Chapter 1

Curcumin: dose dependent structure and functional effect

Science is always wrong. It never solves a problem without creating ten more.

-George Bernard Shaw-
1.1 Introduction

Turmeric (Curcuma longa L.) is a medicinal plant extensively used in Ayurveda, Unani and Siddha medicine as home remedy for various diseases. Turmeric was described as *C. longa* by Linnaeus and its taxonomic position is as follows:

- **Class**: Liliopsida
- **Subclass**: Commelinids
- **Order**: Zingiberales
- **Family**: Zingiberaceae
- **Genus**: Curcuma
- **Species**: Curcuma longa

The wild turmeric is called *C. aromatica* and the domestic species is called *C. longa*. Curcumin (1, 7-bis (4-hydroxy- 3-methoxyphenyl)-1, 6-hepadiene-3, 5-dione) is a major active component from *Curcuma longa Linn* (Zingiberaceae), commonly known as turmeric. Curcumin has a wide spectrum of biological actions like anti-oxidative and anti-inflammatory effects (Ammon and Wahl, 1991; Brouet and Ohshima, 1995; Nirmala and Puvanakrishnan, 1996; Sreejayan and Rao, 1996; Kohli et al., 2005). Extensive research within the past decade has confirmed that curcumin is a therapeutic agent for various diseases, in particular cancer, wound healing, diabetes, neurodegenerative, cardiovascular and pulmonary diseases and arthritis (Mazumder et al., 1996; Allen et al., 1998; Chan et al., 1998; Vlietinck et al., 1998; Ono et al., 2004; Goel et al., 2008).

Several studies have indicated that curcumin has preventive effects against cerebral ischemia in rats middle cerebral artery model when given before reperfusion (Ghoneim et al., 2002; Thiyagarajan and Sharma, 2004; Jiang et al., 2007); in the global ischemic model in gerbils (Wang et al., 2005a) and in rat 1hr ischemia, followed by reperfusion for 1 hr (Ghoneim et al., 2002). The effect of delayed treatment by curcumin after fifteen minutes of ischemia in rat global model has been reported recently (Al-Omar et al., 2006). However, no effort had been made to explore the therapeutic time window of curcumin in cerebral stroke model. The purpose was firstly; to evaluate the effect of curcumin upon the functional deficits induced by MCA occlusion, to determine whether these deficits correlate with histological damage. The secondly aim was to assess the neuroprotective efficacy of
Chapter 1

curcumin at different doses and the therapeutic time window for using curcumin in a rat middle cerebral artery model of cerebral stroke.

1.2 Results

1.2.1 Neurological examination

The rats in the ischemia group showed significant neurological deficits (Median 2.75; 95% CI 2.64-2.87) including hemiplegia and postural abnormalities, such as forelimb flexion, extreme body twisting, and circling behavior. In contrast, hemiplegia and postural abnormalities were milder in the curcumin treated rats. The scores were (Median 2.6; 95% CI 2.561-2.659), (Median 2.188; 95% CI 1.96-2.4) and (Median 1.602; 95% CI 1.506-1.698) for the groups treated with 0.5, 1 and 2 mg/kg curcumin i.v. at 6 hrs, respectively (Fig.1). Curcumin (dose of 2 mg/kg i.v.) given at 2hrs, 4hrs and 8hrs post ischemia, showed (Median 1.270; 95% CI 1.157-1.383), (Median 1.46; 95% CI 1.341-1.579), (Median 1.926; 95% CI 1.773-2.079) improvement in the neurological deficits. Curcumin significantly reversed the decrease of neurological scores caused by the MCAo. The curcumin (2 mg/kg i.v. 6hrs) treated group showed a statistically significant reduction of ischemia-induced motor impairment compared with the ischemia group the 0.5 and 1 mg/kg curcumin treated group.

Fig.1: Effect of different doses of curcumin on neurological deficits score after 24hrs. Sham operated group, ischemia with no treatment, ischemia with curcumin (0.5mg/Kg i.v. at 6hrs), ischemia with curcumin (1 mg/Kg i.v. at 6 hrs), ischemia with curcumin (2 mg/Kg i.v. at 6 hrs), ischemia with curcumin (2 mg/Kg i.v. at 2 hrs), ischemia with curcumin (2 mg/Kg i.v. at 4 hrs), and ischemia with curcumin (2 mg/Kg i.v. at 8 hrs).
Number of animals used were five in each group \( *P<0.001 \) as compared with the ischemic group.

**1.2.2 Dose response of Curcumin in MCAo model**

The extent of infarction caused by MCAo was quantified in ischemia and curcumin treated rats. Coronal sections (2 mm) were obtained by slicing the brain from the rostral extremity of the frontal cortex and staining with TTC. TTC stained viable brain tissue as red and the infarcted regions did not take up TTC stain so remained white in colour. After 24hrs the infarcted area extended from the core regions (caudate putamen, parietal cortex and temporal cortex) to the peripheral regions (penumbra).

Attenuation of the infarct volume was observed with three ascending doses of curcumin 0.5, 1 and 2 mg/Kg by intra venous route. The attenuation of infarct was 276.480 ± 45.53 mm\(^3\) for 0.5mg and 248.490 ± 34.86 mm\(^3\) for 1mg/kg dose of curcumin given after 6hrs of reflow. These values were not statically significant though a trend of reduction was observed against the sham operated (354.98 ± 25.89 mm\(^3\)). Curcumin (2mg/Kg) caused a significant attenuation in the infarct volume as compared to I/R group (178.240 ± 36.4 mm\(^3\)). Curcumin offer a dose related effect on the infarct volume (Fig.2 & 3). In other set of experimentation curcumin (2 mg/Kg) was given at different time of reflow to explore its therapeutic time window. When fixed dose of curcumin 2mg/kg was give at different time of reflow different degree of reduction in volume of infarct was observed. After 2hrs (98.450 ± 42.0 mm\(^3\)), 4hrs (143.790 ± 34.5 mm\(^3\)), 6hrs (178.240 ± 36.4 mm\(^3\)) and 8 hrs (245.780±13.90). Curcumin was given after 8hrs of reflow failed to offer protection. When 2mg/kg dose of curcumin was given after 6hrs of reflow a statistically significant attenuation of infarct volume was observed. The entire work on curcumin was based on these dose and time titration experiments. In the present study 2mg/kg dose of curcumin was used and administration of curcumin was 6 after reflow.
Fig. 2: Representative TTC stained brain sections of sham operated group (1), ischemia with no treatment (2), ischemia with curcumin (0.5mg/Kg i.v. at 6hrs) (3), ischemia with curcumin (1 mg/Kg i.v. at 6 hrs) (4), ischemia with curcumin (2 mg/Kg i.v. at 6 hrs) (5), ischemia with curcumin (2 mg/Kg i.v. at 2 hrs) (6), ischemia with curcumin (2 mg/Kg i.v. at 4 hrs) (7), and ischemia with curcumin (2 mg/Kg i.v. at 8 hrs) (8) group of rats after 24 hrs of reflow. Unstained areas represent the infarcted brain tissue.

Fig. 3: Effect of different doses of curcumin on infarction volume after 24hrs. Sham operated group, ischemia with no treatment, ischemia with curcumin (0.5mg/Kg i.v. at 6hrs), ischemia with curcumin (1 mg/Kg i.v. at 6 hrs), ischemia with curcumin (2 mg/Kg i.v. at 6 hrs), ischemia with curcumin (2 mg/Kg i.v. at 2 hrs), ischemia with curcumin (2 mg/Kg i.v. at 4 hrs), and ischemia with curcumin (2 mg/Kg i.v. at 8 hrs). Number of animals used were five in each group *P<0.001 as compared with the ischemic group.
1.2.3 Effect of Curcumin on edema volume

The edema formation caused by MCAo was quantified in ischemia and curcumin treated rats. Attenuation of the edema volume was observed with different doses of curcumin 0.5, 1 and 2 mg/Kg given by intra venous route. In the 0.5 mg and 1mg/kg curcumin post treated (at 6hrs) group, the infarct volume was not significantly smaller (96.8 ± 8.2; 88.19 ± 6.9 mm$^3$) compared with that of the control group (104.8 ±11.7 mm$^3$). It was found that the group that received post treatment with curcumin at 2mg/Kg caused a significant protective effect against MCAo and curcumin had significantly reduced the infarct volume than the I/R group (68.240 ± 9.8 mm$^3$), curcumin showed a dose related effect on the infarct volume (Fig.4). Curcumin had a good effect at reducing the volume of infarct at the doses of 2 mg/Kg at 2hrs (59.6 ± 11.6 mm$^3$) and 4hrs (70.5 ± 9.45 mm$^3$), but the difference was not statistically significant when given at 8 hrs (81.230 ± 11.90).

Fig.4: Effect of different doses of curcumin on edema volume after 24hrs. Sham operated group, ischemia with no treatment, ischemia with curcumin (0.5mg/Kg i.v. at 6hrs), ischemia with curcumin (1 mg/Kg i.v. at 6 hrs), ischemia with curcumin (2 mg/Kg i.v. at 6 hrs), ischemia with curcumin (2 mg/Kg i.v. at 2 hrs), ischemia with curcumin (2 mg/Kg i.v. at 4 hrs), and ischemia with curcumin (2 mg/Kg i.v. at 8 hrs). Data are expressed as mean ± S.E.M of five animals per group. (*P<0.05) was considered significant when comparisons were made with the ischemia/reflow group by one way ANOVA followed by Newman Keuls post hoc test.
1.2.4 Effect of Curcumin post treatment on the Rota rod test

The mean value of the time spent on the spindle or latency to fall in pre MCAo rats was observed (119 ± 1.01). This served as baseline value. The reduction in average time spent on the rotarod by the MCAo rats (*P value<0.05) when compared with the sham operated animals after 24 h was highly significant. Average time spent on rotarod was significantly improved in the Curcumin treated group (97.05 ± 3.29) in comparison to ischemic animals (65.05 ± 4.59) (Fig.5).

![Bar chart showing the fall latency on an accelerating Rotarod from sham operated, ischemia/reflow (24h of reflow) and curcumin post treated -I/R (24h of reflow) rats on day 1 day 2 and day 7 after induction of ischemia/reflow. Time (in sec) expressed as Mean ± S.E.M. (n=5). Effects of curcumin treated ischemic vs. ischemic rats, ***p<0.001.](image)

Fig.5: Bar chart showing the fall latency on an accelerating Rotarod from sham operated, ischemia/reflow (24h of reflow) and curcumin post treated -I/R (24h of reflow) rats on day 1 day 2 and day7 after induction of ischemia/reflow. Time (in sec) expressed as Mean ± S.E.M. (n=5). Effects of curcumin treated ischemic vs. ischemic rats, **p<0.001.

1.2.5 Effect of Curcumin post treatment on the water maze test

I/R and curcumin post treated rats showed reduction in the latency time to reach platform. Significant decrease in latency time occurred on the day 1, day 2 and day 7 was observed in I/R group of rats. There was a significant improvement in the water-maze latency (*P<0.001) to reach the platform in I/R-curcumin rats on all the days (1, 2 and day 7) the rats were exposed (Fig.6).

The neurological deficit score (Fig.7) and infarct volume (Fig.8) in all the groups of rats further confirmed this behavior and curcumin significantly decreases the infarct volume as compared to ischemic group of rats on day 7 (*P<0.001).
Fig. 6: Effect of curcumin post treatment on ischemia-induced deficit in learning and memory behavior by water maze test in the sham operated, ischemic, curcumin post treated group. Curcumin post treatment resulted in significant improvement in latency time to reach the platform when compared to ischemic group.

Fig. 7: Effect of Curcumin on neurological deficits score after day 7; sham operated group, ischemia, curcumin treated group. Number of animals used were five in each group *P<0.001 as compared with the ischemic group.

Fig. 8: Effect of curcumin on volume of infarct after day 7 in the sham operated group, ischemia, and curcumin treated group. Volume of infarct was significantly reduced by curcumin treatment on day 7. Values are mean ± S.E.M. ***P<0.001 as compared with the ischemic group.
1.3 Discussion

Ischemic stroke has a complex pathophysiology and the lack of total correlation between the measurements of the different outcomes indicates that measurements of behavioral, neurological, and histological endpoints are necessary for effectively and comprehensively examining the putative protective effect of a drug. After an ischemic attack, brain infarction tends to spread out circumferentially from the core of the infarct and the penumbral regions undergo delayed energy-dependent apoptosis (Nicotera and Lipton, 1999) and inflammation (Phillips et al., 2000). A significant decrease in infarct volume was observed with curcumin treatment. For this the histopathological brain infarct measurement was done using a TTC stain at 7 and 24hrs after ischemia. As TTC is reduced to a red-formazan product in the presence of active mitochondrial oxidative enzymes, thus denotes the viable tissue. A non stained TTC white coloured area, the necrotic tissue are consistent as observed in other histopathological stains, such as H&E (Park et al., 1988). The measurements of TTC injury were similar to those reported previously by other investigators. The present data demonstrate a progression of the early lesion (7hrs) develops into a mature irreversible infarct with time. Curcumin treatment reduced the infarct volume (TTC) that reflected the maturation for injury over period of time from 5 hrs to 24 hrs post ischemia.

Curcumin has a dose-dependent neuroprotective effect on ischemia in the rats. Recent studies have shown the preventive effect of curcumin against cerebral ischemia when given 30 min post ischemia (Ghoneim et al., 2002; Thiyagarajan and Sharma, 2004; Jiang et al., 2007) and in gerbil global ischemic model (Wang et al., 2005a). There are no reports on the post treatment of curcumin. To this end the therapeutic time window, with curcumin (2 mg/Kg) was given at different time of reflow, it offered wide therapeutic window of 6 hrs.

Curcumin can cross the blood-brain barrier as its levels in the brain reaches a peak within 1 hr after intra-peritonial injection and declined to basal levels within 2 days (Wang et al., 2005a). Estimations were made on two time points one after 1 hr and the other after 24hrs of MCA.

Over the years, the pharmacokinetics and pharmacodynamics of curcumin have not been widely appreciated owing to its poor oral absorption. Curcumin is readily absorbed from the peritoneal cavity (Pan et al., 1999). Further, various authors (Ravindranath and Chandrasekhar, 1980; Pan et al., 1999) have reported that the metabolism of curcumin in
rats, mice, and gerbils differ significantly from one another. This species difference might be responsible for the anomalies in (pharmacological) physiological action reported by Ammon and Wahl (1991).

Cerebral ischemia, cause cytogenic deterioration and aggravate brain edema formation during reperfusion (Yan and Greene, 1998). Brain edema formation, is one of the most dangerous consequences of ischemic brain injury. Thus, one important factor of brain edema formation is the toxicity due to free radicals generated from lipid peroxidation. A significant suppression was observed in edema volume (as judged by hemispheric enlargement).

Further substantiating the above finding an improvement in the neurological score was observed with curcumin treatment. Occlusion of the middle cerebral artery results in significant impairment of the motor performance of the rats, which was evident from the increased neurological deficits and in the reduced dwell-time on the rotarod. This could be due to neuronal damage in the striatum that regulates motor coordination. Fore-limb flexion, spontaneous motility was affected by ischemia as rat walked on their digits instead of on their foot pads and produced a crawling walk. This can be well correlated to damage of the cortical motor area of rats. Curcumin attenuated all these ischemia induced ill effects, i.e. behavioral deficits by preventing neuronal loss in this region, the results of TTC staining supports this conclusion. Despite the lower bioavailability, therapeutic efficacy of curcumin against various human diseases, including cancer, cardiovascular diseases, diabetes, arthritis, neurodegenerative diseases and Crohn's disease, has been very well documented (Goel et al., 2008).