Effects of curcumin on memory, hippocampal acetylcholine level and neuroapoptosis in repeated cerebral ischemia rat model

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Abstract: The aim of this study was to determine the protective effects of curcumin on memory, hippocampal acetylcholine level and apoptosis in a rat model of repeated cerebral ischemia. Male Wistar rats were divided into sham rats that received saline and the other 3 groups underwent 4-vessel occlusion brain ischemia (4VOI), received oral administration of either saline or curcumin at doses rate of 25mg/kg/day and 50mg/kg/day for 7 days. Memory function was evaluated by eight-arm radial maze task and Morris water maze (MWM) test, Acetylcholine release (ACh) in the dorsal hippocampus was evaluated by microdialysis-HPLC and neuron apoptosis was investigated by terminal deoxynucleotidyltransferase mediated fluorescein-deoxyuridine triphosphate nick-end labeling. 4VOI test reviled impaired memory, reduced dorsal hippocampus Ach level and induced apoptosis in the Repeated Cerebral Ischemia rat model. Curcumin significantly improved the memory deficit (p<0.001), increased Ach level (p<0.001) and prevented hippocampal neuron apoptosis (p<0.001). Curcumin may be suggested as a promising therapy for ischemic cerebrovascular dementia and its beneficial effect is due to its memory preserving, ACh-increasing and neuroprotective effects in the rat.

Keywords: Curcumin, neuroprotective, memory, acetylcholine, apoptosis.

INTRODUCTION

The human brain is an organ with a very high rate of oxygen consumption due to its high metabolism (Watts et al., 2018). The brain consumes oxygen nearly 10 times more than the average body oxygen consumption corrected for the organ weight (Furtado et al., 2018). This high level of oxygen dependence causes severe brain damage in case of ischemia caused by any pathology (Jurcau and Simion, 2021). There is a complex mechanism to regulate cerebral homeostasis and blood flow to supply enough oxygen for the metabolic needs of the brain, which is called cerebrovascular autoregulation (Nogueira et al., 2022). Reduction of cerebral blood flow is one of the most common causes of disruption of cerebrovascular autoregulation, which can lead to serious damage to brain function (Megjhani et al., 2021). Cerebral ischemia caused by stroke is the most common clinical presentation of this disorder (Kolouri et al., 2016).

Curcuma longa L. (Turmeric) is traditionally used as a medicinal spice in different ancient civilizations like China, Egypt, Persia and India (Prasad and Aggarwal, 2011, Khiljhee et al., 2015, Mosavat et al., 2018). It was used for different ailments including age-related memory loss (Sumathi et al., 2017, Ayati et al., 2019, Debjit Bhowmik et al., 2009). Curcumin is known as the main constituent responsible for turmeric’s medicinal effects including anti-inflammatory (Ferguson et al., 2021), anti-cancer (Wong et al., 2021), antibiotic (Teow and Ali, 2015), antioxidant (Miao et al., 2015), anti-atherosclerotic (Singh et al., 2021), anti-hyperlipidemic (Singh et al., 2021) and anti-diabetic effects (Quispe et al., 2022).

Recent evidence has demonstrated the neuroprotective effects of curcumin in various experimental and clinical models of neurologic disorders (Wynn et al., 2018, Small et al., 2018, Sarraf et al., 2019). Curcumin’s molecular structure provides it with the potential to cross the blood-brain barrier. Curcumin showed to have promising effects against Alzheimer's disease, memory impairment and cerebral ischemia (Ovbiagele, 2008, Bhat et al., 2019). Experimental studies indicate the protective effects of curcumin against ischemic reperfusion injury in a rat model of stroke (Wang and Xu, 2020, Wu et al., 2020, Zhou et al., 2020). It is also shown that curcumin can affect molecular pathways involved in memory performance including dopamine, serotonin, as well as acetylcholine-esterase activity Amiri et al., 2021). In general, these reports show that curcumin has potential effects in reducing neuronal and brain injury in an experimental model of ischemia. However, its therapeutic effect in reducing hippocampal neuronal apoptosis and preserving the acetylcholine level as well as memory is not yet demonstrated in the experimental model of repeated cerebral ischemia. Therefore, the aim of this study was to determine the protective effects of various doses of curcumin on memory, hippocampal acetylcholine level and apoptosis in a rat model of repeated cerebral ischemia.
MATERIALS AND METHODS

Animals
Forty male Wistar rats aged 8-10 weeks old (weighing 200-250 g) were housed under a temperature of 23±2 °C and humidity of 60±5% with a 12 hours light/dark cycle. Food and water were available ad libitum. Animal housing and experimental procedures were compatible with the Guidelines for Ethical Conduct in the Care and Use of Animals. All procedures and experimental protocols were approved by the local Animal Ethics Committee (Approval number EC-2021-754).

Forty selected rats were divided into four random equal groups (n=10) after training sessions. The three active experimental groups were 4VOI rats that received either distilled water or curcumin (Santa Cruz Biotechnology, CAS 458-37-7, Purity≥95%) 25 or 50 mg/kg/day orally for 7 days. The rats in the sham group underwent vertebral artery cauterization while receiving distilled water. The occluders were inserted into the common carotid arteries in this group without occlusion. No mortality was observed.

Radial 8-arm maze (RAM) performance
The RAM performance was evaluated as previously explained in detail (Shukitt-Hale et al., 2004). Each behavioral testing session was conducted in a standard RAM consisting of a central platform 25 cm in diameter with eight arms of 70 cm (length)×10 cm (width)×15 cm (height) each, radiating equiangular from the central platform that served as a starting base. The white-painted maze was placed at a fixed position to reduce the variability of each test (Nogami-Hara et al., 2018).

The rats were pre-trained and trained before the assessment of the effects of 4VOI and interventions on memory as explained previously (Traystman, 2003). Briefly, the animals (in 5 rat groups) were adapted to the apparatus and the food pellets in 3 daily sessions of ten minutes for three days as pre-training. After that, the fourteen days training course was started. In this stage, the rats were permitted to freely move to the arms. The tests were finished on entering all arms eating the baits, or after 10 minutes were passed. The performance of the animals was assessed using the number of correct and error choices in the first eight chosen arms. Only rats that made less than two errors were enrolled with further steps of the experiment. This protocol was selected to evaluate working memory that is mainly affected by the 4VOI.

Morris water maze (MWM) test
MWM was also used to evaluate learning and memory performance seven days after 4VOI as described previously (Barnhart et al., 2015). Briefly, a 94 cm round white swimming pool filled with 25±1°C opaque water with 30 cm depth was used as a maze. The escape platform (25 cm²) was positioned in the center of one quadrant of the pool and submerged at 1 cm beneath the water's surface. Rats were allowed to find the platform (60 sec/trial). Escape latencies are defined as the time spent to locate the platform through the test.

Induction of 4VOI
4VOI experimental model was induced as explained previously (Deng and Xu, 2009). 4VOI was performed after the completion of the training. Intraperitoneal sodium pentobarbital with a dose of 50 mg/kg was used for anesthetizing. Stereotoxic apparatus was applied for the immobilization of rats. Electrocauterization of vertebral arteries was done with a bipolar coagulator. Common carotid arteries were exposed. Three days after electrocauterization, both common carotid arteries were occluded using aneurysm clips. The occlusion was performed by awakening rats repeatedly for 10 minutes at 60-minute intervals. The temperature was stabilized at 37°C, using a heating lamp and pad during anesthesia and 4VOI procedure. The rats with saved righting reflex after arterial occlusion were excluded from further experiments. Sham rats underwent cauterization of the vertebral arteries and fitting common carotid arteries occluders without occlusion.

Microdialysis procedures
The Acetylcholine (ACh) levels were evaluated in the extracellular space of the dorsal hippocampus by microdialysis in post-4VOI rats before and after the intervention as explained in detail previously (Bianchi et al., 2003). Microdialysis probes (CMA Microdialysis, North Chelmsford, Mass) with the size of 13*0.6*0.5 mm were used. An active U shape dialysis membrane made from cellulose fiber was applied. The probes were inserted through a guide into the dorsal hippocampus penetrating up to 2 mm into the brain. Probes were primed with Ringer and then implanted into the dorsal hippocampus. Perfusion fluid was pumped into the probe via a perfusion pump at a rate of 2 μl/min. The dialysate was collected into a collection device. It was then investigated by high-performance liquid chromatography (HPLC) (Shimadzu 10ADvp HPLC system) for ACh level.

Histochemical studies
Histochemical analysis was performed as previously explained in detail (Nogami-Hara et al., 2018). Fifteen days post-4VOI intervention, the rats were perfused transcardially with heparinized saline followed by 4% paraformaldehyde under sodium pentobarbital anesthesia (50 mg/kg Intraperitoneally). The brains were removed and fixed by paraformaldehyde. After lipid clearance and dehydration using an auto-degreasing machine, the brain samples were embedded in paraffin. Five μm thickness coronal sections were mounted on slides. The samples underwent deparaffinization and rehydration and then...
they were incubated in proteinase K buffer for 10 minutes. Terminal deoxynucleotidyl transferase UTP nick end labeling (TUNEL) was used for the detection of apoptotic cells of the dorsal hippocampus. The cross-sections were examined for TUNEL-positive cells by fluorescence microscopy. CA1 cells were stained using propidium iodide and cresyl violet staining. NIS-Elements software (Nikon, Tokyo, Japan) was used for quantitative analysis of TUNEL staining results.

**STATISTICAL ANALYSIS**

Quantitative outcomes including the level of ACh and RAM performance were evaluated by one-way ANOVA after confirmation of homogeneity of variance. Tukey post hoc test was used for multiple comparisons with significant differences among groups in the one-way ANOVA test. p<0.05 was considered statistically significant. Data are presented as the mean±standard deviation. IBM SPSS Statistics version 25 was used for the analysis of data. GraphPad Prism 8 was used for graph plotting.

**RESULTS**

**Morris water maze test**

The MWM test demonstrated that 4VOI significantly increased the escape latency whereas curcumin in both (25mg/kg/day and 50mg/kg/day) doses significantly improved the escape latency (F=4.992, P<0.0001). The data also demonstrated a significant decrease in the percentage of time spent in the target quadrants with 4VOI which was improved by curcumin (F=51.20; P<0.001). No significant difference was observed between different doses of curcumin in MWM test results. (fig. 2)

**RAM performance**

The RAM performance study demonstrated that 4VOI disturbed this function, showing as decreased corrected choices, whereas curcumin improved memory [F=14.82; P<0.001]. As shown in fig. 3A, corrected choices were decreased by 31% (P<0.001) in 4VOI, while correct choices increased in curcumin (25mg/kg/day and 50mg/kg/day) receiving rats by 23%, (P<0.028) and 34% (P<0.001), respectively. The data also demonstrated an increase in error choices with 4VOI which was improved by curcumin (F = 93.64; P<0.001). fig. 3B demonstrates increased error choices by 359% (P<0.001) in 4VOI rats which was decreased by 32% (P<0.001) and 47% (P<0.001) in curcumin (25mg/kg/day and 50mg/kg/day) groups, respectively.

**Acetylcholine level**

The basal ACh levels were 89.90±21.25 and 102.10±7.31 fmol/100 μL in the sham rats before and after the vehicle, respectively. No significant difference was observed between the vehicle and curcumin groups before the intervention (P=1.000, P=0.953). There was a significant difference between the ACh levels in different groups [F=67.36; P<0.001] after the intervention. 4VOI group showed a 51% reduction of ACh to 50.1000±7.03fmol/100μL and a significant increase (P<0.001) by curcumin (25mg/kg/day and 50mg/kg/day) to 73.50±7.17fmol/100μL (P<0.001) and 79.70±6.77fmol/100μL (P<0.001) (fig. 4). Hippocampus neuronal apoptosis

The cellular structure of CA1 of the hippocampus of the sham rats is demonstrated in fig. 4A. 4VOI induced neuronal damage in the CA1 (fig. 5B). Sections of rats that received different doses of curcumin (fig. 5C and 5D) showed more preserved cellular architecture. It can be seen in fig. 5B that there are more apoptotic cells (TUNEL-positive) in the CA1 of the 4VOI rats than sham group (fig. 5E). On the other hand, the neuronal features and expression of TUNEL-positive cells of curcumin-treated 4VOI rats are relatively similar to the sham rats (fig. 5F, 5H). Quantitative analysis of the number of TUNEL-positive cells in each group showed statistically significant less apoptosis in both curcumin groups compared to the sham group (F =103.40; P<0.001) (fig. 5 graph).

**DISCUSSION**

Although the memory-improving effect of curcumin has been suggested by previous studies, the associated role of acetylcholine release and apoptosis in the hippocampus is not well investigated. Therefore, in the current study, the effects of curcumin on 4VOI-induced memory impairment with a focus on the ACh level and apoptosis in the CA1 region of the dorsal hippocampus were investigated.

The study demonstrated that 4VOI-induced neuronal apoptosis in the CA1 region of the dorsal hippocampus decreased ACh in this area and provoked memory impairment. Results showed a greater increase in the number of error choices caused by 4VOI compared to the observed decline in the correct choices. This could be explained by more impairment of working memory compared to reference memory in 4VOI(Davis et al., 1987). The animal model of ischemia used in this investigation showed previously to induce apoptosis of the pyramidal neurons of the dorsal hippocampusaCA1 region (Haraguchi et al., 2010). This study confirmed the apoptosis induction in the dorsal hippocampus neurons by 4VOI and also showed the preventive effect of curcumin in 25mg/kg/day and 50mg/kg/day doses against the apoptosis. The experiment also showed a protective effect of curcumin on 4VOI-induced decreased ACh level in the dorsal hippocampus.

Memory-improving effects of curcumin were investigated and supported by multiple studies previously.
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**Fig. 1:** The experiment timeline

**Fig. 2:** Escape latency in Morris water maze test from day 1 to day 5 (A). Percentage of time spent in the target quadrants during the Morris water maze test (B). One-way ANOVA is used for statistical comparison among groups.

**Fig. 3:** Correct (A) and error choices (B) in the Radial 8-arm maze performance. The results show significant decrease in correct choices and significant increase in error choices in 4VOI rats (vehicle group) which was significantly improved by curcumin in 25 and 50 mg/kg/day doses. No significant difference was observed between two different doses of curcumin. One-way ANOVA is used for statistical comparison among groups.

**Fig. 4:** Effect of curcumin on the ACh level in the dorsal hippocampus before (Ach1) and after (Ach2) final dose of drug administration. The Ach1 chart shows the ACh level after 4VOI and before the intervention. Significant decrease in ACh level by 4VOI in 3 intervention groups compared to sham group. The Ach2 chart shows ACh level after intervention. Significant increase in Ach level by curcumin in 25 and 50 mg/kg/day doses is observed. No significant difference was observed between two different doses of curcumin. One-way ANOVA is used for statistical comparison among groups.
Sanei et al. systematically reviewed all the evidence and showed that curcumin improved memory in different experimental models such as stress, anticonvulsant, benzodiazepine and age-induced memory impairment (Sanei and Saberi-Demneh, 2019).

A wide range of dosages of curcumin (from 5 to 480 mg/kg/day) and duration of administration (from 1-84 days) were evaluated in these studies. However, no study has evaluated the association of the preventive effect of curcumin on memory impairment with hippocampal acetylcholine level and neuroapoptosis which is well illustrated in our study.

The result of our study was compatible with those of previous reports that showed supplementation with curcumin in an animal model of brain ischemia can reduce neuronal injury (Liu et al., 2013). It is shown that this effect can be explained by increased expression in PPARγ by curcumin (Liu et al., 2013). The expression of PPARγ was also demonstrated to decrease the inflammatory response mediated by COX-2, TNF-α and IL-1β, which has a critical role in cerebral ischemia (Liu et al., 2013). Since curcumin is the main active constituent of turmeric, these findings are also compatible with the reports on the neuroprotective effect of turmeric supplementation in rats (Siahaan et al., 2018, Yuliani et al., 2017, Yuliani et al., 2019).

Fig. 5: Effect of oral administration of curcumin on neuronal apoptosis in the CA1 region of the dorsal hippocampus. Cresyl violet staining. A: sham group, B: vehicle group, C: curcumin 25mg/kg, D: curcumin 50mg/kg. TUNEL staining. E: sham group, F: vehicle group, G: curcumin 25mg/kg, H: curcumin 50mg/kg. Apoptotic cells shows green florescence. The black arrow shows the disorganization of neuronal cells in dorsal hippocampus. The green dots indicated by white arrow show the apoptotic cells marked by immune-staining.

The graph shows of quantitative analysis of neuronal apoptosis in the CA1 region of dorsal hippocampus by TUNEL staining by NIS-Elements software in different study groups. There was a significant increase in cell apoptosis by 4VOI in vehicle group compared to sham group which was partially prevented by curcumin. One-way ANOVA is used for statistical comparison among groups.
To the best of our knowledge, this is the first report which demonstrates curcumin is able to increase the ACh level in the dorsal hippocampus of ischemic rats. The ACh level in the brain can be raised through an increase in choline acetyltransferase function or a decrease in acetylcholinesterase activity (Vijayaraghavan et al., 2013). Which mechanism corresponds to the observed increase in the ACh level should be evaluated in further studies. Impairment in cholinergic activity is an important pathway in the development of ischemic-associated pathologies in the brain (Wang et al., 2009, Martin et al., 2018). Pyramidal neuron apoptosis in 4VOI can damage the ACh system, resulting in impairment in memory function (Volpe et al., 1992, Bendel et al., 2005). Accordingly, it can be suggested that the increase of ACh by curcumin can be responsible for its memory-enhancing activity. However, the increase in ACh level may be only due to the preventive effect on apoptosis. In other words, this study cannot prove that curcumin has a specific effect on the activity of cholinergic neurons in the hippocampus. Further study is needed to evaluate this issue.

It has previously been demonstrated that curcumin can prevent apoptotic cell death in the pyramidal CA3 region induced by methamphetamine neurotoxicity in the rat. It is shown that curcumin can reduce apoptosis in the caspase-3 positive neuron and GFAP positive astrocyte in streptozotocin-induced diabetes rats.

The study faced some important limitations which should be recognized in the interpretation of the results. First, we did not evaluate the long-term preventive effects of curcumin on memory, learning, hippocampal acetylcholine level and neuroapoptosis. The second limitation was that we only evaluate hippocampal acetylcholine level and neuroapoptosis among all the potential underlying cellular mechanisms which are involved in memory impairment and ischemic injury in the brain.

CONCLUSION

The finding of the study suggests that curcumin can increase ACh levels and prevent apoptosis in the dorsal hippocampus in an experimental model of brain ischemia. Curcumin also prevents memory impairment induced by 4VOI. Further investigations are needed to further clarify the pathophysiologic basis of the curcumin effects in neurodegenerative disorders resulting from cerebral ischemia.

REFERENCES


Khiljee S, Rehman N, Khiljee T, Loebenberg R and Ahmad RS (2015). Formulation and clinical evaluation of topical dosage forms of Indian Penny Wort, walnut...


