A preliminary evaluation of the effects of *Camellia sinensis* on stroke induced rat model

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Abstract

Introduction: The objectives of current study are to test for Neuroprotective activity of *Camellia sinensis* in rat model of stroke and to evaluate the effect of *Camellia Sinensis* as anti-thrombolytic agent and in lowering the impact of disease with the behavioural changes before and after the induction of Stroke. Methods: Forty male albino rats were subjected to middle cerebral artery occlusion method for induction of stroke. *Camellia sinensis* extract was administered orally for 21 consecutive days prophylactically. Ischaemic rats administered the same volume of tap water were used as a control group. Functional outcome tests (Pasta, forelimb flexion, cylinder, staircase) were performed. Rats were subjected to surgical procedures after 21 days’ treatment for analysis of stroke recovery. Results: Treatment with *Camellia sinensis* extract of 400 mg/kg PO significantly (*P=0.000*) enhanced neurological recovery in all tests performed. There was no significant difference of infarct volume among the experimental groups treated with *Camellia sinensis* extract 200 mg/kg PO. Conclusion: The outcomes of this study was vivid that *Camellia sinensis* extract is safe and effective mediator in clot dissolution and stroke reversal in rat model. It is the first agent found effective in no behavioural modification or adverse effects using its extract. Therefore, there is a need to evaluate, assess and appraise its desired characteristics and therapeutics in human subjects.
Keywords

_Camellia sinensis_, Pasta, Forelimb flexion, Cylinder, Staircase

Introduction

Stroke is the third leading cause of death in the developed world. Stroke is characterised by the World Health Organisation (WHO) as “the fast interruption of brain capacity with indications of central (or global) development with side effects enduring 24 hours or more, or that prompts passing with no other obvious cause other than that of vascular starting point.” In industrialised nations, this wellbeing issue is the essential driver of building up a long haul handicap and the third driving reason for death; the primary spot being taken by coronary illness and second place of all other diseases together (Warlow et al., 2011). Stroke comprises of two obsessive subtypes: Ischaemic and haemorrhagic (Gomes and Wachsman, 2013). Atherosclerosis is the major risk factor; ischaemic stroke and its risk elements are in this manner imparted to all other infectious states brought on by atherosclerosis including myocardial dead tissue (Gorelick, 1993).

David Suzuki said, “The medical literature tells us the most effective way to reduce the risk of heart disease, cancer, stroke, diabetes, Alzheimer’s and many more problems is through diet and exercise.” One of these is green tea (_Camellia sinensis_). The therapeutic impacts of tea have a history going back right around 5000 years. The concoction parts of green tea primarily incorporate polyphenols, caffeine and amino acids. _Radix Salviae Miltiorrhizae_ (RSM) is a powder which is extracted and processed from dried root and rhizome of _Salvia miltiorrhiza Bunge_, family _Labiatae_. It has been used for increasing the cerebral blood flow and therefore has a potential against cerebral ischaemia (Tang et al., 2002). It has been tried against various models of cerebral ischaemia like ligation of the carotid artery in gerbils and 4-vessel occlusion model in rats and has been found to be effective. Kuang et al demonstrated that RSM reduced the lipid peroxidation and afforded cerebral protection against reperfusion injury (Kuang et al., 1996). Recently, it has been shown that RSM has the actions of improving blood circulation and resolving stasis to promote regeneration in traumatic intracranial haematoma (Sun et al., 2009) and also has been used for the management of cardiovascular disease (O’Brien et al., 2011). Tetramethylpyrazine (TMP) is widely used in the treatment of ischaemic stroke by Chinese herbalists and is one of the most important active ingredients of the traditional Chinese herbal medicine _Ligusticum wallichii_ Franchat (Chung Xiong). However, the mechanism by which TMP protects the brain is still not clear,
although neuroprotective effects of TMP against ischaemic brain injury might involve its anti-inflammatory potential (Liao et al., 2004). Experimentally, TMP has been shown to induce vasodilatation, to increase coronary blood flow and inhibit ADP induced platelet aggregation. These properties of TMP apparently account for its efficacy in the treatment of disorders associated with blood vessel occlusion like cerebral ischaemia (Luo et al., 1994). Ginseng, the root of Panax ginseng, is a well-known traditional Chinese herbal medicine. It is a slow-growing perennial plant with fleshy roots, in the Panax genus, in the family Araliaceae. Ginsenoside Rd (GSRd), one of the main active ingredients in Panax ginseng, exhibited remarkable neuroprotection when presented during oxygen glucose deprivation and reoxygenation, which may be ascribed to its antioxidative properties by reducing the intracellular reactive oxygen species and malondialdehyde production; increasing glutathione content; and enhancing the antioxidant enzymatic activities of catalase, superoxide dismutase and glutathione peroxidase. These findings suggest that it may be a potential neuroprotective agent for cerebral ischaemic injury and further studies are required to explore the potential neuroprotective efficacy of GSRd (Son et al., 2009).

Tea likewise contains flavonoids, mixes answered to have hostile to oxidant properties having numerous valuable impacts. Tea flavonoids decrease irritation, has antimicrobial impacts and counteracts tooth rot. A related compound found in tea is theophylline, an authorised prescription for the treatment of respiratory maladies, for example asthma. Today's PC driven world creates muddled way of life and utilisation of certain characteristic item like tea might just supplant the evil impacts of substance medications prompting a more secure world with more joyful life (Sharangi, 2009). The water concentrate of green tea and lemon grass was examined for their cancer prevention agent, impacts on pale skinned person, rats with 100 mg/kg body weight of green tea (Ojo et al., 2006).

The objectives of the present study are to assess the neuroprotective activity of Camellia sinensis in a rat model of Stroke.

**Materials-Methods**

Total forty male albino rats, aged 12 months, weighed ranging from 290 – 300 g were taken and kept in standard cages (L = 595 mm x W = 380 mm x H = 200 mm), ten rats per cage. Animal cages were maintained in a controlled environment at a temperature of 22° C, humidity 40 - 60% and light between the hours 07.00 - 19.00. This study was conducted in three phases.

During Phase-I, rats were acclimatised for seven days and behavioural modulation of the rats was done. Four tests were implemented to rats. These include cylinder test, staircase test, pasta test and forelimb flexion test. The Cylinder test is designed to evaluate locomotor asymmetry in rat models having
CNS disorders. The staircase test is designed for the measurement of side-specific deficits in coordinated paw reaching in rats and has been shown to reveal impairments on the contralateral side following unilateral lesions in a wide range of motor structures of the brain. Pasta test develops a simple quantitative measure of forepaw dexterity that is sensitive to lateralised impairment changes. Similarly, a very basic assessment used to detect neurological deficits is a test of forelimb flexion.

In Phase-II these rats were trained and divided into four groups, each comprising of 10 rats. Group A received normal saline, while Group B was Sham group. Sham surgery (placebo surgery) group is a fake surgical intervention that omits the step that is believed to be therapeutically necessary. In clinical trials of surgical interventions, Sham surgery is an important scientific control. This is because it isolates the specific effects of the treatment as opposed to the incidental effects caused by anaesthesia, the incisional trauma, pre- and post-operative care and the patient’s perception of having had a regular operation. Thus, Sham surgery serves an analogous purpose to placebo drugs, neutralising biases such as the placebo effect. Group C received *Camellia sinensis* 200 mg/kg, and group D received 400 mg/kg *Camellia sinensis* extract orally. Extract of *Camellia sinensis* was prepared as specified by Cheruiyot et al (2015) (Sigei et al., 2015). Prepared soluble granules of both black and green tea samples sealed in silver lined sachets stored at room temperature were obtained. Cold aqueous crude extracts were made by soaking weighed amount of dry soluble granules of tea (10 g) in 100 mL of sterile distilled water and shaken for half an hour in an electric shaker. The extracts were filtered using Whatman No. 1 filter paper to exclude any suspending particles. Crude extract, supernatant was then transferred to sterile screw cap bottles, labelled and stored under refrigerated condition (40°C) until use. Crude extract, filtrate was then transferred to sterile screw cap bottles, labelled and stored under refrigerated condition (40°C) until use. Only fresh extracts were used in the experiment, as marked chemical changes occurred when tea was allowed to stand (Sigei et al., 2015).

During Phase-III, ischaemic stroke was induced through Middle Cerebral Artery Occlusion (MCAO) method. The MCAO method involves the threading of carotid artery resulting in cessation of blood flow and subsequent brain infarction in the MCA territory. This technique was used for transient occlusion. The suture was removed after 60 minutes, reperfusion was achieved. The highlights of surgical procedure are shown in Fig. 1. To evaluate the extent of cerebral infarction, we stained brain slices with 2, 3, 5-triphenyltetrazolium chloride (TTC) to identify ischaemic brain area as shown in Fig. 2.

Data was analysed on SPSS statistical software version 22.0 using One-Way ANOVA. Tukey range Post Hoc test was applied considering $P < 0.05$ as significant.
Ethical Committee Statement

Research protocol and use of animals (Rats) was approved by the Committee on Animal Ethics, Hajvery University, Lahore-Pakistan.

FLOW CHART FOR RESEARCH METHODOLOGY

Figure 1. Surgical procedure for induction of ischemic stroke.

Figure 2. Brain slicing of rats. Area shown in white shows ischemic region as clearly seen in Group B.
Results

This study was designed for the evaluation of neuroprotective activity of *Camellia sinensis* extract (CSE) in rat model of stroke. Behavioural modulation of rats was performed and following four tests were applied. These include cylinder test, forelimb flexion test, staircase test and pasta test.

Overall performance was measured as the total number of falls, movements and grips in relation to time was observed and recorded in a row of 1st, 4th, 7th, 10th and 13th day. All four tests were performed and their behaviour was observed as shown in Fig. 03.

Figure 3. Shows the rat behavior and task performance before induction of stroke.

It is shown from Fig. 3 that all rats acquired the basic task. However, few rats in control and Sham groups in Forelimb Flexion and Pasta tests showed quicker response and behaviour. It may be caused by their physiological function. It was also noted that those rats that were administered with CSE showed a little aggressive behaviour, which may be further evaluated in future research. In short, our finding has no significant difference in behaviours of animals of all the groups and they were in appropriate condition for surgical procedure.

Cylinder Test

Cylinder test is intended to evaluate locomotor asymmetry in rat models having CNS disorders. It was observed during experimentation that there was significant difference between CSE 200 mg (0.714 ± 0.420, 1.142 ± 0.260, 1.142
and control (5.142 ± 0.340) as well as Sham groups (1.142 ± 0.260, 0.857 ± 0.260, 1.285 ± 0.285) in 24, 48 and 72 hours intervals (p = 0.000, p = 0.000, p = 0.000) as shown in Fig. 4. However, results of CSE 400 mg (5.142 ± 0.260, 3.857 ± 0.670, 5.571 ± 0.297) and control (5.142 ± 0.340) are not significant (p = 1.000, p = 0.072, p = 0.953). This indicates that CSE 400 mg has high influence in the recovery of stroke at all three time intervals among all defined treatment groups. Moreover, CSE 200 mg (0.714 ± 0.420, 1.142 ± 0.260, 1.142 ± 0.142) did not produce an exemplary effect on stroke and remained almost parallel to Sham (1.142 ± 0.260, 0.857 ± 0.260, 1.285 ± 0.285) group. Data also show that homogeneity of variance in the cylinder test (p = 0.459) is far from the level of significance declared, i.e. 0.05.

**FIGURE 4. CYLINDER TEST**

!![Cylinder Test Graph]

Figure 4. Cylinder test. Figure shows that the cylinder test results at 24, 48 and 72 hrs intervals. This indicates that CSE 400 mg has high influence in recovery of stroke at all three time intervals among all defined treatment groups.

**Forelimb Flexion Test**

A very elementary evaluation used to distinguish neurological discrepancies is the test of Forelimb Flexion. Significant difference was found among CSE 200 mg (1.428 ± 0.202, 1.428 ± 0.202, 1.571 ± 0.202), control (6.285 ± 1.267) and Sham (0.571 ± 0.202, 0.428 ± 0.202, 0.714 ± 0.184) groups in 24, 48 and 72 hours intervals (p = 0.000) respectively as demonstrated in Fig. 05. However, CSE 400 mg (4.428 ± 0.202, 5.000 ± 0.308, 5.428 ± 0.368) and control (6.285 ± 1.267) groups are non-significant (p = 0.040, p = 0.275, p = 0.710), which shows that CSE 400 mg is very effective in induced stroke at all levels of treatment intervals. On the other hand, CSE 200 mg (1.428 ± 0.202, 1.428 ± 0.202, 1.571 ± 0.202) showed little improvement in stroke recovery. Data of Forelimb Flexion show homogeneity of variance (p = 0.000).
Figure 5. Forelimb flexion test. Figure demonstrates the Forelimb Flexion test results at 24, 48 and 72 hours intervals. CSE 400mg found effective in induced stroke at all levels of treatment intervals.

Figure 6. Staircase test. Figure illustrates the Staircase test results at 24, 48 and 72 hours intervals. CSE 400mg had a positive response in nervous coordination.
**Staircase Test**

Staircase test is a well-known test for the measurement of deficits in coordinated paw reaching in rats and shown to expose damages on the contralateral side following unilateral lesions in a wide range of motor structures of the brain. When we statistically analysed the control group with all other three groups, we found no really significant results with CSE 200 mg \((p = 0.163, p = 0.134, p = 0.904)\) and CSE 400 mg \((p = 0.904, p = 1.000, p = 0.695)\) irrespective of Sham that was highly significant \((p = 0.000, p = 0.000, p = 0.027)\). Staircase test showed that although the results are not significant in all experimental groups, but CSE 400 mg had a positive response in nervous coordination as illustrated in Fig. 6.

**Pasta Test**

Pasta test is used for simple quantitative extent of forepaw dexterity that is sensitive to lateral impaired changes. Statistical analysis showed significant results between Control \((7.000 \pm 1.573)\) and Sham \((20.428 \pm 2.158, 18.285 \pm 1.569, 18.571 \pm 1.411)\) group \((p = 0.000, p = 0.000, p = 0.000)\), while other groups showed insignificant results in comparison to control. As the Fig. 7 demonstrated, CSE 400 mg \((5.428 \pm 0.368, 6.428 \pm 0.368, 5.285 \pm 0.285)\) showed the best strength in comparison to all groups. In addition, level of homogeneity of variance is highly significant in Pasta test \((p = 0.000)\).
Discussion

The objective of the present research study was to evaluate the neuroprotective activity of *Camellia sinensis* extract (CSE) in stroke induced rat model. The data was subjected to analysis at different levels of complexity according to the requirements of the specific experimental investigations. After administration of CSE for the specific period of time not more than 21 days, rats were subjected to surgery according to the procedure as specified in previous sections. CSE administered was 200 mg and 400 mg/kg. Two doses were selected on the basis of dose ranging study. A dose-ranging study is a clinical trial where different doses of an agent (e.g. a drug) are tested against each other to establish which dose works best and/or is least harmful. Typically, a dose ranging study will include a placebo group of subjects and a few groups that receive different doses of the test drug. For instance, a typical dose-ranging study may include four groups: a placebo group, low-dose group, medium-dose group and a high-dose group. The maximum tolerable dose information is necessary to be able to design such groups and therefore dose-ranging studies are usually designed (Ting, 2006).

The subjects were evaluated with different tests and their behaviour was observed post-operatively. Cylinder test is aimed to assess locomotor asymmetry in rat models having CNS disorders. This test has additionally been found to be able to recognise even shallow neurological impedance. It was observed during experimentation that there was much significant difference between CSE 200 mg and control as well as Sham groups at 24, 48 and 72 hours intervals (p = 0.000). However, results of CSE 400 mg and control are not significant (p = 1.000, p = 0.072, p = 0.953). This indicates that CSE 400 mg has high influence in the recovery of stroke at all three time intervals among all defined treatment groups. Moreover, CSE 200 mg did not produce an exemplary effect on stroke and remained almost parallel to the Sham group. CSE composition has flavonoids that are involved in improvement of brain function and reduction in stroke damage. The neuroprotective actions of dietary flavonoids involve a number of effects within the brain including a potential to protect neurons against injury induced by neurotoxins, an ability to suppress neuro-inflammation and the potential to promote memory, learning and cognitive function. This multiplicity of effects appears to be underpinned by two processes. Firstly, they interact with important neuronal signalling cascades leading to an inhibition of apoptosis triggered by neurotoxic species and to a promotion of neuronal survival and differentiation. These interactions include selective actions on a number of protein kinase and lipid kinase signalling cascades, most notably the PI3K/Akt and MAP kinase pathways, which regulate pro-survival transcription factors and gene expression. Secondly, they induce peripheral and cerebral vascular blood flow in a manner which may lead to the induction of angiogenesis and new nerve cell growth in the hippocampus. Therefore, the consumption of flavonoid-rich foods such as berries and cocoa throughout life holds a potential to limit the neuro-degeneration associated with a variety of neurological
disorders and to prevent or reverse normal or abnormal deteriorations in cognitive performance (Spencer, 2009). In relation to this study, our vivisection found effective as CSE 400 mg installed a stroke reversal program in rat brain.

Forelimb Flexion test is an elementary evaluation for detection of neurological dysfunction. It is one of the sensorimotor tests used to assess brain function with limb coordination. Significant difference was found among CSE 200 mg, control and Sham groups at 24, 48 and 72 hours intervals \((p = 0.000)\) respectively. However, CSE 400 mg and control groups are non-significant \((p = 0.040, p = 0.275, p = 0.710)\), which shows that CSE 400 mg is very effective as surgical assault in induced stroke at all levels of treatment intervals. On the other hand, CSE 200 mg showed little improvement in stroke recovery. Parallel research is piloted by Zhang et al (2004) for the investigation of neuroprotective effect. The effect of Crataegus flavonoids on brain ischaemic insults were investigated in Mongolian gerbil stroke model and results suggest that oral administration of this antioxidant increases the antioxidant level in the brain and protects the brain against delayed cell death caused by ischaemia/reperfusion injury (Zhang et al., 2004; Zhao, 2005). Our exploration established the better bridge among paws’ grasping and brain function in rat in association with previous studies.

Staircase test is recognised for the evaluation of side-specific discrepancies in synchronised paw reaching in rats, which show the impairments on the contralateral side following individual lesions in an extensive range of motor structures of the brain. No really significant results were found with CSE 200 mg \((p = 0.163, p = 0.134, p = 0.904)\) and CSE 400 mg \((p = 0.904, p = 1.000, p = 0.695)\) irrespective of Sham that was highly noteworthy \((p = 0.000, p = 0.000, p = 0.027)\). Staircase test showed that although the results are not significant in all experimental groups, but CSE 400 mg had positive response in nervous coordination. CSE 400 mg positively declared the sensory and forelimb reaching capacities along with motor coordination. Similar results was also observed by Freret et al (2006). They used staircase test for the evaluation of above defined boundaries (Freret et al., 2006). Another vital indicator observed in our study was the ability to assess practiced use of the limbs autonomously following impairment. CSE 400 mg made more sensitive restoration efforts for unilateral brain damage when evaluating independent limb use of the contralateral limb. This feature was also observed by Grabowski (Grabowski et al., 1993) with staircase test that optimises our study.

Pasta test is used to measure a modest quantitative measure of forepaw dexterity that is delicate to lateral impaired changes. Statistical analysis showed significant results between Control and Sham group \((p = 0.000)\), while other groups showed insignificant results in comparison to control. CSE 400 mg showed the best strength in comparison to all groups. However, caution should be used in attempting to identify neuroprotective drugs. Neural tissue is complex and a large number of animals/samples are needed before meaningful results can be obtained.
Conclusion

This research was conducted with the aim to evaluate the neuroprotective activity of *Camellia sinensis* extract prophylactically in surgically induced stroke in rat model. The outcomes of this study point out to the fact that *Camellia sinensis* extract is safe and effective mediator in clot dissolution and stroke reversal in rat model. It is the first agent found effective with out behavioural modification or adverse effects using its extract. Therefore, there is a need to evaluate, assess and appraise its desired characteristics and therapeutics in human subjects.

List of Abbreviations

RSM: Radix Salviae Miltiorrhizae; TMP : Tetramethylpyrazine; GSRd : Ginsenoside Rd; CSE: Camellia sinensis extract; MCAO : Middle Cerebral Artery Occlusion; TTC : 2, 3, 5-triphenyltetrazolium chloride

Author’s Contribution

Arsalan Ali designed the study and performed surgery. Tanveer Ahmed Khan wrote the draft of manuscript and statistically analysed the data. Lubna Shakir and Mehtab Ahmad Khan supervised the study. Komal Najam and Fouzia Karim performed the tests. Muhammad Yousaf and Atif Saeed extracted the *Camellia sinensis*. Saad Nabeel collected the data. Awais Ali Zaidi reviewed the manuscript.
References


