Original Article

The Neuroprotective Effects of Memantine, and Curcumin after Cerebral Ischemia-reperfusion Injury in Elderly Rats

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ABSTRACT

Background: Some researches reveal that pharmacologic and phytotherapeutic agents have benefits on neurological disorders associated with the effects of reactive oxygen species. **Aim:** The purpose of this research was to evaluate the protective effects of curcumin and memantine in a cerebral ischemia/reperfusion model in rats. **Materials and Methods:** This experimental study was conducted at the Bagcilar Training and Research Hospital, Istanbul, Turkey. Rats were separated into five experimental groups ($n=8$) as follows: Curcumin (Group I), Memantine (Group II), Curcumin + Memantine (Group III), ischemia (Group IV), and sham (Group V). Cerebral ischemia was performed surgically with bilateral common carotid artery occlusion for 30 min, followed by reperfusion for 72 hr. Tissue and serum samples were collected and assessed for tumor necrosis factor-α, interleukin-6, lactate dehydrogenase, catalase, glutathione peroxidase, xanthine dehydrogenase, superoxide dismutase, and malondialdehyde levels. Further, in tissue samples, both neuronal loss and caspase-3 levels were determined. **Results:** Serum and tissue levels of interleukin-6, tumor necrosis factor-α, malondialdehyde, and lactate dehydrogenase were found significantly lower in the Group I, II, and III compared to Group IV ($p<0.001$). Glutathione peroxidase, superoxide dismutase, and catalase levels in Group I, II, and III were significantly higher than those of Group IV ($p<0.001$). **Conclusion:** Curcumin, Memantine, and Curcumin + Memantine treatment were found to be efficacious in decreasing oxidative damage in cerebral ischemia but failed to prevent tissue damage.

Keywords: Brain ischemia, Curcumin, Memantine, Oxidative injury, Reperfusion.

INTRODUCTION

Ischemia related free oxygen radicals and proinflammatory cytokines increase calcium (Ca^{2+}) entry into the cell leading to cell membrane damage. Free oxygen radicals denature cell enzymes and consequently initiate cell coagulation necrosis.[1] Apoptosis is an important mechanism implicated in post-ischemic reperfusion and secondary brain parenchyma injury.[2] Both *in vivo* and *in vitro* experiments, as well as clinical studies, revealed several promising antioxidants, and antiapoptotic agents which reduce stroke-related injuries.[3]

Curcumin (CUR) is a yellow-colored substance derived from turmeric (*Curcuma longa*) from the ginger family. A clinical and experimental study has shown that CUR exhibits antioxidant, anti-inflammatory, anti-fibrotic, apoptotic, and anti-cancer features.[4] CUR reduces the production of

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cytokines and chemokines by inhibiting nuclear factor-kappa B (NF-κB) activation, caspase-3, cyclooxygenase, and lipoxygenase pathways.[5] Further, a study showed that CUR inhibited the peroxynitrite anion ($ONOO^-$) mediated blood-brain barrier damage resulting in the alleviation of Cerebral Ischemic/Reperfusion (I/R).[6] Both CUR and Memantine (MEM) inhibit the N-methyl-D-aspartic acid (NMDA) receptor/ion channel that leads to an increase in Ca^{2+} influx into the cell resulting in cell death.[7,8] The NMDA receptor antagonist has a strong protective and apoptosis-inhibiting effect over the cerebral I/R pathway.[9] MEM is used in the treatment of many clinical conditions.[10]

Therefore, the purpose of this research was to investigate cerebral ischemic injury and to compare the neuroprotective efficacy of MEM and CUR in a rodent model.

MATERIALS AND METHODS

Ethical Statement

This study was conducted at the Bagcilar Training and Research Hospital, Istanbul, Turkey according to the approval from the local ethical committee (2013/11).

Rats

A total of forty, 22-month old Wistar Hannover rats weighing 400 - 500 g were included. Rats were cared for in accordance with the guidelines.

Experimental Setup and Administration of CUR and MEM *in vivo*

The following experimental set up assessed the effects of CUR and MEM treatment in rats with ischemic brain induced damage. Rats were separated into five groups: Group I CUR (n = 8) and were administrated via oral gavage with a 2 ml freshly prepared solution of 9 % ethanol and 150 mg/2ml of CUR for 20 days prior to ischemia induction.[11] A total of 300 mg/kg of CUR (Sigma, St. Louis, Mo., USA) was administered across 20 days. On day 21, ischemic brain damage was performed via surgery to the rats, and after 150 mg/ml of CUR was administered intraperitoneally (i.p.) once a day for three days, to avoid the risk of aspiration pneumonia due to loss of consciousness related to dysphagia. At the end of these three days, 10 mg/kg intramuscular (i.m.) of xylazine and 65 mg/kg of ketamine were injected to animals for anesthesia, and surgery was performed to collect intracardiac blood samples. All rats were sacrificed, when the experimental endpoint was reached. In Group II MEM (n = 8) rats were not treated in the 20 days leading up to ischemia induction. On day 21, a midline neck incision was performed under anesthesia resulting in an ischemic brain injury. A total of 25 mg/kg of MEM (Melanda®, Ali Raif, Istanbul, Turkey) was injected intravenous (i.v.) into the jugular vein once a day for three days following ischemia.[12] Afterward, animals were sacrificed according to ethical guidelines. In Group III CUR + MEM (n = 8) 150 mg/ml of CUR was administered prior to ischemia for 20 days using the same protocol as applied to Group I. CUR administration was continued for three days i.p. after ischemic damage was induced on day 21. Additionally, 25 mg/kg of MEM was injected i.v. once a day for three days following ischemia induction as carried out for Group II. Animals were sacrificed according to ethical guidelines. In Group IV Ischemia (n = 8), animals were only subjected to a midline incision resulting in ischemic brain injury and were sacrificed three days after the surgical procedure. Lastly, in Group V Sham (n = 8), 9 % ethanol was administered via oral gavage to rats for 20 days before a surgical midline incision. Anesthesia was given to animals on day 21, but no ischemia was established, and after three days, all animals were sacrificed according to ethical guidelines.

Surgical Procedure

Bilateral common carotid artery occlusion (BCCAO) method was used for the induction of ischemic brain injury under sterile conditions for the first four experimental groups.[7] Briefly, a midline neck incision was made under sterile conditions. A cranial pedicle flap was used to lift fatty tissue away. The sternomastoid muscle (SM) was then pulled away from the cranial and caudal sites. Underneath the SM, the omohyoid muscle, which covers the common carotid artery (CCA), was also moved aside. Both the CCA and the vagus nerve were dissected from the surrounding tissues. Reperfusion was carried out for 72 hr after the clamping of both CCAs for 30 min.

Histopathological and TUNEL Analysis

Brain tissues dissected from rats post-experimental procedure were stained with Hematoxylin Eosin (H&E). The histopathological scoring method was modified according to that proposed by Jacobs MA and Dereski MO *et al.* Ischemia was assessed using the criteria of neuronal degeneration and chromatolysis, neuronal shrinkage, and neuron loss. Each parameter was scored on a 4-point scale as follows: no criteria=0, mild=1, moderate=2, and severe=3.[13,14] Detection of neuronal loss in these sections was assessed using the Apoptotic Index (AI) given as a percentage of apoptotic cells amongst the total cell population.[15]

Apoptotic cells were identified by assessing fractures to DNA using the TUNEL technique in paraffin sections (Apop Tag® Peroxidase *in situ* Apoptosis Detection Kit, EMD Millipore).

Biochemical Analysis

Blood samples were acquired by intracardiac punching. These samples were spun down 2500 RPM 10 min. After, serum was separated and keeped at -80°C until further analysis. The levels of MDA, CAT, IL-6, TNF-α, GSPx, SOD, XDH, and LDH in serum were determined. Brain tissue samples were homogenized (Velp Scientifica UV-5 D500), and protein contents were measured according to the Biuret method. MDA (detection range [dr]: 0.312-20 nmol/mL), SOD (dr: 1.56-100 U/mL), CAT (dr: 0.625-40 ng/mL), IL-6 (dr: 15.6-1000 pg/mL), TNF-α (dr: 15.6-1000 pg/mL), GSPx (dr: 0.78-50 ng/mL), and XDH (dr: 0.156-10 ng/mL) levels were measured using the enzyme-linked immunosorbent assay (ELISA, Biotek Instruments USA, kits: EIAab Wuhan Science Co. Ltd. branded immunoassay) according to manufacturer's recommendations. A competitive binding enzyme immunoassay based ELISA was used to determine levels of MDA and SOD, and a classical ELISA was performed to identify other proteins levels of interest.

Statistical Analysis

Results represent the mean±standard error. The Kruskal-Wallis test, the one-way ANOVA, the Duncan test, and the Mann-Whitney U test were used with SPSS 18 program (SPSS Inc., Chicago, USA) for the data analyses. Non-normal distributed variables were evaluated with the Kruskal-Wallis test and the Mann-Whitney U test. For variables showing normal distribution, the one-way ANOVA and the Duncan test were used instead. Statistical significance occurred when a $P<0.001$ was observed for biochemical findings and apoptosis. Statistical significance was confirmed when a $P<0.05$ was found for histopathological scores.

RESULTS

Tissue and serum IL-6, TNF-α, GSPx, XDH, SOD, CAT, MDA, and LDH concentrations for all animals are listed in Table 1. Tissue and serum IL-6, TNF-α, MDA, and LDH levels were significantly lower in groups I, II, and III compared to group IV ($P<0.001$). GSPx, SOD, and CAT concentrations were significantly higher in the treated groups compared to group IV ($P<0.001$). However, serum XDH levels in group I and III were significantly lower than that of group IV ($P<0.05$).

Table 1: IL-6, TNF-α, GSPx, XHD, SOD, CAT, MDA and LDH values.

	Biochemical parameters	Groups (Mean±SD)					Significance
		Curcumin	Memantine	Curcumin + Memantine	Ischemia	Sham	
Tissue	IL-6 (pg/g)	81±4.33^{c}	89±4.18^{b}	60±5.57^{d}	117±6.07^{a}	23.92±3.32^{e}	$p<0.001$
	TNF-α (pg/g)	79±4.64^{bc}	83±6.82^{b}	76±6.42^{c}	155±7.47^{a}	52.58±5.71^{d}	$p<0.001$
	GSPx (ng/g)	191±5.86^{c}	167±4.4^{d}	198±6.51^{b}	117±6.18^{e}	250±7.58^{a}	$p<0.001$
	XDH (ng/g)	1,96±0.61	2.47±1.47	2.3±0.54	2.32±0.67	2.33±1.65	NS
	SOD (U/g)	22±2.93^{c}	19±2.4^{d}	28.5±3.43^{b}	11.56±3.02^{e}	38.38±2.66^{a}	$p<0.001$
	CAT (ng/g)	0.4±0.06^{c}	0.41±0.06^{d}	0.65±0.02^{b}	0.19±0.04^{e}	0.88±0.04^{a}	$p<0.001$
	MDA (nmol/g)	2.5±0.17^{c}	3.52±0.3^{b}	2.24±0.3^{c}	4.72±0.37^{a}	1.4±0.23^{d}	$p<0.001$
	LDH (U/g)	117±13.13^{b}	183.23±10.76^{a}	100±7.18^{c}	181.6±7.54^{a}	76.8±8.86^{d}	$p<0.001$
Serum	IL-6 (pg/mL)	17.28±0.061^{bc}	17.34±1.53^{b}	17.26±1.32^{c}	17.67±0.52^{a}	17.16±1.07^{d}	$p<0.001$
	TNF-α (pg/mL)	86.44±1.80^{c}	86.67±2.07^{b}	86.13±1.82^{d}	88.02±1.16^{a}	85.99±1.73^{e}	$p<0.001$
	GSPx (ng/mL)	2.64±0.09^{c}	2.34±0.07^{d}	3.11±0.19^{b}	1.18±0.11^{e}	3.71±0.15^{a}	$p<0.001$
	XDH (ng/mL)	0.57±0.23^{b}	0.84±0.15^{b}	0.58±0.31^{b}	1.15±0.71^{a}	0.61±0.18^{ab}	$p<0.05$
	SOD (U/mL)	32±2.73^{b}	25±2.43^{c}	33.23±3.66^{b}	11.83±2.04^{d}	39.60±2.24^{a}	$p<0.001$
	CAT (ng/mL)	2.42±0.06^{a}	2.4±0.04^{a}	2.4±0.04^{a}	1.43±0.06^{b}	2.38±0.02^{a}	$p<0.001$
	MDA (nmol/mL)	4.61±0.35^{bc}	4.87±0.52^{b}	4.11±0.36^{c}	8.38±1.02^{a}	3.34±0.17^{d}	$p<0.001$
	LDH (U/L)	343±24.66^{b}	321±21.22^{c}	329±18.06^{bc}	402.62±10.19^{d}	196.9±11.78^{a}	$p<0.001$

^{a,b,c,d,e} Mean values marked with different superscripts in the same line are significantly different from each other ($p<0.001$), N.S: Non spesific. One-way Anova-Duncan test.

No histopathological differences were identified in the sham group (Figure 1a). Massive necrotic areas were observed in both the ischemic and treated groups (Figure 1c). In particular, lesions found in the substantia alba layer included, spongiosis of neuropil, degeneration in neurons, chromatolysis, and pyknotic changes to cell nuclei. In addition to edematous, a granular and sparse appearance of the axonal structures was identified, with an occasional whipping of the cells (Figure 2a, c, and e). In the substantia grisea, ischemic insult areas were found to have increased perineural space, cell shrinkage, cell degeneration, and necrosis. These findings were identified in the neuropil, along with both granular and vacuolar changes. However, there was a statistically significant difference between the treated and untreated groups compared with the sham group ($P<0.05$). Apoptotic cell assessment by TUNEL staining was not carried out in the sham group (Figure 1b). However, in both the untreated and treated groups, apoptotic cells were identified in areas of ischemic damage (Figure 1d and Figure 2b, d, and f). AI was determined for each individual by counting all positive cells (Table 2).

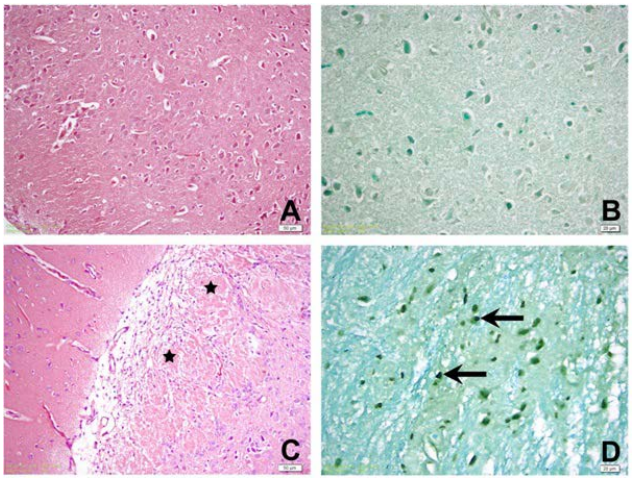

Figure 1: Normal and ischemic tissue appearance from sham and ischemia groups.

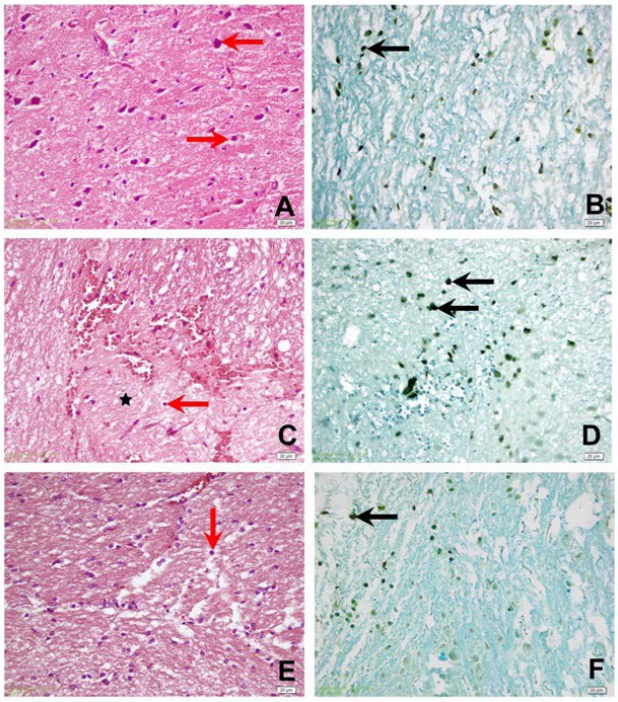

Figure 2: Apoptotic cells in curcumin and memantine administered groups.

DISCUSSION

Memantine exerts a neuroprotective effect on vascular dementia and global cerebral ischemia.[10] CUR is the main compound found in turmeric, which is used as a traditional spice by eastern countries and is already well known for its immunomodulatory properties in and outside of science.[4] CUR has been assessed and documented for its protective effects against cerebral ischemia. Prior research carried out evaluated the neuroprotective effect of both CUR and MEM in an experimental cerebral ischemia model.[6,9] However, there are no studies that investigate the combined effect of MEM and CUR in ischemia prevention. We investigated both the individual and combined contributions of CUR and MEM in an experimentally induced rat ischemia model. In tissue IL-6, TNF-α, MDA, and LDH concentrations and serum IL-6, TNF-α, MDA, LDH, and XDH concentrations were significantly lower in both treated groups compared to the ischemia only group. Furthermore, the concentrations of GSPx, SOD, and CAT were significantly lower in the ischemia groups compared to the treated group.

MEM works as an excitotoxic drug by binding to and inhibiting the flow of Ca^{2+} through NMDA receptors, which leads to reduced Ca^{2+} and subsequent apoptosis.[8,16] NMDA receptors are more abundant in the cerebral cortex compared to the striatum.[17] In focal

Table 2: Total histopathologic scores.

Groups (Mean±SD)	Curcumin	Memantine	Curcumin+Memantine	Ischemia	Sham	P values
Scores	1.71±1.25	1.86±1.86[a]	1.71±1.38[a]	2±1.41	0.29±0.48[b]	*

[a,b,c,d,e] Mean values marked with different superscripts in the same column are significantly different from each other ($P<0.05$). Kruskal-Wallis and Mann-Whitney U Tests.

cerebral ischemia models, early reperfusion occurs during the beginning of neuronal apoptosis. In contrast, apoptosis after transient global cerebral ischemia is delayed by 48 hr by the hippocampal pyramidal neurons.[18] These differences may be due to increased neuroprotective effects within focal ischemic models than in global ischemic models. The group of Chen *et al.* identified that the neuroprotective effect by MEM was significantly higher in the focal ischemia model in both clinical and experimental studies.[19] However, the neuroprotective effect observed in these studies may be related to the high MEM doses. In the limbic cortex, notably raised Brain-Derived Neurotrophic Factor (BDNF) mRNA levels were observed during treatment with an appropriate dose of MEM. This effect was more frequent and pronounced in higher MEM doses. Thus, elevated endogenous production of BDNF in the brain may have compromised the neuroprotective features of MEM.[20] Similar MEM treatment dosages used in the study of Yiğit *et al.* were also in this study, and the combined and single effects of MEM and CUR on ischemia were determined.[13]

Several groups have demonstrated that CUR can inhibit cerebral ischemia.[7,21-23] However, there is much that has not yet been revealed regarding the underlying mechanisms. A recent study shows that curcumin has neuroprotective effects due to its antioxidant in detail.[24] Besides antioxidant anti-inflammatory effects of CUR, Zhang *et al.*, showed that the expression of vascular endothelial growth factor (VGEF) protecting neurons from ischemic injury and improving synaptic plasticity was increased by Cur20 which is a CUR derivate.[23] Also, it's been reported that CUR is more effective in neuroprotection, especially when applied as nanoparticles.[8,25] Experimental models of ischemia showed that the route of administration of CUR also affects its activity. Jiang *et al.* assessed different dose administrations of CUR intravenously in the following dose rates 0.5, 1, and 2mg/kg. Improvement of ischemia/reperfusion-induced brain damage, decreased vasogenic edema, inhibition of iNOS from astrocytes, and inhibition of ONOO−induced brain capillary endothelial cell injury was reported in a dose-dependent effect. CUR i.v. has a higher potency than i.p. and oral administration.[6] Prior research revealed that CUR also is weakly absorbed by the gastrointestinal tract. Thus, the effective dose ranges observed may be due to the absorption and pharmacokinetics of CUR.[26] In a study conducted by Ghoneim *et al.*, tissue sampling of SOD, GSPx, and LDH enzyme activities were found to be significantly lower than CAT levels in subjects treated i.p. with 200mg/kg CUR.[21] We observed a significant decrease upon biochemical analysis of XO, O2•-, MDA, levels compared to the control group and no significant change was found in the GSPx level. In our study, CUR was administered via an intragastric probe before ischemia and again i.p. following ischemia. GSPx, SOD, and CAT concentrations were significantly lower in the untreated group compared to the treated groups. In contrast, MDA and LDH concentrations were lower in the treated groups than the untreated group.

Regarding the LDH activity, ischemia gives rise to lactate and H^+, which accumulates in the ischemic forebrain of rats confirmed with the elevation of LDH activity, which is the enzyme that converts pyruvate into lactate.[27] Ghoneim *et al.* showed that CUR (200 mg/kg) proved to be beneficial in restoring decreased LDH activities and suppressive effects on xanthine dehydrogenase/xanthine oxidase (XD/XO).[21] In our study, rats were administrated with 300 mg/kg of CUR. Tissue and serum LDH and XDH concentrations were significantly decreased in the treated groups than the untreated group.

To date, no study has investigated the combined neuroprotective effect of CUR and MEM. Here we investigated for the first time if these two drugs can protect ischemic injury. However, there are studies regarding the protective effect of CUR and MEM in addition to other drugs. In a study that used both MEM and galantamine to treat a gerbil model of transient global ischemia, there was an observed increase in raised living pyramidal neurons, diminished TUNEL, activation of caspase-3 and SOD-2 immunoreactivity, and preserved spatial memory. However, the outcomes of the glutamine alone were similar when used in combination with MEM.[28] Kılıc *et al.* investigated the neuroprotective effect of the combination of MEM with melatonin in a rat model of focal cerebral ischemia. Interestingly, they identified that MEM elevated the activation of the ERK-1/2 pathway, while melatonin decreased its activation. Accordingly, single drug treatment for multiple pathological events following stroke has not been discovered; however, existing neuroprotectant combinations are used for current therapy.[29] The synergistic effect presented in our study may be due to increased activation of the ERK-1/2 pathway by MEM and the inhibition of iNOS by CUR, althought this has yet to be proved.

The antioxidant enzyme levels suggest the presence of a synergistic effect by the two antioxidant agents. In our study, in addition to significantly lower oxidative damage and proinflammatory cytokine levels in the CUR and MEM groups compared to those of the untreated

group, there were no differences identified in tissue damage between treated and untreated groups. Still, the presence of tissue necrosis is not always consistent with biochemical findings,[4] as the complex cellular processes leading to cell death (necrosis) are very complicated, and has yet to be fully elucidated.[1] Further, the reduction of enzymes in the intercellular fluid and the occurrence of cell membrane damage by antioxidant agents such as blood serum CUR presents a more straightforward method to prevent the cell from irreversible necrosis. This hypothesis is supported by Gulcubuk *et al*., which use an experimental model of acute pancreatitis, to show that the various applications of ciprofloxacin/metronidazole and CUR in acute pancreatitis did not impede tissue injury, but markedly reduced damage induced by free radicals and frequency of bacterial translocation.[4]

CUR and MEM did not reduce tissue damage despite reducing oxidative and cytokine damage in transient global ischemic brain injury-induced rats. However, both CUR and MEM present as good candidates for the treatment of diseases, which induce oxidative and cytokine damage. In the future, we believe that ultrastructural electron microscopic studies for tissue damage might provide a better understanding of the underlying complex pathological mechanisms.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

ABBREVIATIONS

AI: Apoptotic Index; **BCCAO:** Bilateral common carotid artery occlusion; **Ca:** Calcium; **CAT:** Catalase; **CCA:** Common carotid artery; **CUR:** Curcumin; **ELISA:** Enzyme-linked immunosorbent assay; **GSPx:** Glutathione peroxidase; **H&E:** Hematoxylin Eosin; **IL:** Interleukin; **I/R:** Ischemic/Reperfusion; **i.v.:** intravenous; **LDH:** Lactate dehydrogenase; **MDA:** Malondialdehyde; **MEM:** Memantine; **NF-κB:** nuclear factor-kappa B; **NMDA:** N-methyl-D-aspartic acid; **ONOO-:** Peroxynitrite anion; **SM:** Sternomastoid muscle; **SOD:** Superoxide dismutase; **TNF:** Tumor necrosis factor; **VEGF:** Vascular endothelial growth factor; **XDH:** Xanthine dehydrogenase; **XO:** Xanthine oxidase.

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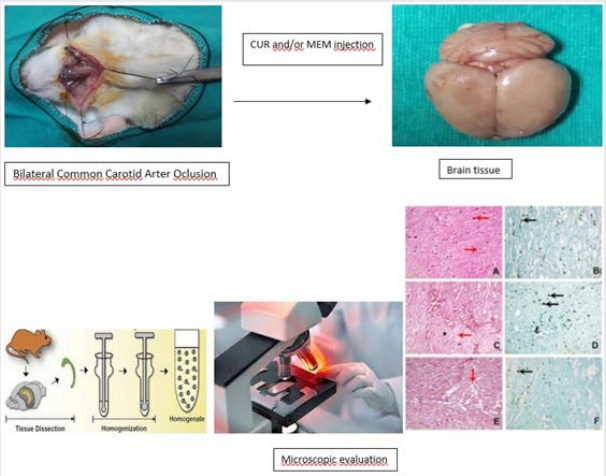
PICTORIAL ABSTRACT



SUMMARY

Curcumin is a constituent of the traditional medicine known as turmeric. CUR has been assessed and documented for its protective effects against cerebral ischemia. Memantine exerts a neuroprotective effect on vascular dementia and global cerebral ischemia. CUR and MEM did not reduce tissue damage despite reducing oxidative and cytokine damage in transient global ischemic brain injury-induced rats. However, both CUR and MEM present as good candidates for the treatment of diseases, which induce oxidative and cytokine damage. In the future, we believe that ultrastructural electron microscopic studies for tissue damage might provide a better understanding of the underlying complex pathological mechanisms.

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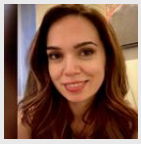

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