Neuroprotective effect of curcumin in middle cerebral artery occlusion (MCAO) induced focal cerebral ischemia in rats

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Introduction

• Oxidative stress was suggested to be involved in the pathogenesis of ischemia/reperfusion injury.
Oxidants:

- Superoxide, Hydrogen peroxide,
- hydroxyl radical, nitric oxide, peroxynitrite
- can damage to lipids, protein, DNA

Antioxidants:

Antioxidant:

Enzymatic (endogenous)
- enzyme e.g. superoxide dismutase (SOD), glutathione peroxidase (GPx), catalase

Non-enzymatic
- vitamin E,C
- beta-carotene
- flavonoid
After ischemic-reperfusion injury, there was an imbalance between oxidants and antioxidants, resulting in oxidative stress and cell damage and death.
Xanthine dehydrogenase (XD)

Irreversible

Xanthine oxidase (XO)

ATP breakdown

Inosine ↔ Adenosine ↔ AMP ↔ ADP ↔ ATP breakdown

Hypoxanthine and xanthine

Substrate

Inhibition of the enzyme

Induction of the enzyme

Scavenging activity

Nitration of aromatic residues of cytosolic proteins

Neuronal injury

Lipid peroxidation

Fenton reaction

O$_2$ ↔ NO$^*$ → ONOO$^*$ → SOD → H$_2$O$_2$ → OH$^*$ → Lipid peroxidation

H$_2$O + O$_2$
At present, there is no effective neuroprotective therapy for treatment of ischemia-reperfusion.
Many antioxidants have been reported to be neuroprotective in experimental model of cerebral ischemia.
Antioxidant

Reduction of ROS mediated reactions

would rescue the neurons from reperfusion induced neuronal loss in animal model of cerebral ischemia

Curcumin is the most active ingredient among the three curcuminoids that found in curcuma longa Linn. (or turmeric or Ka-Min-Chan)
Curcumin

- antioxidant and neuroprotective properties
- potent scavenger of oxygen radicals
- inhibitor of lipid peroxidation and xanthine oxidase-induced $\text{O}_2^-$ production
Kunchandy and Rao (1990), Shalini and Srinivas (1987), Subramanian et al. (1994)

Curcuma longa was demonstrated to have antioxidant potential

Dikshit et al. (1995), Jones and Shoskes (2000)

Curcumin have cytoprotective activity in cardiac and renal ischemia
Aim of study

To evaluate the neuroprotective potential of curcumin in middle cerebral artery occlusion induced focal cerebral ischemia in rat model.
Experimental study

Reperfusion injury

generated ROS

assessed by measuring these parameters

• Lipid peroxidation
• Superoxide dismutase (SOD) activity
• Glutathione peroxidase (GPx) activity
• Peroxynitrite
• Tyrosine nitration
• Infarct and edema volume
• Neurological deficit

induced by MCAO
Materials and methods
Preparation of the animals

- 92 Male Sprague-Dawley rats
- weight 240-260 g.

- The rats were housed in room at 22±1°C
- 12 h. dark and light cycle
- Standard rat chow pellets and water was allowed ad libitum
92 rats after induced MCAO temporarily, were randomly divided into vehicle and curcumin treated groups.

- Vehicle (NaCMC + tween 80)
- Curcumin 30 mg/kg
- Curcumin 100 mg/kg
- Curcumin 300 mg/kg
Vehicle (CMC)  
Curcumin 30 mg/kg  
Curcumin 100 mg/kg  
Curcumin 300 mg/kg

Brain infarct and edema volume, neurological deficit

Lipid peroxidation, SOD, GPx, peroxynitrite, tyrosine nitration

were measured
Materials and methods

❤ Preparations of animals

Temorally focal cerebral ischemia

induced by

Middle cerebral artery occlusion

❤ Measure all parameters of ischemic reperfusion injury
Cerebral ischemia/reperfusion injury

Preanaesthetic medication:
- atropine sulphate

Anaesthetic medication:
- chloral hydrate

Midline incision of the neck to expose trachea and left common carotid artery
Cerebral ischemia/reperfusion injury

Monitored physiological parameters
- Respiratory rate
- Rectal temperature
- Blood pressure
- Heart rate

Investigate effect of curcumin 300 mg/kg

In sham operated group, the filament was introduce into the external carotid artery only but not advanced further.
MCAO

reperfusion

0 2 h. 4 h. 12 h. 24 h.

pulled out the filament

For measure all parameters of oxidative stress

1 ml. of 5% dextrose solution

Sacrifice rats and brains were removed

Summarized diagram of the experiment
Parameters measured

- Infarct and edema volume
- Neurological deficit
- Lipid peroxidation
- Superoxide dismutase (SOD) activity
- Glutathione peroxidase (GPx) activity
- Peroxynitrite
- Tyrosine nitration by immunofluorescense

The rats, not shown neurological deficits immediately after reperfusion (neurological score < 3) were excluded from the study.
Infarct and edema volume

2 mm. thick

10 min. 37°C

2% triphenyl tetrazolium chloride (TTC) solution in saline

overnight

10% formal saline

Stained

Fixed

Measured the area of infarction by using “Leica Qwin” image analysis software
Infarct and edema volume

Infarct volume (mm³) (in occlude side)

= total infarct area × thickness of brain section

Edema volume (mm³)

= infarct volume of ipsilateral side × volume of contralateral side

Volume of Ipsilateral side
Neurological deficits

scored on a 5-point scale

0 = No neurological deficit
1 = failure to extend right paw fully
2 = circling to right
3 = falling to right
4 = did not walk spontaneously and had depressed levels of consciousness
Lipid peroxidation was measured in the formed malondialdehyde (MDA) by spectrophotometer.

Quantification based on generate the standard curve by using authentic MDA.
Estimation of lipid peroxidation

Contralateral brain homogenate → ipsilateral brain homogenate

5% aqueous trichloroacetic acid

0.1 ml. of methanolic butylated hydroxy toluene solution → Boiling water 30 min. spinning

supernatant

1 ml. of thiobarbituric acid reagent → Boiling water 30 min.

spectrophotometer

532 nM.
Estimation of superoxide dismutase (SOD) activity

SOD activity was estimated in brain homogenate by calculated the rate inhibition of nucleotide oxidation.
Estimation of superoxide dismutase (SOD) activity

- 800 μl of triethanolamine-diethanolamine buffer (pH 7.4)
- 40 μl of 7.5 mM NADH
- 25 μl of 100-50 mM EDTA-MnCl₂ (pH 7)
- 100 μl of brain homogenate
- 800 μl of triethanolamine-diethanolamine buffer (pH 7.4)
- 40 μl of 7.5 mM NADH
- 25 μl of 100-50 mM EDTA-MnCl₂ (pH 7)
- 50 mM phosphate buffer

Spectrophotometer at 340 nm

100 μl of 10 mM mercaptoethanol

Calculated rate of nucleotide oxidation (in control) and inhibition of nucleotide oxidation in homogenate.
Estimation of superoxide dismutase (SOD) activity

One unit of the SOD activity

amount of enzyme required to inhibit the rate of NADH oxidation of the control 50 %
Estimation of glutathione peroxidase (GPx) activity

GPx activity was estimated in brain homogenates and measured the change in NADH absorbance by using spectrophotometer.
Estimation of glutathione peroxidase (GPx) activity

1 mM of glutathione, 0.2 mM of NADH, 1.4 U glutathione reductase, brain homogenate equivalent to 0.1 mg of protein in 50 mM phosphate buffer.

0.25 mM hydrogen peroxide

Spectrophotometer

340 nm

measure the change in NADH absorbance
Estimation of glutathione peroxidase (GPx) activity

One unit of GPx activity

amount of sample required to oxidize 1 µM of NADH per min. based on the molar absorbance of $6.22 \times 10^6$ for NADH.

All values which get from this experiment was subtracted with the basal reaction rate which used PBS instead of brain homogenate.
Estimation of peroxynitrite

\[ \text{O}_2 \xrightarrow{\text{e}^-} \text{O}_2^- \]

Superoxide anion

\[ \text{NO}^- \]

Nitric Oxide

\[ \text{OONO}^- \]

Peroxynitrite
Peroxynitrite was estimated by using a fluorescent dye dihydrorhodamine 123. Peroxynitrite oxidized to rhodamine123 in a peroxynitrite dependent manner. Level of rhodamine123 was calculated from the standard curve obtained from authentic rhodamine123 (in a concentration 0-10 nM which prepared in plasma obtained from the untreated rat.)
Estimation of peroxynitrite

MCAO  dye injection  reperfusion

Collect the blood by cardiac puncture
Estimation of peroxynitrite

Excitation wavelength 500 nM.
Emission wavelength 536 nM.

Spectrofluorometer

All values derived from this experiment were subtracted with the basal reaction rate.
Peroxynitrite mediated tyrosine nitration was estimated by using anti-nitrotyrosine antibody by immunofluorescence technique
Estimation of tyrosine nitration by immunofluorescence

1. Reperfusion
2. Perfuse the brain with ice-cold PBS for 4 h.
3. Reanaesthetize with chloral hydrate transcardially.
4. Fix the brain in 10% buffered formal saline.
5. Embed in wax.
6. Wash with distilled water.
7. Incubate in proteinase K for 20 min.
8. Wash with PBS.
9. Incubate in blocking buffer for 120 min.
10. Incubate in avidin for 30 min.
11. Incubate in biotin for overnight.
12. Incubate in 1° Ab at 4°C overnight.
13. Incubate in anti-nitrotyrosine in blocking buffer.
Estimation of tyrosine nitration by immunofluorescence

1° Ab at 4°C overnight

anti-nitrotyrosine in blocking buffer

1° Ab washed

anti-nitrotyrosine in blocking buffer

incubated

2 h.

PBS 3 times washed

PBS

biotin

anti-mouse IgG

blocking buffer

Detection specific labelling

avidin conjugated FITC system

image analysis software “Leica Qwin”.

CCD camera 0.23 S.

Fluorescence microscope
Statistical evaluation

- Means ± S.E.M
- Student t-test
- ANOVA followed by a Tukey test
- Neurological deficit $\rightarrow$ median±95% CI
Results
Physiological parameters

Rectal temperature (°C)

- After MCAO 6 h.
- After MCAO 9 h.
- After MCAO 12 h.
Physiological parameters

Curcumin-treatment
300 mg/kg

Arterial blood pressure
(mm.Hg)
Heart rate (beats/min.)

Before
After 30 min.
After 60 min.
Effect of curcumin on cerebral infarction

* Represents $p < 0.05$ of respective group as compared to vehicle treated group
Effect of curcumin on cerebral infarction

Vehicle

Curcumin 100 mg/kg

Curcumin 300 mg/kg
Effect of curcumin on cerebral edema

* Represents p < 0.05 of respective group as compared to vehicle treated group
Effect of curcumin on neurological deficit

Neurological deficits

0 = No neurological deficit
1 = failure to extend right paw fully
2 = circling to right
3 = falling to right
4 = did not walk spontaneously and had depressed levels of consciousness
Effect of curcumin on lipid peroxidation

MDA level (nM)

Vehicle treated group

Curcumin 300 mg/kg

ipsilateral side
ccontralateral side

* p<0.05
Effect of curcumin on superoxide dismutase activity

- Ipsilateral side
- Contralateral side

SOD (U)

- Vehicle treated group
- Curcumin 300 mg/kg
Effect of curcumin on glutathione peroxidase activity

- **Vehicle treated group**
- **Curcumin 300 mg/kg**

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<th>Ipsilateral Side</th>
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<td>Curcumin</td>
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* * p<0.05
Effect of curcumin on peroxynitrite

- Sham operated n=3
- Vehicle treated n=4
- Curcumin mg/kg n=3 (300 mg/kg)
Effect of curcumin on peroxynitrite mediated tyrosine nitration

vehicle

Curcumin 300 mg/kg
Discussion
Curcumin-treatment (i.p.) showed dose dependent reduction in infarct volume in MCAO induced focal cerebral ischemia model of stroke in rats.
Cytoprotective activity of curcumin is also reported in model of cytotoxicity

- reduces renal damage and inflammation in renal ischemia reperfusion injury (Jones and Shoskes; 2000)
- protective effect in isoproterenol induced myocardial ischemia in rats (Nirmala and Puvanakrishnan; 1996)

Suggested mechanism of cytoprotective effect:

- inhibit activity over oxidative stress induced activation of AP1 and NF-kB, upregulation of c-fos, c-jun and phosphorylation of c-jun proteins. (Luo et al.; 1999)
- inhibit JNK, PKC, iNOS and COX-2 enzymes (Huang et al., 1991; Rao et al., 1999)
Cerebral edema occurred as a result of

- Ionic imbalance
- Immune-activation (inflammation)
  - infiltration of macrophage
  - secondary reaction

Reduction of cerebral edema

Curcumin

Rao et al.; 1999
After ischemic-reperfusion injury, imbalance resulted in oxidative stress, which led to cell damage and death. Antioxidants and oxidative protection help maintain balance.
Oxidants

Antioxidants

lipid peroxidation

Vehicle treated group
Curcumin 300 mg/kg

MDA level (nM)

Vehicle treated group
Curcumin 300 mg/kg

ipsilateral side
contralateral side

* *
Oxidants

Antioxidants

lipid peroxidation

peroxynitrite formation

in form the level of rhodamine 123 and immunofluorescence method
Oxidants

lipid peroxidation
peroxynitrite formation

Antioxidants

endogenous antioxidant defense enzymes
SOD
GPx
Curcumin

ROS scavenging activity and enhanced transcription of SOD and GPx.
(Piper et al., 1998, Shahed et al., 2001).

increased the activity of SOD and GPx in liver homogenate
(Reedy and Lokesh, 1994).

Curcumin up-regulates the SOD gene expression in rat kidney after ischemia-reperfusion injury
(Shahed et al., 2001).

increased glutathione peroxidase
Curcumin offered significant neuroprotection in middle cerebral artery occlusion induced focal cerebral ischemia.
Oxidants

lipid peroxidation

peroxynitrite formation

Antioxidants

endogenous antioxidant defense enzymes

SOD

GPx
Thank you for your attention..