pm 1.516 \mu$mol/mg (*$P<0.05$). Increase in nitrite levels was observed in the I/R group of rats 24hrs after reflow $40.70 \pm 2.325 \mu$mol/mg as compared to the sham-operated rats $13.70 \pm 0.0 \mu$mol/mg. Post treatment with Curcumin reduced the nitrite formation after 24 hrs of reflow significantly $40.61 \pm 0.9522 \mu$mol/mg (*$P<0.05$) (Fig.10).
Fig. 10: Tissue nitrite content in sham, I/R and Curcumin post treatment group measured at 7hrs and 24hrs after I/R. Increased tissue nitrite level in I/R was significantly reduced by Curcumin post treatment.

Increase in nitrate levels was observed in the I/R group of rats. After 7hrs of reflow a significant rise was observed (12.39 ± 1.217 μmol/mg) as compared to the sham-operated rats (3.1 ± 0.435 μmol/mg). Post treatment with Curcumin reduced the nitrate formation significantly (8.330 ± 1.51 μmol/mg; *P<0.05). The I/R rats showed an increase in the nitrate levels 24hrs after reflow (13.57 ± 0.47 μmol/mg) as compared to that from the tissue from the sham-operated rats. Post treatment with Curcumin reduced the nitrate formation significantly (7.49 ± 0.92 μmol/mg; *P<0.05) (Fig. 11).

Fig. 11: Tissue nitrate content in sham, I/R and Curcumin post treatment I/R group measured at 7hrs and 24hrs after I/R. Increased tissue nitrate level in I/R was significantly reduced by Curcumin post treatment.
2.2.3 NOS isoform immunoblotting and immunolabeling in brain cryosections

The immunolabeling patterns (in the brain cryosections) of iNOS (Fig.12), nNOS (Fig.13) and eNOS (Fig.14), expression were examined in brain tissue sampled after 7hrs and 24hrs of reflow. An increase in the expression of iNOS, nNOS and eNOS was observed in the I/R group. Curcumin post treatment decreased the nNOS, iNOS and eNOS immunoreactivity with well-defined cells in the cerebral cortex region. However, co-localization in the section from the Curcumin 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.15), nNOS (Fig.16) and eNOS (Fig.17) was observed in I/R group after 24hrs in the immunoblot, which was decreased in the Curcumin treated group.
Fig. 12: Immunolabeling (in brain cryosections) of nitric oxide synthase isoforms. (Five animals per group were used). Photomicrographs (representative of 5 photomicrographs) showing inducible nitric oxide synthase (iNOS) immunoreactivity in brain cryosections from sham group (A-C) cerebral tissue subjected to 1 hr of transient ischemia followed by 7 hrs and 24 hrs of reflow in I/R, and I/R-Curcumin treated groups. Sections were immunolabeled with a polyclonal anti-iNOS antibody, which was observed as green fluorescence (B, E, H, K & N) and neuron specific marker NeuN shown as red fluorescence (A, D, G, J & M). The merger is shown in C, F, I, L & O. Arrows in the cortical region 24 hrs indicate the fluorescence after ischemia. (A, B & C) represents sham; (D, E & F) as ischemia reflow at 7 h (I/R-7h); (G, H & I) represent I/R-Curcumin group at 7 h (Cur 7h), (J, K & L) as ischemia reflow.
at 24 h (I/R-24h); (M,N&O) represent I/R-Curcumin group at 24 h (Cur 24h), rat sections. Magnification: 40X (Bar 20µm)

Fig.13: Photomicrographs (representative of 5 photomicrographs) showing neuronal nitric oxide synthase (nNOS) immunoreactivity in brain cryosections from sham group (A-C) cerebral tissue subjected to 1hr of transient ischemia followed by 7hrs and 24hrs of reflow in I/R, and I/R-Curcumin treated groups. (Five animals per group were used). Sections were immunolabeled with a polyclonal anti-nNOS antibody, which was observed as green fluorescence (B, E, H, K& N) and neuron specific marker NeuN shown as red fluorescence (A, D,G, J& M). The merger is shown in C, F, I, L& O. Arrows in the cortical region 24hrs indicate the fluorescence after ischemia. (A, B & C) represents sham; (D, E & F) as ischemia reflow at 7 h (I/R-7h); (G, H&I) represent I/R-Curcumin group at 7 h (Cur 7h), (J,K & L) as ischemia reflow at 24 h (I/R-24h); (M,N&O) represent I/R-Curcumin group at 24 h (Cur 24h), rat sections. Magnification: 40X (Bar 20µm)
Fig. 14: 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 7 hrs and 24 hrs of reflow in I/R, and I/R-Curcumin treated groups. (Five animals per group were used). Sections were immunolabeled with a polyclonal anti-eNOS antibody, which was observed as green fluorescence (B, E, H, K & N) and neuron specific marker NeuN shown as red fluorescence (A, D, G, J & M). The merger is shown in C, F, I, L & O. Arrows in the cortical region 24 hrs indicate the fluorescence after ischemia. (A, B & C) represents sham; (D, E & F) as ischemia reflow at 7 h (I/R-7h); (G, H & I) represent I/R-Curcumin group at 7 h (Cur 7h); (J, K & L) as ischemia reflow at 24 h (I/R-24h); (M, N & O) represent I/R-Curcumin group at 24 h (Cur 24h), rat sections. Magnification: 40X (Bar 20 μm)
**Fig. 15:** 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 (7hrs); lane 3: I/R (24hrs), lane 4: I/R with Curcumin post treatment (7hrs) and lane 5: I/R with Curcumin post treatment (24hrs), (50 μg/lane). I/R induced iNOS expression was greatly reduced by Curcumin post treatment.

**Fig. 16:** 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 Curcumin post treatment (7hrs); lane 3: I/R with Curcumin post treatment (24hrs), lane 4: I/R (7hrs); and lane 5: I/R (24hrs) (50 μg/lane). I/R induced nNOS expression was greatly reduced by Curcumin post treatment.
Fig. 17: eNOS expression by immunoblotting. Bar chart shows the optical density of bands in eNOS/β-actin ratio. Lower Panel showing the bands for eNOS and β-Actin as lane 1: sham, lane 2: I/R with Curcumin post treatment (7hrs); lane 3: I/R with Curcumin post treatment (24 hrs), lane 4: I/R (7hrs); and lane 5: I/R (24hrs) (50 μg/lane). I/R induced eNOS expression was greatly reduced by Curcumin post treatment.

2.2.4 Neuronal NO, ROS, calcium, peroxynitrite and mitochondrial membrane potential (ΔΨm) 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. Curcumin post treatment showed a significant decrease in NO content (*P<0.05) in the neuronal rich populations isolated (ex vivo) (Fig.18)

Fig. 18: Mean fluorescence of neuronal rich populations (10^6/ml) from sham, I/R and Curcumin post treatment 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. Mean
fluorescence of DAF-2DA from neurons (10^6/ml) of sham, I/R and Curcumin post treatment I/R was done.

A significant increase in the levels of ROS in the neuronal rich populations from I/R was observed as compared to that from the sham-operated rats. Curcumin treated I/R group showed significant decrease in ROS content (*P<0.001) (Fig.19).

Fig.19: Mean fluorescence of neuronal rich populations (10^6/ml) from sham, I/R and Curcumin post treatment 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 ROS estimation the mean fluorescence of DHDCF-DA in isolated neuronal rich populations from sham, I/R and Curcumin post treatment 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. Curcumin post treatment showed a significant decrease in calcium overload (*P<0.05). Results are presented in (Fig.20).

Fig.20: Mean fluorescence of neuronal rich populations (10^6/ml) from sham, I/R and Curcumin post treatment 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 measurements of intracellular calcium, fluorescence of Fluo-3AM was obtained from neuronal rich populations of sham, I/R and Curcumin post treatment I/R groups of rats.

An increase in mean fluorescence for mitochondrial membrane potential (∆Ψm) was observed in the in the neuronal rich populations from I/R group. Curcumin post treatment offered significant reduction in the mean fluorescence was decreased (*P<0.05) (Fig.21).

![Fig.21: Mean fluorescence of neuronal rich populations (10⁶/ml) from sham, I/R and Curcumin post treatment 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 Curcumin post treatment I/R groups of rats was used.

A sharp increase in the level of peroxynitrite was observed in the in the neuronal rich populations from I/R group as compared to sham group. Curcumin post treatment significantly decreases the ischemia induced peroxynitrite content (*P<0.05) (Fig.22).
**Fig.22:** Mean fluorescence of neuronal rich populations (10⁶/ml) from sham, I/R and Curcumin post treatment 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 peroxynitrite, the mean fluorescence of DHR 123 in neuronal rich populations from sham, I/R and Curcumin post treatment I/R groups of rats was used.

### 2.2.5 Determination of neuronal mode of death

Significantly large numbers of neurons were Annexin-V⁺ and PI⁺ from the I/R group of rats after 24hrs of reflow indicating an apoptotic population in the neuronal rich cell preparation as compared to sham-operated rats. Curcumin post treatment on one hand significantly reduced the number both type (apoptotic and necrotic) of cell and on other improved the percentage of viable cells significantly (**Fig.23**) (*P < 0.05*).
Fig. 23: Histogram showing the viable and apoptotic population in neurons were obtained from sham, I/R and Curcumin post treatment I/R groups of rats. (f) 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 Curcumin post treatment 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).

2.2.6 TUNEL staining

TUNEL-positive nuclei showing chromatin condensation and fragmentation into apoptotic bodies could be detected in the I/R group after 7hrs and 24hrs of reflow. The TUNEL-positive cells seemed to have disintegrated membranes. Curcumin post treatment greatly reduced the TUNEL-positive cells at both time point of investigation. These cells might have been diffusely stained pointing towards attenuation in damage caused by ischemia/reflow (Fig. 24).
Fig. 24: Representative of Laser confocal images of in situ DNA fragmentation as detected by the TUNEL method in the ischemic cortex at 7 hrs (D-F) and at 24 hrs (J-L). Comparisons were made among sham control and ischemia with or without Curcumin (2mg/kg i.v.), which was injected after 6hrs of MCAo. Cell death was demonstrated by TUNEL staining appears in green fluorescence (B, E, H, K& N), indicated by arrowheads and all nuclei are visible with Propidium iodide as red fluorescence (A, D, G, J& M) and merger of both is shown in C, F, I, L& O. Arrows in the cortical region 24hrs indicate the fluorescence after ischemia. (A, B & C) represents sham; (D, E & F) as ischemia reflow at 7 h (I/R-7h); (G, H&I) represent I/R-Curcumin group at 7 h (Cur 7h), (J, K & L) as ischemia reflow at 24 h (I/R-24h); (M, N&O) represent I/R-Curcumin group at 24 h (Cur 24h), rat sections. Magnification; 40X (Bar 20μm)

2.2.7 Caspase-3 activity in brain tissue

After 7 hrs of reflow ischemia induced a significant increase in mean fluorescence obtained from the caspase-3 activity (0.408 ± 0.018) in the brain tissue when compared with
sham (0.29 ± 0.06). Curcumin post treatment significantly reduced the ischemia induced increase in caspase-3 activity (0.293 ± 0.007) (*P<0.05) close to the level of the sham group. After 24 hrs of reflow ischemia induced a significant increase in mean fluorescence obtained from the caspase-3 activity (0.593 ± 0.019) in the brain tissue when compared with sham. Curcumin post treatment significantly reduced the ischemia induced increase in caspase-3 activity (0.372± 0.019) (*P<0.05) close to the level of the sham group of rats (Fig.25).

![Fig.25: Mean fluorescence of fluorometric substrate Ac-DEVD-AMC, in the brain tissue from sham, I/R and Curcumin post treatment groups of rats (n=5) was estimated. Curcumin post treatment decreased the Caspase-3 levels after 7hrs and 24hrs of ischemia/reflow. 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.](image-url)
Fig.14: Photomicrographs (representative of 5 photomicrographs) showing Caspase-3 immunoreactivity in brain cryosections from sham group (A-C) cerebral tissue subjected to 1hr of transient ischemia followed by 7hrs and 24hrs of reflow in I/R, and I/R-Curcumin treated groups. (Five animals per group were used). Sections were immunolabeled with a polyclonal anti-Caspase-3 antibody, which was observed as green fluorescence (B, E, H, K& N) and neuron specific marker NeuN shown as red fluorescence (A, D,G, J& M). The merger is shown in C, F, I, L& O. Arrows in the cortical region 24hrs indicate the fluorescence after ischemia. (A, B & C) represents sham; (D, E & F) as ischemia reflow at 7 h (I/R-7h); (G, H & I) represent I/R-Curcumin group at 7 h (Cur 7h); (J, K & L) as ischemia reflow at 24 h (I/R-24h); (M, N & O) represent I/R-Curcumin group at 24 h (Cur 24h), rat sections. Magnification: 40X (Bar 20μm)

The increased levels of cleaved caspase-3 (17 &19 kDa) in I/R group of rats were further confirmed by immunoblotting. The expression of cleaved caspase-3 was reduced by Curcumin post treatment as comparable to sham group. Since the antibody used in the present study labels cleaved caspase-3 (~17 &19 kDa), the caspase-3 expression was greater in the
I/R group as compared to sham and was decreased in the Curcumin post treatment significantly reduced the expression (Fig.26).

![Relative activated Caspase-3 level (versus sham)](image)

**Fig.26:** Caspase-3 expression by immunoblotting. Bar chart shows the optical density of bands. Lower Panel shows the bands for cleaved caspase-3 (17 & 19 kDa). Lane 1: sham, lane 2: I/R with Curcumin post treatment (7hrs), lane 3: I/R with Curcumin post treatment (24hrs); lane 4: I/R (7hrs) and lane 5: I/R (24 hrs) (65 µg/lane). I/R induced cleaved caspase-3 expression was greatly reduced by Curcumin post treatment. The cleaved caspase-3 levels were similar to sham group in Curcumin post treatment group the level was lowered as compared to I/R groups. 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.

### 2.2.8 Caspase-9 activity in brain tissue

After 7 hrs of reflow ischemia induced a significant increase in mean fluorescence obtained from the caspase-9 activity (0.341± 0.032) as compared with sham (0.21 ± 0.0213). Curcumin post treatment significantly reduced the ischemia induced increase in caspase-9 activity (0.148 ± 0.0397) (*P<0.05) bringing it close to the level of the sham group. Twenty-four hours of reflow after MCAo a significant increase in mean fluorescence of caspase-9 activity (0.370 ± 0.026) was observed as compared with sham. Curcumin post treatment significantly reduced the ischemia induced increase in caspase 9 activity (0.187 ± 0.029) (*P<0.05) (Fig.27) as compared to I/R rats.
Fig.27: Mean fluorescence of fluorometric substrate for Caspase 9, in the brain tissue from sham, I/R and Curcumin post treatment groups of rats (n=5) was estimated. Curcumin post treatment decreased the Caspase-9 levels after 7hrs and 24hrs of ischemia/reflow. 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.

2.2.9 Caspase-1& 4 activity in brain tissue

After 7 hrs of reflow ischemia induced a significant increase in mean fluorescence obtained from the caspase-1& 4 activity (0.330± 0.02) in the brain tissue when compared with sham (0.186± 0.028). Curcumin post treatment significantly reduced the ischemia induced increase in caspase1& 4 activity (0.258 ± 0.021) (*P<0.05) close to the level of the sham group. After 24 hrs of reflow ischemia induced a significant increase in mean fluorescence obtained from the caspase1& 4 activity (0.372± 0.014) in the brain tissue when compared with sham. Curcumin post treatment significantly reduced the ischemia induced increase in caspase1& 4 activity (0.238± 0.032) (*P<0.05) (Fig.28).
**Fig.28:** Mean fluorescence of fluorometric substrate for caspase 1&4, in the brain tissue from sham, I/R and Curcumin post treatment groups of rats (n=5) was estimated. Curcumin post treatment decreased the Caspase-1&4 levels after 7hrs and 24hrs of ischemia/reflow. 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.

2.2.10 **Cytochrome c, Bax, Bcl-2, TNFα, IL6 and p53 immunoblotting and immunolabeling in brain cryosections**

2.2.10.1 **Expression of cytochrome c**

Expression of cytochrome c in the brain cryosections was increased in the I/R groups after 7hrs and 24 hrs. Double labeling (in the brain cryosections) using neuronal marker NeuN determined that cytochrome c positive cells were neurons (Fig.29). Curcumin 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 was increased in the I/R group after 24hrs, which was decreased in the Curcumin treatment group (Fig.30).
Fig. 29: Photomicrographs (representative of 5 photomicrographs) showing cytochrome c immunoreactivity in brain cryosections from sham group (A-C) cerebral tissue subjected to 1hr of transient ischemia followed by 7hrs and 24hrs of reflow in I/R, and I/R-Curcumin treated groups. (Five animals per group were used). Sections were immunolabeled with a polyclonal anti-cytochrome c antibody, which was observed as green fluorescence (B, E, H, K& N) and neuron specific marker NeuN shown as red fluorescence (A, D,G, J& M). The merger is shown in C, F, I, L& O. Arrows in the cortical region 24hrs indicate the fluorescence after ischemia. (A, B & C) represents sham; (D, E & F) as ischemia reflow at 7 h (I/R-7h); (G, H & I) represent I/R-Curcumin group at 7 h (Cur 7h); (J, K & L) as ischemia reflow at 24 h (I/R-24h); (M, N & O) represent I/R-Curcumin group at 24 h (Cur 24h), rat sections. Magnification: 40X (Bar 20μm)
Fig. 30: Cytochrome c expression by immunoblotting. Bar chart shows the optical density of bands in cytochrome c/β-actin ratio. Lower Panel showing the bands for cytochrome c and β-Actin as lane 1: sham, lane 2: I/R (7hrs); lane 3: I/R (24hrs) lane 4: I/R with Curcumin post treatment (7hrs) and lane 5: I/R with Curcumin post treatment (24hrs), (50 μg/lane). I/R induced cytochrome c expression was greatly reduced by Curcumin post treatment.

2.2.10.2 Expression of p53

Expression of p53 in the brain cryosections was increased in the I/R groups after 7hrs and 24 hrs. Double labeling (in the brain cryosections) using neuronal marker NeuN determined that p53 positive cells were neurons. Curcumin treatment reduced p53 immunoreactivity as compared to that in the ischemia/reflow group (Fig. 31). Expression of p53 in the immunoblot from the I/R group was increased in the I/R group after 24hrs, which was decreased in the Curcumin treatment group. A significant increase in the expression of p53 in the immunoblot was observed from the I/R group at both time point of study i.e. 7hrs and 24hrs. Curcumin treatment at both time points reduced significantly and bringing the value close to the sham group operated group of rats (Fig. 32).
Fig. 31: Photomicrographs (representative of 5 photomicrographs) showing p53 immunoreactivity in brain cryosections from sham group (A-C) cerebral tissue subjected to 1 hr of transient ischemia followed by 7 hrs and 24 hrs of reflow in I/R, and I/R-Curcumin treated groups. (Five animals per group were used). Sections were immunolabeled with a polyclonal anti-p53 antibody, which was observed as green fluorescence (B, E, H, K & N) and neuron specific marker NeuN shown as red fluorescence (A, D, G, J & M). The merger is shown in C, F, I, L & O. Arrows in the cortical region 24 hrs indicate the fluorescence after ischemia. (A, B & C) represents sham; (D, E & F) as ischemia reflow at 7 h (I/R-7h); (G, H & I) represent I/R-Curcumin group at 7 h (Cur 7h); (J, K & L) as ischemia reflow at 24 h (I/R-24h); (M, N & O) represent I/R-Curcumin group at 24 h (Cur 24h), rat sections. Magnification: 40X (Bar 20 μm)
Fig. 32: p53 expression by immunoblotting. Bar chart shows the optical density of bands in p53/β-actin ratio. Lower Panel showing the bands for p53 and β-Actin as lane 1: sham, lane 2: I/R (7hrs); lane 3: I/R (24hrs), lane 4: I/R with Curcumin post treatment (7hrs) and lane 5: I/R with Curcumin post treatment (24hrs), (50 μg/lane). I/R induced p53 expression was greatly reduced by Curcumin post treatment.

2.2.10.3 Expression of Bax

Expression of Bax in the brain cryosections was increased in the I/R groups after 7hrs and 24 hrs. Double labeling (in the brain cryosections) using neuronal marker NeuN determined that Bax positive cells were neurons (Fig. 33). Curcumin treatment reduced Bax immunoreactivity as compared to that in the ischemia/reflow group. Expression of Bax in the immunoblot from the I/R group was increased in the I/R group after 24hrs, which was decreased in the Curcumin treatment group. Bax expression was significantly increased in the ischemia/reflow group after 7hrs and 24hrs, which was lowered in the Curcumin treatment group but the amount, was not equal to that in the sham group (Fig. 34).
Fig. 33: Photomicrographs (representative of 5 photomicrographs) showing Bax immunoreactivity in brain cryosections from sham group (A-C) cerebral tissue subjected to 1 hr of transient ischemia followed by 7 hrs and 24 hrs of reflow in I/R, and I/R-Curcumin treated groups. (Five animals per group were used). Sections were immunolabeled with a monoclonal anti-Bax antibody, which was observed as green fluorescence (B, E, H, K & N) and neuron specific marker NeuN shown as red fluorescence (A, D, G, J & M). The merger is shown in C, F, I, L & O. Arrows in the cortical region 24 hrs indicate the fluorescence after ischemia. (A, B & C) represents sham; (D, E & F) as ischemia reflow at 7 hr (I/R-7h); (G, H & I) represent I/R-Curcumin group at 7 hr (Cur 7h); (J, K & L) as ischemia reflow at 24 hr (I/R-24h); (M, N & O) represent I/R-Curcumin group at 24 hr (Cur 24h), rat sections. Magnification: 40X (Bar 20 μm)
Chapter 2

Fig. 34: Bax expression by immunoblotting. Bar chart shows the optical density of bands in Bax/β-actin ratio. Lower Panel showing the bands for Bax and β-Actin as lane 1: sham, lane 2: I/R (7hrs); lane 3: I/R (24hrs), lane 4: I/R with Curcumin post treatment (7hrs) and lane 5: I/R with Curcumin post treatment (24hrs), (50 μg/lane). I/R induced Bax expression was greatly reduced by Curcumin post treatment.

2.2.10.4 Expression of Bcl-2

Brain cryosections from I/R showed reduction in expression of Bcl-2 at 7hrs and 24 hrs of reflow after MCAo. Double labeling (in the brain cryosections) using neuronal marker NeuN determined that Bcl-2 positive cells were neurons (Fig. 35). Curcumin treatment up regulated Bcl-2 expression as compared to that in the ischemia/reflow group at both time point of reflow 7 and 24 hrs. Expression of Bcl-2 in the immunoblot from the I/R group was increased after 24hrs. Bcl-2 expression was significantly increased in the Curcumin treatment group. (Fig. 36).
Fig. 35: Photomicrographs (representative of 5 photomicrographs) showing Bcl-2 immunoreactivity in brain cryosections from sham group (A-C) cerebral tissue subjected to 1hr of transient ischemia followed by 7hrs and 24hrs of reflow in I/R, and I/R-Curcumin treated groups. (Five animals per group were used). Sections were immunolabeled with a monoclonal anti-Bcl-2 antibody, which was observed as green fluorescence (B, E, H, K& N) and neuron specific marker NeuN shown as red fluorescence (A, D, G, J& M). The merger is shown in C, F, I, L& O. Arrows in the cortical region 24hrs indicate the fluorescence after ischemia. (A, B & C) represents sham; (D, E & F) as ischemia reflow at 7 h (I/R-7h); (G, H & I) represent I/R-Curcumin group at 7 h (Cur 7h); (J, K & L) as ischemia reflow at 24 h (I/R-24h); (M, N & O) represent I/R-Curcumin group at 24 h (Cur 24h), rat sections. Magnification: 40X (Bar 20μm)
Fig. 36: Bcl-2 expression by immunoblotting. Bar chart shows the optical density of bands in Bcl-2/β-actin ratio. Lower Panel showing the bands for Bcl-2 and β-Actin as lane 1: sham, lane 2: I/R with Curcumin post treatment (7hrs); lane 3: I/R with Curcumin post treatment (24hrs), lane 4: I/R (7hrs); and lane 5: I/R (24hrs) (50 μg/lane). Bcl-2 expression was greatly increased by Curcumin post treatment.

2.2.10.5 Expression of TNF α

Expression of TNF α in the brain cryosections was increased in the I/R groups after 7hrs and 24 hrs. Double labeling (in the brain cryosections) using neuronal marker NeuN determined that TNF α positive cells were neurons (Fig. 37). Curcumin treatment reduced TNF α immunoreactivity as compared to that in the ischemia/reflow group. Expression of TNF α in the immunoblot from the I/R group was increased in the I/R group after 24hrs, which was decreased in the Curcumin treatment group (Fig. 38).
Fig. 37: Photomicrographs (representative of 5 photomicrographs) showing TNF α immunoreactivity in brain cryosections from sham group (A-C) cerebral tissue subjected to 1hr of transient ischemia followed by 7hrs and 24hrs of reflow in I/R, and I/R-Curcumin treated groups. (Five animals per group were used). Sections were immunolabeled with a polyclonal anti-TNF α antibody, which was observed as green fluorescence (B, E, H, K & N) and neuron specific marker NeuN shown as red fluorescence (A, D, G, J & M). The merger is shown in C, F, I, L & O. Arrows in the cortical region 24hrs indicate the fluorescence after ischemia. (A, B & C) represents sham; (D, E & F) as ischemia reflow at 7 h (I/R-7h); (G, H & I) represent I/R-Curcumin group at 7 h (Cur 7h); (J, K & L) as ischemia reflow at 24 h (I/R-24h); (M, N & O) represent I/R-Curcumin group at 24 h (Cur 24h), rat sections. Magnification: 40X (Bar 20μm)
Fig. 38: TNF α expression by immunoblotting. Bar chart shows the optical density of bands in TNF α/ β-actin ratio. Lower Panel showing the bands for TNF α and β-Actin as lane 1: sham, lane 2: I/R with Curcumin post treatment (7hrs); lane 3: I/R with Curcumin post treatment (24 hrs), lane 4: I/R (7hrs); and lane 5: I/R (24hrs) (50 μg/lane). I/R induced TNF α expression was greatly reduced by Curcumin post treatment.

2.2.10.6 Expression of IL6

Expression of IL6 in the brain cryosections was increased in the I/R groups after 7hrs and 24 hrs. Double labeling (in the brain cryosections) using neuronal marker NeuN determined that IL6 positive cells were neurons (Fig. 39). Curcumin treatment reduced IL6 immunoreactivity as compared to that in the ischemia/reflow group. Expression of IL6 in the immunoblot from the I/R group was increased in the I/R group after 24hrs, which was decreased in the Curcumin treatment group (Fig. 40).
Fig. 39: Photomicrographs (representative of 5 photomicrographs) showing IL-6 immunoreactivity in brain cryosections from sham group (A-C) cerebral tissue subjected to 1 hr of transient ischemia followed by 7 hrs and 24 hrs of reflow in I/R, and I/R-Curcumin treated groups. (Five animals per group were used). Sections were immunolabeled with a polyclonal anti-IL-6 antibody, which was observed as green fluorescence (B, E, H, K & N) and neuron specific marker NeuN shown as red fluorescence (A, D, G, J & M). The merger is shown in C, F, I, L & O. Arrows in the cortical region 24 hrs indicate the fluorescence after ischemia. (A, B & C) represents sham; (D, E & F) as ischemia reflow at 7 hrs (I/R-7h); (G, H & I) represent I/R-Curcumin group at 7 hrs (Cur 7h); (J, K & L) as ischemia reflow at 24 hrs (I/R-24h); (M, N & O) represent I/R-Curcumin group at 24 hrs (Cur 24h), rat sections. Magnification: 40X (Bar 20μm)
Fig. 40: IL-6 expression by immunoblotting. Bar chart shows the optical density of bands in IL-6/β-actin ratio. Lower Panel showing the bands for IL-6 and β-Actin as lane 1: sham, lane 2: I/R (7hrs); lane 3: I/R (24hrs), lane 4: I/R with Curcumin post treatment (7hrs) and lane 5: I/R with Curcumin post treatment (24hrs), (50 μg/lane). I/R induced IL-6 expression was greatly reduced by Curcumin post treatment.

2.3 Discussion

The role of NO as it relates to the evolution of core and penumbral injury is complex. Within this context, it is relevant to mention, the initial rise in NO starts within 3–24 min and rises significantly up to 1 μM from <10nM during ischemia (Malinski et al., 1993) due to activation of constitutive NOS. In the extra cellular milieu, NO reacts with oxygen and water to form nitrates and nitrites. It was found that NOx levels were increased in the brain 24 after ischemic injury, consistent with results from other laboratories in animal studies (Gamez et al., 2003) and in patients following stroke (El Kossi and Zakhary, 2000). The delayed increase in nitrite and nitrate levels (7 to 24hrs) in the brain tissue after cerebral ischemia may be injurious or, at the very least, reflect injury. Curcumin blunts the detrimental rise in nitrite and nitrate at 7 and 24hrs after ischemic injury. The present results showed an increase in nitrite levels in the ischemic rats and treatment with curcumin significantly decreased brain nitrite levels. It is well documented that curcumin has NO scavenging effect because of its potential to inhibit expression of inducible nitric oxide synthase (Chan et al., 1998) and nitric oxide scavenging effects (Nanji et al., 2003). The increase in nitrosative stress in MCAo model of stroke is presumably mediated by peroxynitrite. NO combines with super oxide anions (O2-) to form peroxynitrite (ONOO-), an oxidizing free radical that causes DNA
fragmentation and lipid oxidation (Beckman, 1994). Especially it hits severely mitochondrion by acting on the respiratory chain complex leading to collapse of the energy production.

The present study showed that curcumin attenuates NO formation as shown indirectly by estimation of tissue nitrite and nitrate and NO levels. As curcumin reduces NO content at the injury site it may in turn stops the generation of peroxynitrite thereby salvaging tissue injury. One possible explanation can be that at one-hand curcumin functions as a direct scavenger of nitric oxide and on the other hand it inhibits ROS production as shown by present results. It is more pertinent to consider the formation of peroxynitrite due to the lack of availability of NO and ROS. Curcumin reduces the production of NO and ROS. Interestingly the resultant effect of ischemia the neuronal calcium overload was significantly reduced by curcumin. Further a significant rise in peroxynitrite levels was observed as previously reported. Curcumin significantly attenuated this rise. A rise in peroxynitrite levels can induce apoptotic cell death in ischemic injury (Virag et al., 1998). If so, peroxynitrite-mediated apoptotic may also be inhibited by curcumin. However, it is important to mention that, in addition to interacting with calcium, curcumin has other properties that may also contribute to neuroprotection.

Particularly during the ischemia eNOS expression is augmented, while nNOS increases during reperfusion. It has been suggested that eNOS-derived NO may protect against ischemia by contributing to vasodilatation and by inhibiting aggregation and adherence of platelets or leukocytes (Loscalzo and Welch, 1995). This striking burst of NO synthesis is not clearly evident whether endothelial NOS, neuronal NOS, or both caused the elevation of the NO end products seen after ischemic insults. Where as iNOS-mediated NO increase occur around 4 hrs after MCAo (Nagafuji et al., 1994). In transient forebrain ischemia model, iNOS expression peaks 12 hrs after I/R insult (Iadecola et al., 1996). Greater NOS activity in core regions could explain in part the increased vulnerability of that region to ischemia and could theoretically contribute to the progression of the infarct over time (Ashwal et al., 1998). Neuroprotection offered by curcumin could primarily be due to inhibition of iNOS because the curcumin was given after 6 hrs of initial ischemic insult and previous studies have shown that expression of iNOS starts after 4-6 hrs of initial ischemic insult. This observation was further substantiated by highly significant rise in nitrite and nitrate levels at 24hrs further, it is well documented that expression of nNOS peaks after 1 hr of initial ischemic insult so at the time of treatment this target is already lost.
NO triggers apoptosis when it binds to cytochrome $c$ oxidase and induces the formation of $O_2^-$ in the mitochondria, generating $\text{ONOO}^-$, which damages the mitochondrial complexes, membrane, DNA and mitochondrial SOD, and induces $\text{Ca}^{2+}$ release, transient permeability and mitochondrial swelling (Brown, 1999). Moreover, NO-induced apoptosis is related to the increase in the Bax/Bcl-xL rate, the release of cytochrome $c$ and caspase activation (Kolb, 2000).

Cytochrome $c$ along with Apaf-1 begins the activation of cascade of activation of caspases. Increased expression of caspase-1, -3, -8, and -9, and of cleaved caspase-8, has been reported in the penumbra. Caspase-1 has the peculiarity of being involved in the activation of both apoptosis and inflammation, through the inter-mediate of the pro-inflammatory cytokine IL-1$\beta$ (Rothwell et al., 1997). Although the present data demonstrated attenuation of caspase-1&4 and 3 by curcumin in transient focal cerebral ischemic injury, a role for cytokines in the regulation of cell death was not excluded.

Cerebral stroke follows complex cascade of event in ischemic injury. The action of some of events can lead to cell survival or cell death depending on the duration or severity of the insult (Resnick and Fennell, 2004). There is also disagreement as to the specific role of some of these pathways (Martindale and Holbrook, 2002) and the complex interactions (‘cross-talk’) that exist between these pathways. In brief, curcumin significantly reduces the ill effect of ischemia by attenuating nitrosative and oxidative stress and followed by decrease in mitochondrial membrane potential, increased cytochrome $c$ release, and subsequently iNOS expression, caspases activation and apoptosis. Within this context, it is relevant to mention that as curcumin attenuates the Bax and up-regulates Bcl-2 expression these possibly block the release of mitochondrial cytochrome $c$ and prevent apoptosis. Curcumin might therefore modify biochemical pathways that lead to cell death or survival. Perhaps action of curcumin in controlling both apoptosis and necrosis through up or down regulation is advantageous as a way to ensure immediate and complete elimination of damaged cells.

Cerebral edema, in the focal ischemic model, is known to occur as a result of ionic imbalance (and hence, the osmotic pressure) across the cellular membrane. Chemotactic recruitment of leukocyte is one of the initial events of the injury starts within hours of I/R insult. It was observed that curcumin treatment had significantly reduced the edema volume (please see chapter 1 of curcumin). MPO is a marker enzyme of PMNs and their infiltration at the ischemic site. On one hand activated leukocytes produces a secondary occlusion and on
the other hand generate a burst of significant amount of superoxide adding to the injury. The tissue MPO levels were significantly attenuated by post treatment with curcumin at both the time point of study i.e. 7 and 24hrs of reflow.

Thus significant reduction in MPO activity indicates the potent anti-inflammatory action of curcumin in focal cerebral ischemia.

Recent study indicated the importance of immune/inflammatory proteins (cytokines) in neuronal death due to cerebral ischemia. Despite numerous reports of increased expression of various cytokines after experimentally induced cerebral ischemia, relatively few (e.g., interleukin-1 [IL-1]; tumor necrosis factor; nerve growth factor; transforming growth factor-B) have been directly implicated in ischemic brain damage (Rothwell et al., 1995; Feuerstein et al., 1998).

Following stroke, the cytokine tumor necrosis factor-α (TNF-α) is up regulated in injured brain regions of rodents (Liu et al., 1994; Botschkina et al., 1997). Several experimental approaches to inhibit the effects of TNF-α in acute stroke, such as intraventricular infusion of antibodies against TNF-α (Dawson et al., 1996; Barone et al., 1997; Nawashiro et al., 1997) and systemic administration of an antagonist to TNF-α converting enzyme (Wang et al., 2004), have reduced the degree of ischemic injury in animal models. These findings indicate that TNF-α contributes to the progression of brain damage after stroke. However, the actions of TNF-α in stroke are complex. Tumor necrosis factor-alpha (TNF-alpha) is upregulated in stroke-damaged brain and is down regulated by curcumin post treatment in the present study.

Another cytokines IL-6, is also synthesized within neurons and glia, and cerebral expression is increased in a wide variety of CNS disorders, including ischemia (Woodroofe et al., 1991; Yan et al., 1992; Taupin et al., 1993; Wang et al., 1995). Furthermore, IL-6 prevents learning disability and delayed neuronal loss in gerbils after forebrain ischemia (Matsuda et al., 1996). Recently, (Wang et al., 1995) reported that cerebral ischemia, produced by permanent occlusion of the middle cerebral artery (MCAO) causes increased cortical expression of IL-6 mRNA. MCAO produced a rapid, dramatic and sustained increase in IL-6 expression in the ischemic hemisphere consistent with a previous observation of increased IL-6 expression in both hemispheres after mechanical brain injury (Taupin et al., 1993). IL-6 expression after MCAO in the rat was observed in the brain cryosections. The expression of IL-6 was identified in the neuron in the contralateral hemisphere. The reason
for this synthesis is unknown, but it may be due to physical deformation of tissue resulting from swelling of the ischemic hemisphere. Curcumin treatment further decreased the expression of IL-6 in the present study at both the time periods of reflow 7 and 24 hrs. However, curcumin might be involved suppressing the IL-6 expression of the neuron and this in turn offer neuroprotection. However, there are reports that IL-6 protects hippocampal neurons *in vivo* without affecting hippocampal blood flow (Matsuda et al., 1996) and protects against excitotoxicity *in vitro* (Yamada and Hatanaka, 1994) suggesting a direct action of IL-6 on neuronal viability. In conclusion, the present study demonstrated that curcumin was consistently effective when evaluated for neuronal damage and behavior deficit. The brain edema, tissue NOx (nitrate + nitrite) level, and myeloperoxidase activity were significantly attenuated by curcumin given 6hrs post ischemia. In the view of present observations, it can be concluded that delayed treatment of curcumin is neuroprotective and has a wide therapeutic window in the focal cerebral ischemia in rat. The next part of the study was planned to explore the possible effects of curcumin on neural stem cells (NSCs). Proliferation after ischemia is advantageous only if the new cell ultimately repopulate the brain region which is depleted of cells as a consequence of stroke.