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ORIGINAL ARTICLE

Protective effect of trans-anethole on brain ischaemia reperfusion injury in rats induced by bilateral common carotid artery occlusion

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ABSTRACT
Stroke is the major cause for the mortality of most people around the world and is associated with multiple etiologies like injury, insults, ischaemia etc. There are numerous mechanisms by which brain injury occurs out of which oxidative stress, cerebral ischaemia, inflammation are emphasized. There was no clear understanding of the pathophysiology of ischemic stroke. Drugs of herbal origin or isolated from medicinal plants often display no side effects and are proven very effective in treating strokes caused by various factors. Most importantly, herbal drugs are known to fight against free radicals that are generated due to ischaemia and result in brain damage. So the ischaemia prevention potential of trans-anethole which was also notable for its antioxidant properties was tested in present work. Investigation was made to correlate the antioxidant property and the anti-ischemic potential of the compound in bilateral common carotid artery occlusion method in albino wistar rats. The data proved that there was a significant lowering of the peroxide enzymes and elevation of the antioxidant enzymes in trans-anethole treated groups. Histopathology of brain tissue revealed the presence of inflammatory process behind the injury which was reduced significantly with the treatment of trans-anethole. When tested at two doses 250, 500 mg/kg p.o. trans-anethole showed a dose dependent activity in lowering the oxidative stress, excitatory mediators and sodium potassium ATPase in the brain caused due to ischaemia thereby preventing the injury of the brain. It was also revealed that trans-anethole had an anti-inflammatory activity responsible for prevention of brain injury.
***Keywords**: BCCAO, ischaemia, trans-anethole, reperfusion, antioxidant*

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INTRODUCTION

Stroke is the major cause for the mortality of people around the world and it also responsible for the patients end up having long term and permanent disabilities [1]. It is also ranked the 2nd leading cause for the mortality in the world estimating about 5.5million deaths annually. It affects every 2 in a 1000 individuals in a year. Elderly patients are known to be more affected due to stroke and the data predicts that there is a drastic rise in the curve from 0.5 billion affected people in 2009 to 1.5 billion in 2050 [2].

There are numerous causative factors and mechanisms by which stroke occurs. The list includes inflammation in brain, oxidative stress, cerebral artery occlusion leading to ischaemia, and nerve-cell apoptosis and necrosis. There is no clear understanding about the etiologies and pathophysiology of stroke. This facilitated the development of advanced technologies to effectively manage and treat stroke. But even with the medical advancement, treating ischemic stroke remains challenging and options for managing acute ischemic stroke are limited [3].

There had been investigations to design and synthesize drugs and neuroprotective agents that displayed the best preclinical outcomes. Such drugs often fail in the clinical stage due to the side effects and adverse effects that arise from the stroke management. Stroke is caused in several mechanisms and usually the drugs that are being investigated only manage stroke in one pathway [4,5]. As there are multifactorial

etiologies for development of stroke and its comorbidities, there is need to be investigations to develop drugs that are effective in managing stroke multidirectional. There is an urgent need to understand and establish all the possible mechanisms by which stroke occurs and to develop new drugs that acts effectively in multiple pathways. There is a need to also make sure the drugs do not cause side effects in clinical usage.

Drugs of herbal origin or isolated from medicinal plants often display no side effects and are proven very effective in treating strokes caused by various factors. Most importantly, herbal drugs are known to fight the free radicals that are generated due to ischaemia and result in brain damage. Anethole is one of such potent drugs and is less investigated in this regard. It is chemically called as 1-Methoxy-4-[(1E)-prop-1-en-1-yl]benzene. It is an organic moiety that is used a lot as a flavouring agent and is a derivative of Phenylpropene which commonly found in volatile oils. Major families that contain trans-anethole are *iliaceae, apiaceae, myrtaceae, fabaceae, schisandraceae* [6]. It possess antioxidant, antimicrobial, antifungal anti-inflammatory, anticancer, diuretic, anti rheumatic, treatment of alzhemier's disease [7] and protection against neuronal cells *(in-vitro*) [8]. It is also proven to have antipyretic activity, analgesic activity and brain related activities like hypnotic and anticonvulsant activity. Following the literature, this investigation was designed to assess the role of antioxidant potential of trans-anethole in controlling the injury caused due to occlusion of Bilateral Common Carotid Artery (BCCA) in laboratory animals.

MATERIAL AND METHODS

Drugs and chemicals

Trans-anethole was procured from Sigma Aldrich, trichloroacetic acid, 2-Thobarbituric acid, epinephrine, 2,3,5 triphenyltetrazolium chloride. Analytical grade chemicals were used in all the experiments.

Experimental rats

Healthy Albino rats (200–250g) were procured from supplier from Bengaluru. Rats were maintained under normal lab conditions, and were maintained with12hlight/dark and allowed to have freely fed pellet feed and water. Animals were allowed to acclimatize to laboratory conditions for 1 week prior to study. Approval was received from IAEC for the procedures involving animals (Ethical committee. No. 1016/PO/Re/S/06/ CPCSEA/2019/010). Maintenance and handling of animals were done as per CPCSEA guidelines.

Study protocol

Animals were divided into four groups which contained 9 rats in each group. 1stgroup (sham control) was administered with 2% tween 80 orally, 2nd group was treated as disease control (ischaemic reperfusion after BCCA occlusion) and was administered with 2% tween 80 orally. 3rd and 4thgroup of rats received trans-anethole at dose of 250 and 500 mg/kg *p.o.* **[**9, 10] for 14 days. After 14 days of treatment with trans-anethole, rats were prepared for BCCA occlusion for 1 hour followed by 22 hrs reperfusion and were subjected for biochemical and histopathological studies.

Experimental design

The cerebral ischaemia reperfusion injury was induced by the method of Iwasaki *et al* (1989) [11]**.** The rats were anesthetized using ketamine injection (100mg/kg/IM) and xylazine injection.(10mg/kg/IM).The carotid arteries on both the sides were exposed over the midline and were dissected between sternocleidomastoid muscle and sternohyoid muscle parallel to the trachea. The ischaemia was caused by occluding both carotid arteries with a sterile thread for 1 hour. After 1hr occlusion knots were released and blood flow was allowed for 22 hours as a part of reperfusion. During surgery, the body temperature was maintained around 37^{o}C±0.5^{o}C with a thermo statistically controlled infrared lamp. Rats were sutured and povidone iodine ointment was applied with sterile cotton on sutured area. Sham control animals were also processed with surgery but without causing the occlusion of arteries. After observing for 22 hours reperfusion phase, animals were sacrificed for biochemical and histological assessment.

Biochemical Estimation

Brain homogenization

The animals were sacrificed by decapitation after inducing anesthesia after 22 h of reperfusion. The head was dissected to isolate brain. A part of the brain tissue was subjected to histopathological studies where the imaging was carried out to identify the abnormalities in brain caused due to the reperfusion. Remaining tissue was homogenized in 5mM phosphate buffer mixed with 0.1mM EDTA to yield 10%w/v brain homogenate. This homogenate was subjected to centrifugation at 4000rpm for15 min and the supernatant liquid was separated and stored in refrigerator at -5ºC and is used for biochemical estimation [12].

Lipid peroxidation level (LPO)

LPO level was measured using 200 µl of supernatant. This was added to 50µl of 8.1% SDS, (sodium dodecyl sulphate), mixed well and let it react for 10 min at normal temperature. 375µl of 0.6% w/v thiobarbituric acid mixed with the solution and boiled in a water bath for about 1hr. 1.25 ml of mixture of butanol:pyridine in a ratio of 3:2 was mixed with the solution and centrifuged at 1000rpm for about 5mins. The OD was measured at 532 nm in spectrophotometer [13].

Superoxide Dismutase level (SOD)

Dismutase levels were estimated using the standard procedures by Misra and Fridovich (1972) [14]. 100µl of the supernatant liquid was mixed with 880 µl of 0.05M carbonate buffer and 20µl of 30Mm epinephrine pH adjusted with 0.05% acetic acid and 0.1mM EDTA solution. It was allowed to react for 5mins and the OD was measured at 480nm.

Catalase level (CAT)

100µl of the supernatant liquid was mixed with 10µl of ethanol and cooled in an icebath for 30min. The test tubes were let to rest in room conditions and triton of 10µl was added. 50µl of the solution was combined further with 200µl of phosphate buffer in 250µl of hydrogen peroxide (0.66M). The reaction was allowed to carry on for about 60 sec and the OD values were measured at 240nm [15].

Reduced Glutathione (GSH)

0.75ml of the supernatant liquid was mixed in equal part of 4% sulfosalicylic acid at room conditions and was subjected to centrifugation at 1200rpm for 5min. The mixture was let to cool and 0.5ml of supernatant liquid was mixed with 4.5ml of 0.01M DTNB solution. The OD was measured at 412 nm [16].

Total Calcium level

Calcium levels were estimated by using the commercially brought calcium estimating kits (Span diagnostic Ltd., India).

Glutamate levels

1ml of the tissue supernatant liquid was mixed with 2ml of perchloric acid and pH at 9 was adjusted with phosphate buffer. This mixture was centrifuged at 1500 rpm for 15min and allowed to cool for another 10 min in ice bath. The mixture was filtered and absorbance was measured at 340nm [17].

Sodium-potassium ATPase (Na^+K^+ATPase) activity

A reaction mixture was prepared using 0.5 ml of 0.2M potassium chloride, 0.5 ml of 1M sodium chloride, 0.5 ml of 0.2M TrisHCl buffer, 0.5 ml of 0.1M magnesium chloride and 0.2 ml of brain homogenate supernatant to make a final volume of 2.2 ml. Another mixture was prepared by mixing 0.5 ml of 0.1M magnesium chloride, 0.5 ml of 0.2 M Tris HCl buffer, 0.5ml of 0.1M sodium chloride, 0.5 ml of 10mM of Oubain and 0.2ml of brain homogenate supernatant to make a final volume of 2.2 ml. Now 0.4ml of 25mM ATP solution was added to both the mixtures at the same time and the temperature was maintained at 37ºC. They were allowed to react for 15min. 0.1ml of 10% trichloroacetic acid was added to the mixtures to stop further reaction. This was chilled and centrifuged at 1000rpm for 5mins and the supernatant was collected to estimate the phosphorus by following the method suggested by Fiske and Subbarow [18].

Statistical Procedure

All the data obtained is represented as the Means of the values and their standard errors. They were analyzed using one way ANOVA and significance of differences was estimated using Dunnet's test. 'P' value less than 0.05 and 0.001 were treated as significant.

RESULTS

Effect of trans-anethole on antioxidant and pro-oxidant enzyme levels

Bilateral Common Carotid Artery (BCCA) Occlusion caused a notable brain damage which is evident by estimating the antioxidant enzymes. The ischemic reperfusion injury group lowered the antioxidant levels like SOD, CAT and GSH enzymes. There was a significant rise in LPO levels when compared with the Sham control group. There was significant lowering of the LPO levels with the treatment of trans-anethole at 250 and 500 mg/kg *p.o*. doses. There was a spike in SOD, GSH and CAT levels in trans-anethole treated groups and at a higher dose trans-anethole showed an activity comparably similar to the sham group. The values were tabulated in table 1 and represented in figure 1.

Effect of trans-anethole on excitatory mediators and Na^+k^+-ATPase activity

The results in table 2 showed significant activity of trans-anethole in lowering the calcium and glutamate which were elevated due to the ischemic perfusion injury. There was a lowering in the Na^+ and K^+ ATPase activity in the ischemic injury group and the values were significantly elevated in the treatment groups with trans-anethole. Interestingly trans-anethole at 500 mg/kg *p.o* showed a significantly similar activity to that of the sham control in increasing the sodium and potassium ATPase activity in the brain tissue.

Histopathology

Pictures of the brain tissue of I/R group clearly showed neutrophil infiltration into the intracellular spaces and the vacuole number and size was elevated. There was significant scarcity of cells in the section showed in figure 2. There was a remarkable lowering of the neutrophil counts and the intracellular spaces in the trans-anethole treated groups. This was similar to the sham group. It represents the trans-anethole treated animals significantly resisted and recovered the brain damage due to the ischaemia and reperfusion.

Table 1: Effect of trans-anethole on anti oxidative and pro-oxidant enzyme levels in rats

S.NO	GROUP	SOD (U/mg protein)	CATALASE (µM/min/mg)	GSH (µM of GSH/mg protein)	LPO- (nM/mg/protein)
1	Sham control	44.72±5.087^{+++}	32.01±5.350^{+++}	19.24±3.724^{+++}	11.82±2.732^{+++}
2	Ischemic & reperfusion (I/R) control	16.04±3.658*	9.98±2.013*	6.416±2.152*	33.01±5.014*
3	Trans-anethole (250 mg/kg *p.o*)	28.11±3.97^{+++}	21.23±3.146^{+++*}	11.37±2.835^{+++}	23.08±4.981^{+++}
4	Trans-anethole (500 mg/kg *p.o*)	35.26±4.765^{+++}	28.10±4.712^{+++}	17.95±3.579^{+++}	15.2±3.140^{+++}

The values were represented as mean and their standard errors; n=9. $^{+++}$P<0.001 significant compared with I/R control group and *P<0.05 significant compared with sham control group

Table 2: Effect of trans-anethole on Calcium, Glutamate and Na^+/K^+ATPase after ischaemic reperfusion injury in rats

S.NO	Group	Calcium (µg/mg protein)	Glutamate (µg/mg protein)	Na^+/K^+ATPase
1	Sham control	9.8±0.369^{+++}	8.12±0.298^{+++}	8.73±0.514^{+++}
2	Ischemic & reperfusion (I/R) control	36.2±1.268*	25.9±0.517*	3.24±0.356*
3	Trans-anethole 250mg/Kg *p.o*	30.5±0.671^{+++*}	19.4±0.402^{+++*}	4.08±0.498^{+++*}
4	Trans-anethole 500mg/Kg *p.o*	17.3±0.482^{+++*}	12.01±0.391^{+++}	6.15±0.612^{+++*}

The values were represented as mean and their standard errors; n=9. $^{+++}$P<0.001 significant compared with I/R control group and *P<0.05 significant compared with sham control group

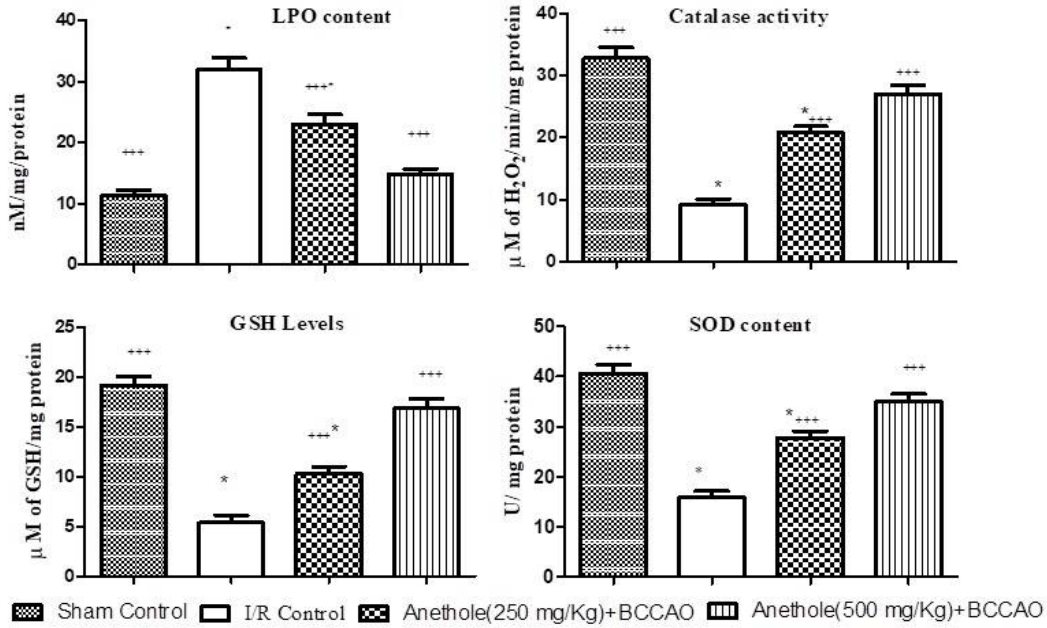

Fig 1: Effect of trans-anethole on antioxidant and pro-oxidant enzyme levels after ischemic reperfusion injury. The values were represented as mean and their standard errors; n=9. $^{+++}$P<0.001 significant compared with I/R control group and *P<0.05 significant compared with sham control group

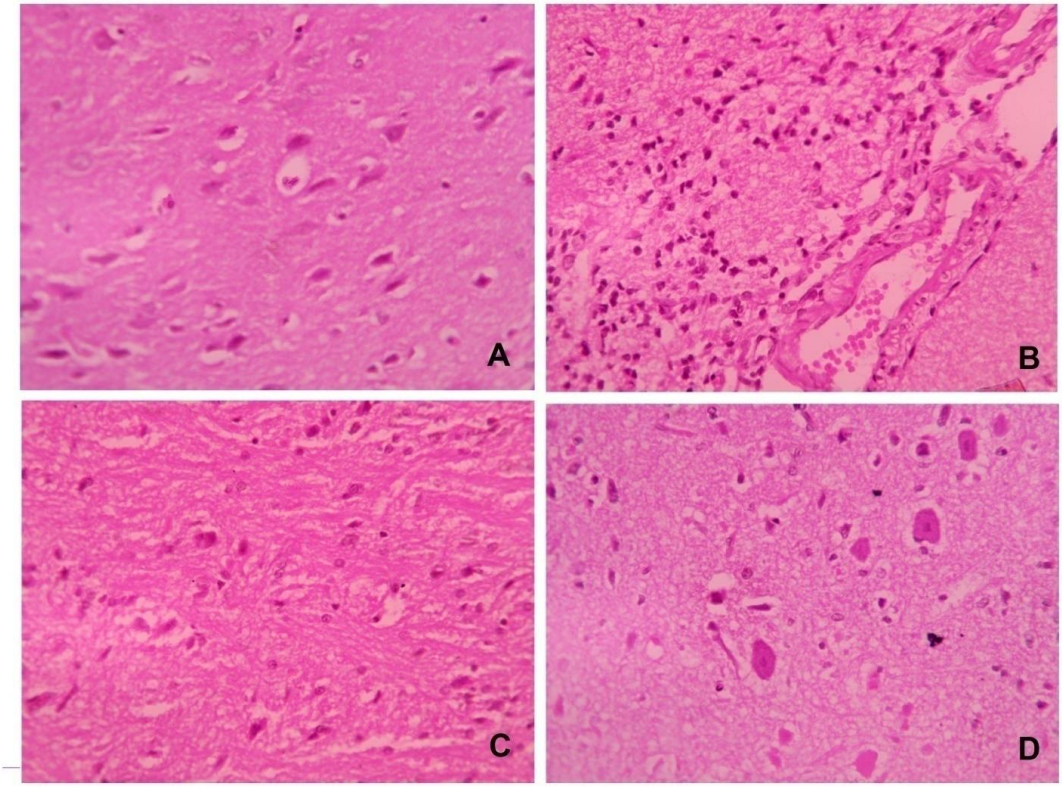

Fig 2: Effect of trans-anethole on the brain tissue injury caused due to ischaemia and reperfusion
A-Sham Control; B: I/R Control; C- trans-anethole250 mg/kg; D- trans-anethole 500 mg/kg

DISCUSSION

Brain damage due to ischaemia occurs in multiple mechanisms and pathways. Oxidative stress, inflammation and apoptosis, excitotoxicity and ion imbalance are most significant among them [19] . In this work, the brain damage was induced by ischaemia and reperfusion of bilateral common carotid arteries (BCCAO) in rats. The activity of trans-anethole was estimated by treated two groups of rats with trans-anethole at two doses of 250 mg/kg and 500 mg/kg *p.o*.

Injury caused by Ischemic reperfusion in brain is associated with the oxidative stress. The release of oxygen free radicals elevated the LPO levels and reduction of antioxidant enzymes like CAT, GSH and SOD. This was indicative of the oxidative damage in the brain. The elevation of the LPO is due to the lipid peroxidation and the cell membrane damage of the nerve cells [20]. The lowering of the antioxidant enzymes is due to the exhaustion of the enzymes in combating the released free radicals. In general these enzymes protect the nerve cells against the damage caused by the oxygen radical species. The catalases and dismutases prevent the formation of hydroxyl free radicals. Their levels were significantly lowered in I/R group which indicates that there might be mechanisms relating to the liberation of the free radicals which were controlled in the groups treated with trans-anethole. Imbalance in the glutathione levels are indicative of neurodegenerative diseases and is contributing factor of oxidative damage due to ischaemia. Trans-anethole significantly attenuates the glutathione levels even after occlusion of bilateral carotid arteries that resulted in the ischemic injury.

As there is an occlusion of the arteries there is a significant reduction in the flow of blood that resulted in the failure of sodium-potassium ATPase pump. Thus failed balance in the energy flow in the brain causes stroke and injury. As there is a lack of ATP in the brain tissue and lowered glucose levels and other nutrients due to low blood flow, accumulation of sodium ions takes place inside the nerve cell [21]. The redistribution of the intracellular Na^+ ions causes the depolarization of the membrane resulting in the excessive neurotransmitters. This also causes increase in the glutamate which leads to the elevation of intracellular calcium levels. This eventually triggers the apoptosis and necrosis of the cells [22]. This was clearly marked in the histopathology of the brain tissue. There are various inflammatory processes also involved in the brain damage like neutrophil migration and increase in the intracellular space that is resulted due to the death of nerve cells. The prophylactic treated trans-anethole groups inhibited the

elevated levels of excitatory mediators, sodium-potassium ATPase levels and decreased the brain damage in dose dependent manner.

CONCLUSION

From the study it is confirmed that there are oxidation mechanisms that is involved in the brain damage caused due to the ischaemia by bilateral common carotid artery occlusion. Trans-anethole is a potent antioxidant, had lowered calcium, glutamate levels and recovered sodium-potassium ATPase and had significantly attenuates the brain damage. The mechanism involved was by lowering the oxidation indicated by lowering LPO levels. Trans-anethole prevented the brain damage by elevating the SOD, CAT and GSH levels which fight against the free radicals there by reducing the brain injury caused by BCCAO. The inflammatory mechanisms like neutrophil proliferation, cellular necrosis are also prevented by trans-anethole which is evident in histopathological studies. Overall trans-anethole showed a dose dependant activity in lowering the brain damage caused by ischaemia.

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COMPETING INTERESTS

The authors have declared that no competing interest exists.

DECLARATION

This manuscript is neither published nor submitted for publication, in whole or in part, either in a serial, professional journal or as a part in a book which is formally published and made available to the public.

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