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ANTIOXIDATIVE ACTIVITY OF CLOVE (SYZYGIUM AROMATICUM) OIL ADMINISTRATION IN MIDDLE CEREBRAL ARTERY OCCLUSION (MCAO) MODELS OF ACUTE FOCAL CEREBRAL ISCHEMIA

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ABSTRACT

Objective: Stroke is a multifactorial neurological deficit syndrome. Oxidative stress is the principal underlying pathophysiological mechanism of ischemic stroke associated with neuronal damage and neuroinflammation.

Methodology: The purpose of present study is to investigate the preventive effects of clove (*Syzygium aromaticum*) oil pre-stroke and post-stroke administration against cerebral ischemia/reperfusion injury. Total of forty Wistar rats were divided into five groups as control, sham, stroke, pre-stroke treated (receive clove oil (33mg/kg body weight) for 15 days once daily, stroke given after completion of 15 days pretreatment regime) and post-stroke treated group (receive two doses of clove oil (33mg/kg body weight) one at 0 h (immediately after stroke induction) and second dose after 6 h of stroke). Stroke was induced via middle cerebral artery occlusion method (MCAO) (15 minutes occlusion followed by 24 h reperfusion). At end of experimentation, animals were tested for sensorimotor functioning via neurological deficit score and brain antioxidants including superoxide dismutase (SOD), catalase (CAT) and glutathione (GSH) were estimated in all groups

Results: Results showed significant betterment in neurological deficit score in treated groups ($P<0.05$) compared to stroke group. MCAO induction significantly ($P<0.01$) increase oxidative stress in brain tissue of stroke group. Pre-stroke and post-stroke treatment regime significantly ($P<0.05$) enhance brain endogenous antioxidants (SOD, CAT, and GSH).

Conclusion: Suggesting the possible preventive role of clove oil against oxidative neuronal damage in acute focal cerebral ischemia.

Keywords: Oxidative stress, eugenol, antioxidants, clove oil, neurological deficit, stroke.

INTRODUCTION:

Cerebrovascåular accidents are highly prevalent global problem. Stroke is the fourth most common cause of death and second most leading cause of disability around the globe [(1)]. Among other forms of stroke, ischemic stroke accounts for the majority (approx. 85%) of all stroke cases. Stroke is the focal neurological deficit syndrome results from interrupted cerebral blood flow (CBF) with subsequent oxygen and glucose deprivation in nervous tissue [(2)]. Reperfusion in ischemic tissue results in free radicals generation which augments neuronal injury followed by initiation of inflammatory mechanism [(3)]. Uncontrolled calcium influx, glutamate excitotoxicity, reactive oxygen species (ROS) over production are the underlying pathophysiological mechanisms of ischemic stroke [(4)]. Among all pathophysiological mechanisms of ischemic stroke, ROS over-production crucially contributes to neuronal oxidative burden, disruption of blood brain barrier, neuroinflammation and activation & recruitment of immune mediators in ischemic tissue [(5,6)]. The only

approved stroke therapy is recombinant tissue plasminogen activator (rtPA) but it has very narrow therapeutic window with high risk of thrombolysis limits its clinical use [7].

Dietary antioxidants and antioxidant therapies has been long investigated for their promising neuroprotective, neuronal survival and restorative potentials[8].

Clove (*Syzygium aromaticum*), an aromatic flower buds harvest from tree belongs of Myrtaceae family. Cloves' essential oil (aromatic, pale yellowish fluid) extracted via distillation from air-dried, un-opened flower buds[9]. For centuries, clove flowers, buds and essential oil of clove have been used in traditional medicinal systems to treat variety of health problems.

A single clove bud contains 14-20% of essential oil. Eugenol is a principal bioactive phytochemical richly found in clove oil[10]. Therapeutic potentials of clove are credited to eugenol including analgesic, anti-spasmodic, antioxidant, anti-inflammatory, anti-thrombotic, neuroprotective, anti-convulsant, anti-carcinogenic, anti-diabetic, anti-bacterial and antifungal properties[9,10].

Therefore, the present study aimed to investigate the antioxidative and possible neuroprotective effects of clove (*Syzygium aromaticum*) oil administration against ischemia-reperfusion (IR) injury in middle cerebral artery occlusion (MCAO) rat models of acute focal cerebral ischemia.

MATERIALS & METHODS:

Ethics Statement: Animal handling, care and all experimental procedures were performed according to the guidelines of animal care and use [11].

ANIMALS:

Adult female Wistar rats (200-250g) were purchased from animal care facility of ICCBS, university of Karachi, Karachi Pakistan.

Wistar Adult Female Rats (200-250gms) were used in all the experimental groups, purchased from the animal house of ICCBS Karachi, Pakistan.

Animals were housed as one animal per cage in a well-aired room (12h light/dark cycles) with food (standard laboratory diet) and water *ad libitum*. Animals were allowed to acclimatize for a week prior to any experimentation.

CLOVE OIL:

Commercially available analytical grade Clove Oil (catalog no. C8392) was purchased from Sigma-Aldrich (St Louis, MO, USA).

Experimental Protocol:

A total of forty animals were randomly divided into following groups:

Control Group: (n=8) Normal, untreated animals.

Stroke Group (n=8): Stroke inducted group

Stroke Induction: Focal acute cerebral ischemia was established via MCAO technique as mentioned earlier (12). Briefly, following anesthesia (via ketamine (50mg/kg body weight) + xylazine (10mg/kg bodyweight) intraperitoneal injection) neck incision was made to expose common carotid artery (CCA), external carotid artery (ECA) and internal carotid artery (ICA). A silicon coated 4/0 nylon filament was introduce in left ECA to the lumen of ICA and advanced until it occluded the origin of middle cerebral artery (MCA). Filament was removed after 15 minutes to allow reperfusion (24 h). To maintain body temperature animal was placed on heating pad and carefully monitored throughout the procedure.

Sham Group (n=8): Sham operated group.

Sham surgery: Sham group animals also underwent identical MCAO procedure but without the occlusion of MCA

Pre-stroke Treated Group (n=8): Animals of this group were intraperitoneally administered with clove oil (33mg/kg body weight per day) for 15 days. After the pre-treatment period, animals were inducted with stroke.

Post-stroke Treated Group (n=8): Animals of this group received two intraperitoneal doses of clove oil (33mg/kg body weight i.p). First dose was administered immediately after stroke induction and second dose was administered 6 hours of stroke induction.

At the end of experimentation period (after 24 h of reperfusion), neurological deficit score and brain tissue antioxidants (superoxide dismutase, catalase and glutathione) were investigated in all groups

NEUROLOGICAL ASSESSMENT

Neurological deficits were evaluated 24 h after reperfusion according the methods as described earlier

(13,14). The neurological deficit (ND) score comprised of three tests: body twisting, forelimb flexion and body balance as described earlier [15].

Final ND score was calculated as follow: ND score= score of Test 1 + Test 2 + Test 3
ND score rates neurological deficit on a scale of 0-9 where 0, no deficit and 9 maximum neurological deficit.

Biochemical Assessment and Analytical Procedures

Tissue Sample

Under deep anesthesia brain was removed immediately rinsed with ice-cold saline (0.9% NaCl) weighed and stored at -80ºC till further investigation.

Preparation of Brain Tissue Homogenate:

Brain tissue homogenate was prepared according to the method as described in previous study [5].

Assessment of Brain Antioxidants:

Brain tissue antioxidants including superoxide dismutase (SOD), catalase (CAT) and glutathione (GSH) was estimated according to the recommended procedures.

Superoxide Dismutase Assay: Tissue SOD concentration was estimated spectrophotometrically by the method of Kono (16) based on nitroblue tetrazolium (NBT) reduction rate recorded at 560nm using Schimadzu UV spectrophotometer. SOD activity was expressed as U/g of tissue. Catalase Assay: Concentration of catalase in brain tissue was determined by the spectrophotometric procedure as mentioned by Sinha, 1972. The reaction mixture comprised of hydrogen peroxide and dichromate acetic acid solution and read at 570nm wavelength. Tissue CAT activity was recorded as μmol/g of tissue.

Glutathione Assay: GSH activity in brain tissue was estimated according to previously described method (18). GSH concentration was recorded on kinetic spectrophotometer at 25ºC and results were represented as U/g of tissue.

Statistical Analysis:
Data were presented as mean ± SEM (standard error of mean) Statistical analysis was performed by one-way analysis of variance (ANOVA) with Tukey's post hoc test using the Statistical Package for the Social Sciences (SPSS) 16.0 software (SPSS Inc., Chicago, IL, USA). Difference with $P<0.05$ was considered statistically significant.

RESULTS

Pre and Post Treatments with Clove Oil Reduces Neurological Functional Deficit in MCAO Rats In the present study, we investigate whether clove oil administration is neuroprotective against acute IR injury by using MCAO rat model and pre and post-treatment regimen. As shown in Figure 1, no neurological deficits were observed in control and sham operated animals. Whereas MCAO mediated IR injury significantly affect sensorimotor function as indicated by the highest neurological scores stroke group animals (ND score=7.83≈8, $P<0.0001$), after MCAO. Compared to stroke group, animals pretreated with clove oil for 15 days showed a significant decrease in neurological deficit score ($P<0.01$) after MCAO. While ND score of animals post-treated with clove oil with two dose after stroke also exhibit significant ($P<0.05$) decrease in ND score when compared with stroke group (Table 1). Comparing with stroke ND score, pre-treated group showed more improvements then post-treated group. However, no statistically significant change was observed in pre and post-treated groups ($P>0.05$) (Figure1, Table 1).

Pre and Post Treatments with Clove Oil Ameliorates Oxidative stress in MCAO rats' Brain Tissue Brain weights of all experimental groups remain close to baseline vale except a nonsignificant increase was observed in brain weights of MCAO stroke group compared to control ($P>0.05$). As shown in Table 2, MCAO mediated IR injury significantly decrease brain antioxidants including superoxide dismutase, catalase and glutathione activities when compared with animals of control (SOD and CAT $P<0.05$; GSH: $P<0.01$) and sham (SOD and CAT $P<0.05$; GSH: $P<0.01$) groups.
Effect of Pretreatment Regimen: Per day i.p. clove oil administration at dose of 33mg/kg body weight for 15 days found to significantly increase brain SOD ($P<0.01$) CAT ($P<0.01$) and GSH ($P<0.05$) concentrations when compared with MCAO untreated rats (Table 2).

Effect of Post-treatment Regimen: Intraperitoneal administration of clove oil doses one at 0 h and second at 6 h post-stroke induction improve brain antioxidant (CAT, SOD, GSH) status in animals of post-treated group (SOD: $P<0.05$) however these increase were not statistically significant for CAT and GSH ($P>0.05$) as compared to stroke group (Table 2). Moreover, pretreatment found to improve tissue antioxidants more effectively then post-stroke clove oil doses (Table 2).

Table 1: Effect of Clove Oil administration on Neurological Deficit Score among different experimental groups

Neurological Deficit Score				
Control	Sham	Stroke	Pre-Treated	Post-Treated
0	0	7.83±1.06	4.83±1.07**	5.83±1.2*/NS

Values are presented as mean±SEM. *P<0.05, **P<0.01, NS: non-significant (compared with stroke/compared with Pre-treated)

Figure 1: ND score comaprision among experimental groups

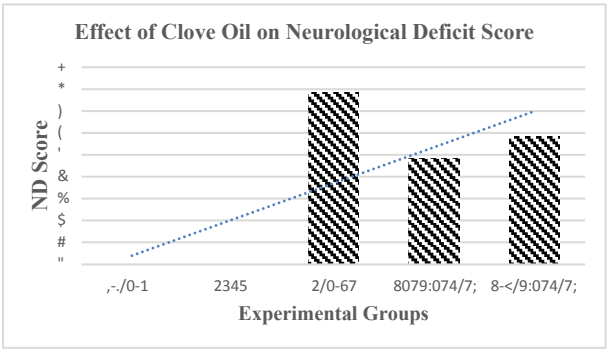

Table 2: Effect of Clove Oil Pre and Post Treatments on Brain organ weight, total protein and Antioxidants (SOD, CAT, and GSH) levels in MCAO Rats

	Control (n=8)	Sham (n=8)	Stroke (n=8)	Pre-Treated (n=8)	Post-Treated (n=8)
Organ Weight (g)	2.015±0.21	2.04±0.23^{NS}	2.29±0.15$^{NS/NS}$	2.07±0.24$^{NS/NS/NS}$	2.14±0.24$^{NS/NS/NS/NS}$
Total Protein (g/dL)	5.19±0.28	5.16±0.23^{NS}	5.27±0.27$^{NS/NS}$	5.07±0.25$^{NS/NS/NS}$	5.15±0.27$^{NS/NS/NS/NS}$
SOD (U/g tissue)	73.99±6.64	71.94±5.99^{NS}	50.842±2.89$^{*/*}$	63.65±1.69$^{NS/NS/**}$	59.89±2.01$^{NS/NS/*/NS}$
CAT (µmol/g tissue)	20.36±1.81	20.53±1.66^{NS}	13.79±1.05$^{*/*}$	18.807±0.86$^{NS/NS/**}$	15.38±1.78$^{NS/NS/NS/NS}$
GSH (U/g tissue)	0.084±0.006	0.083±0.005^{NS}	0.056±0.004$^{*/**}$	0.077±0.006$^{NS/NS/*}$	0.067±0.006$^{NS/NS/NS/NS}$

Values are presented as mean±SEM. *P<0.05, **P<0.01, NS: non-significant (compared with control/compared with sham/compared with stroke/compared with Pre-treated).

DISCUSSION

The global stroke burden has been raised alarmingly in past few years equally affecting young and geriatric population and both genders. Moreover ischemic stroke is the second most leading cause of permanent disabilities in adult [19]. Alteplase (rtPA) is the only available pharmacological therapy for ischemic stroke but it has its own therapeutic limitations. Post-stroke physical disabilities are matter of serious concern as most of the stroke victims suffer some degree of physical disability after stroke incident. Due to side effects and other pharmacological therapeutic tribulations, identification of effective and safe therapy from natural products for stroke or any other disease management is the current horizon of research. Bioactive components from herbal origin and plant polyphenols are multipotent therapeutic agent to treat several health ailments [20].

The current study also designed to investigate the therapeutic potentials of clove oil administration against focal cerebral ischemia induced neuronal damage. To study the protective effects of clove oil against cerebral IR injury, we used MCAO intraluminal technique to reproduce acute focal cerebral ischemia in rats (15 min occlusion and 24 h reperfusion). During reperfusion phase of ischemic stroke, ROS overproduction plays crucial role in destructing brain's anatomical regions (neuronal damage) and its associated sensorimotor dysfunction[15].

In our study we evaluate the post stroke neurological function and effects of treatment regimens via neurological deficit score. ND score is a composite score to assess sensorimotor functioning in rodent models of ischemic stroke or other neurological damage. Results of present study showed that MCAO untreated rats score maximum ND score (Table 1) while neurological functioning get better with pre and post treatments with clove oil possibly attributable to antioxidative and anti-spasmodic potentials of eugenol (bioactive component of clove oil). A study reported that eugenol effectively protect cortical cells from oxidative damage, excitotoxicity and ischemic injury via modulation of N-methyl-D-aspartate (NMDA) receptors & superoxide radicals [21].

Endogenous antioxidants like glutathione, SOD and CAT are necessary to maintain tissue oxidant/antioxidant balance and to protect tissues from ROS damage (5,8). Results of present study showed marked decrease in brain SOD, CAT and GSH concentrations in MCAO animals while 15 days clove oil pretreatment and two doses of clove oil post-stroke both treatments found to significantly improve the antioxidant status following stroke induction. Consistent to our results, several experimental studies also reported the potent antioxidative activity of eugenol against oxidative injury due to eugenol free radical scavenging capabilities[9].

Study of Farias and colleagues reported that that eugenol and eugenol derivatives effectively inhibit protein and lipid peroxidation in liver and brain tissues [22]. According to another study, eugenol effectively inhibiting oxygen glucose deprivation (OGD) and NMDA induced neurotoxicity by rapidly scavenging superoxide

radicals and controlling calcium influx and neuronal apoptosis [23].

CONCLUSION:

In summary, results of the present study demonstrate the antioxidant mediated protective effects of clove oil treatments against IR injury in MCAO rat model of acute focal cerebral ischemia as exhibited by better neurological function and antioxidants activities. Clove oil or eugenol could be a preventive treatment of stroke however further detailed experimental studies at different doses are recommended.

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Hira Jawed; data collection, data analysis, manuscript writing, manuscript review
Mufzala shamim; concept, data collection, data analysis, manuscript writing, manuscript review
Sumera Sohail; data analysis, manuscript writing, manuscript review
Uzma Firdous; contributed to data management and literature review
Nazish Iqbal Khan; data analysis, manuscript writing, manuscript review